Biological Pretreatment of Rice Husks with the White-rot Fungus *Pleurotus ostreatus*: the Role of Laccase Activity

DINARY ELOISA DURÁN SEQUEDA

https://doi.org/10.48035/Tesis/2454/41230

Biological Pretreatment of Rice Husk with the White-rot Fungus *Pleurotus ostreatus*: the Role of Laccase Activity

A thesis submitted in fulfillment of the requirements of the degree of
Doctor in Engineering
Doctor in Biotechnology

Presented by:
Dinary Eloisa Durán Sequeda

Advisor:
Rocio Sierra Ramírez, Ph.D.
Universidad de Los Andes

Co-Advisor:
Antonio Gerardo Pisabarro, Ph.D.
Universidad Pública de Navarra

2021

Universidad de los Andes

upna
Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

EVALUATION COMMITTEE

President

Dra. Lucia Ramírez Nasto

Universidad Pública de Navarra, UPNA

Vocal

Dra. Silvia Restrepo Restrepo

Universidad de los Andes, UNIANDES

Secretary

Dr. Juan Camilo González

Bristol Myers Squibb

Substitutes

Dra. María Jesús Martínez

Centro de Investigaciones Biológicas, CSIC

Dr. Andrés González Barrios

Universidad de los Andes, UNIANDES

Dr. Fernando Martínez Galán

Universidad de Navarra

External reviewers

Dra. María Jesús Martínez

Dr. Juan Camilo González

External reviewer substitute

Dr. Fernando Martínez

Dra. María Isabel Calvo Martínez

Universidad de Navarra

Dña. ROCÍO SIERRA RAMÍREZ, Profesora Asociada del Departamento de Ingeniería Química y de Alimentos de la Universidad de Los Andes de Bogotá (Colombia), y

D. ANTONIO G. PISABARRO DE LUCAS, Catedrático de Microbiología de la Universidad Pública de Navarra,

INFORMAN

que la presente memoria de Tesis doctoral titulada "Pretratamiento Biológico de las cascarillas de arroz con el hongo de podredumbre blanca *Pleurotus ostreatus*: el papel de la actividad lacasa" elaborada por **Dña. DINARY ELOÍSA DURÁN SEQUEDA** ha sido realizada bajo nuestra dirección en la modalidad de **COTUTELA** y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctora.

Y, para que así conste, firman el presente informe en Bogotá y Pamplona a seis de octubre de dos mil veintiuno.

Fdo. Dra. Rocio Sierra Ramírez

Fdo. Dr. Antonio G. Pisabarro

Funding

This thesis has been carried out within the framework of a co-supervision agreement between the University of Los Andes and the Public University of Navarra.

The doctoral student was supported by funds of the Gobernación del Departamento del Cesar by the Ministerio de Ciencia y Tecnología en Innovación de Colombia through 681 of 2014 resolution.

The research carried out during the stay in Spain has been financed with own funds from the Public University of Navarra (UPNA) and with funds from the Research project RTI2018-099371-B-I00 (MCIU, AEI, FEDER / EU).

Acknowledgments

I want to thank my advisors Professor Rocio Sierra and Professor Gerardo Pisabarro; thank you very much to both of you for your patience, advice, and helpful insight during the development of my thesis. Thank you because I always had the certainty of unconditional help that become the light in the dark moments.

I am grateful to the Faculty, administrative and technical staff at Universidad de Los Andes, for their administrative support, and their human and technical laboratory staff for their human and technical quality. In the same way to the Public University of Navarra for the management and support of the co-supervision.

I want to thank Professor Lucia Ramírez for accepting me into her Research group during my internship.

All this way was made enjoyable in large part due to the friends and groups I interacted with during my studies the course of my studies I want to thank my colleagues from the Universidad de Los Andes group: Luis Cruz, Daniel Durán, Carla Cárdenas, and Juan Chiriví for teaching and helping. To Luis and Daniel for their friendship. I want to thank my colleagues from the UPNA: Angélica Navarro, Manuel Alfaro, Gúmer Perez, María Ibañez, Mayte Murillo, Amaya Molinero, Emilia Chuina, Edurne Garde, and Ana Fernández for all their generosity and the unforgettable moments we spent together. To the Vallenatos group, to Jesús Morales, Olga Fuentes, Julio Mario, and Mayra for the joy of their friendship.

I would like to thank my family for all their love and encouragement.
To my dear mother Alba Sequeda, my brothers Luis and Alba, my nephews Sebastian and Alison, and Mommy Ina for their love. To my beloved Eduardo and José for their patience, their love, and all the time they waited for me, to José for his infinite generosity.

Lastly, I am grateful to the Government of Cesar that financed my studies with the Ministry of Science, Technology, and Innovation at request 681 through resources of Science, Technology, and Innovation for higher education.

Agradecimientos

Quiero agradecer a mis asesores la profesora Rocío Sierra y el profesor Gerardo Pisabarro; muchas gracias a ambos por su paciencia, sus consejos y asesoría en el desarrollo de mi tesis doctoral. La certeza de su apoyo incondicional siempre fue luz en los momentos difíciles.

Agradezco a la Universidad de Los Andes, por su apoyo administrativo, a sus profesores y al personal del laboratorio técnico por su calidad humana y técnica, de igual manera a la Universidad Pública de Navarra por la gestión y apoyo de la co-supervisión.

Quiero agradecer a la profesora Lucia Ramírez por aceptarme en su grupo de investigación durante mi pasantía.

Todo este camino se hizo disfrutable en gran parte gracias a los amigos y grupos, quiero agradecer a mis compañeros del grupo Universidad de Los Andes: Luis Cruz, Daniel Durán, Carla Cárdenas, Juan Chiriví por todo su apoyo técnico y ayuda en la etapa inicial del doctorado. A Luis y Daniel por su amistad. A mis compañeros de la UPNa: Angélica Navarro, Manuel Alfaro, Gúmer Pérez, María Ibañez, Mayte, Amaya Molinero, Emilia Chuina, Edurne Garden, Ana Fernández por toda su generosidad y momentos inolvidables. Al combo Vallenatos, a Jesús Morales, Olga Fuentes, Julio Mario, Mayra, y Migue por su alegría y amistad. A Jesús, en especial por las batallas compartidas.

Me gustaría agradecer a mi familia por todo su amor y aliento.
A mí querida madre Alba Sequeda, mis hermanos Luis y Alba, mis sobrinos Sebastián y Alison, y por supuesto a mami Ina por su cariño. A mis amados Eduardo y José por su paciencia, su cariño, y todo el tiempo que me han esperado, a José por su infinita generosidad.

Por último, agradezco a la Gobiernación del Cesar que me financió con el Ministerio de Ciencia, Tecnología e Innovación por la convocatoria 681 de 2014 a través de recursos de Ciencia, Tecnología e Innovación para la educación superior

Abstract

Agro-industrial by-products such as rice husks are rich in lignocellulose, which contains the three most abundant polymers in nature: cellulose, hemicellulose, and lignin. While cellulose and hemicellulose are potentially fermentable carbohydrate stores, lignin is a recalcitrant polymer that prevents access to these carbohydrates. Different strategies have been studied to remove lignin from lignocellulose, such as biological delignification. It uses the natural ability of some organisms, mainly fungi, to break down lignin. This degradation can occur because the fungus secretes enzymes in its environment; an example of these enzymes is laccase.

Pleurotus ostreatus is a white-rot fungus that secretes an amount of laccase that varies in a wide range. The secretion of these multicopper oxidase enzymes by *P. ostreatus* depends on the composition of the culture medium. Consequently, many studies had focused on evaluating various culture media to increase the secretion of these enzymes by the fungus. Although these studies had shown that nutritional factors and some inducing substances influenced the secretion of laccases in *P. ostreatus*, it was unknown how these factors are related to each other and influence the secretion of these enzymes. Therefore, the main objective of this study was to determine the influence of the composition of the culture medium and lignocellulosic compounds on the secretion of laccase enzymes by *P. ostreatus* in submerged cultures. These studies were done using a statistical and systematic approach that allowed the control of the culture media composition. The optimal nutritional conditions were found that simultaneously increased fungal growth and laccase activity in the absence and presence of copper sulfate, a recognized inducer of laccase. Under these conditions, the biochemical aspects of transcripts in *P. ostreatus* related to laccase secretion were evaluated , which revealed the participation of membrane transporters with high affinity for copper (CTRs) as intermediate candidates for the regulation of three laccase genes, *lacc2*, *lacc6,* and *lacc10*. Moreover, the evaluation of the results of the culture media composition suggests that the regulation of these transporters is closely linked to sufficient nutritional conditions in carbon and nitrogen, with central participation of the metabolism of organic nitrogen in this process.

With these findings, it was possible to obtain more profound knowledge of the pretreatment of lignocellulosic biomass by *P. ostreatus* in a submerged culture that was oriented to determine the role of laccase activity in the biological pretreatment of rice husks.

Resumen

Los subproductos agroindustriales como las cascarillas de arroz son ricos en lignocelulosa, que contiene los tres polímeros más abundantes en la naturaleza: celulosa, hemicelulosas y lignina. Mientras que la celulosa y las hemicelulosas son depósitos de carbohidratos potencialmente fermentables, la lignina es un polímero recalcitrante que impide el acceso a estos carbohidratos. Se han estudiado diferentes estrategias de pretratamiento para eliminar la lignina de la lignocelulosa, entre ellas la deslignificación biológica, la cual utiliza la capacidad natural de algunos organismos, principalmente hongos, de degradar la lignina por la acción de oxidasas que secretan a su entorno para tal efecto.

Pleurotus ostreatus es un hongo de podredumbre blanca que secreta lacasas en su entorno de crecimiento. La secreción de estas oxidasas multicobre por este hongo dependen de la composición del medio de cultivo, por tanto, muchos estudios se había enfocado en evaluar diversos sustratos para incrementar la secreción de estas enzimas. Aunque estos estudios habían mostraron que los factores nutricionales carbono y nitrógeno y algunas sustancias inductoras en el medio de cultivo influían en la actividad lacasa de los extractos de cultivos del hongo, se desconocía cómo estos factores se relacionan entre sí en la regulación de la transcripción de genes lacasas. En consecuencia, el objetivo principal de la investigación fue determinar la influencia de la composición del medio de cultivo y los compuestos lignocelulósicos en la secreción de enzimas lacasas por *P. ostreatus* en cultivos sumergidos. Utilizando un enfoque estadístico y sistemático que permitió el control de la composición de los medios de cultivo, se encontraron las condiciones nutricionales óptimas que simultáneamente incrementaban el crecimiento fúngico y la actividad lacasa en ausencia y presencia de sulfato de cobre, un inductor reconocido de esta actividad enzimática. En dichas condiciones se indagó sobre los aspectos bioquímicos transcripciones en P. ostreatus relacionados con la secreción de lacasas, lo cual reveló la participación de transportadores de membrana de alta afinidad por el cobre (CTRs) como candidatos intermediarios de la regulación de tres genes lacasas *lacc2*, *lacc6* and *lacc10*. Los resultados de la evaluación de la composición de los medios de cultivos sugieren que la regulación de estos trasportadores se encuentra estrechamente ligada a condiciones nutricionales suficientes en carbono y nitrógeno, con una participación central del metabolismo del nitrógeno orgánico en dicho proceso.

Este conocimiento fue orientado a determinar el papel de la actividad lacasa en el pretratamiento biológico de la cáscara de arroz con el fin de contribuir a obtener una comprensión más profunda del pretratamiento de la biomasa de lignocelulosa por P. ostreatus en cultivo sumergido.

Table of Contents

Chapter 1

Introduction

In this doctoral thesis, the study of the biological pretreatment of rice husks with the white-rot fungus *Pleurotus ostreatus* was aimed focusing on determining the role of laccase activity in this pretreatment. It was written such as each chapter is aimed for publication; therefore, each is self -contained. This presentation has the advantage of showing clearly the depth and impact of each subject of study but also has the disadvantage of the necessity to repeat some general facts in the introductory and methodology paragraphs of some chapters. Chapter 2, shows how the nutritional requirements were established to favor the growth of the fungus while increasing or inducing laccase activity in synthetic culture media. Once these nutritional requirements were determined, the potential for enzymatic pretreatment of rice husks from different crude extracts produced in synthetic culture media with different laccase inducers was addressed shown in Chapter 3. A second pretreatment strategy is shown in Chapter 4, which used a modified culture media concept; in this part, various biological pretreatment systems for rice husks were evaluated using different concentrations of the same lignocellulosic biomass in synthetic culture media. Finally, Chapter 5 aims to find genes associated with laccase induction or regulation, and the *P. ostreatus* transcriptome was compared in synthetic and modified culture media with different laccase activities.

Optimizing the valorization processes and use of biomass constitutes one of the pillars of the transition to a biobased economy. Under the biorefinery concept, within all the types of biomass available, lignocellulose biomass is versatile because it can be used in at least three platforms based on its constituent polymers, which generate key intermediates such as cellulose, other C6-sugars, hemicellulose, other C5-sugars, and lignin as an intermediate *per se*. These intermediates have a bioenergy potential as second-generation fuels. Alternately, they are feedstocks for bio-based products such as biomolecules, biofertilizers, animal feed, or biomaterials. Although the conversion processes used in these platforms can be classified into mechanical/physical, chemical, thermochemical, or biochemical processes in an actual setting, the mixture or combinations of these processes may be required; however, biochemical processes are usually carried out at low temperatures and pressures, using microorganisms or enzymes. The biochemical processes operating conditions results in a lower operating cost than the alternative mechanical or thermochemical processes and often a reduction in the production of toxic compounds compared to conversion mediated by the action of an external chemical; however, these operational advantages are often hindered by longer processing times and low efficiencies. Even so, overlooked valuable by-products are often obtained using biochemical processes, such as microbial biomass, enzymes with multiple applications, or bioactive molecules formed during microbial metabolism.

Rice husks biomass was used in this work. Rice husk is a lignocellulosic byproduct of the processing of a rice paddy [1], [2]. It has been estimated that this byproduct constitutes around 20%-33% of the weight of the rice product [3]. Given the widespread cultivation of rice worldwide, this means a significant availability of this lignocellulosic biomass; in fact, around 750 million tons (MT) of rice with husk were produced worldwide in 2019 [4], of which around 2.6 MT were produced in Colombia alone [5].

Because the rice husks are formed from the two leaves of the spikelet, that is, the palea that covers the ventral part of the seed and the lemma that covers the dorsal portion, the microstructure of the rice husk is an arrangement of epidermal tissue that follows a parenchymal tissue and ends with a hypodermis, hence its richness in lignocellulose. In addition, other structural characteristics explain its recalcitrance and complex composition. The outer layer forms dome-shaped bumps conspicuously ridged, extremely thick, highly convoluted, and rich in silica and lignin, whereas the inner layer is smooth, uniform, low in silica and lignin [6]. Therefore, rice husks have a composition rich in cellulose, hemicellulose, lignin, ash, and other extractable substances, with varied compositions [7]. Cellulose content varies between 38-50%, hemicellulose between 23-32%, lignin ranges between 15-25%, and ash content in rice husks can reach around 20% of the total weight [8]. Although rice husks ashes could also contain oxides of other elements such as aluminum, iron, magnesium, sodium, calcium, phosphorous, sulfur, and titanium, these ashes are characterized by being rich in silicon dioxide [7], [9], [10]. These compositional characteristics are variable; in any case, they imply a holocellulose content (fraction of cellulose and hemicellulose) of at least 50%. This content is attractive for C6-C5 biorefinery platforms; however, the heterogeneity in composition and high silica and lignin contents make this material difficult to degrade.

Depending on the rice husks conversion pathway selected to recover this lignocellulose biomass, the process steps may change; however, the pretreatment stage is almost standard in several of these processes, regardless of selected conversion [11]. Take, for example, the acid wash pretreatment of rice husks in biomass pyrolysis [12], the alkaline pretreatment of rice husks to produce activated carbon and other value-added products [13], the liquid ionic acid-catalyzed pretreatment for the conversion of rice husks into fermentable sugar and silica [14], or the bioethanol production from rice husks using different chemical pretreatments [15] among others. It is easy to see that these conversion pathways can be mechanical/physical, chemical, thermochemical, or biochemical (usually called biological). The pretreatments can also be of the same nature, sharing the same advantages and disadvantages, and can be used alone or in combination. In any case, the goal of pretreatment is to break down the compact structure of the lignocellulose and expose the cellulose fibers, helping to overcome recalcitrance of the material by combining chemical and structural changes in lignin and carbohydrates [16].

In biological pretreatment, the deconstruction of lignin structures in the cell wall occurs using microbes and/or enzymes as catalysts [16]. Biological pretreatments use wood-degrading microorganisms, including white-rot, brown-rot, soft-rot fungi, and bacteria [17]. Biological pretreatment with fungi has a greater percentage of lignin

removal than pretreatment with bacteria [18]. Moreover, because brown-rot and soft-rot fungi usually attack cellulose and cause minor lignin modifications, white-rot fungi targeting lignin are preferably selected for biological pretreatments [17], [19]. White-rot fungi encompass different ligninolytic basidiomycetes that digest wood, causing its decomposition, including *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, *Phlebia* spp., *Gonoderma* sp. *Oxysporus* sp., *Trametes versicolor*, *Pleurotus* sajor-caju, *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, among others [20], [21]. The highest, most common percentages of cellulose, hemicellulose, and lignin loss reported for plant materials treated with these microorganisms are 49%, 78%, and 72%, respectively [18]. However, *P. ostreatus* is usually preferred over *P. chrysosporium*, *T. versicolor*, and *C. subvermispora* because this fungus selectively degrades the fraction of lignin instead of the holocellulose fraction found in substrates such as rice husks [22], [23].

P. ostreatus is a basidiomycete of the Agaricales order [24], [25]. During most of its life cycle, it has a dikaryon mycelium whose two nuclei come from the corresponding monokaryon parental mycelia that join during plasmogamy, stay together through the vegetative growth but separated in the same cytoplasm [26]. The dikaryotic mycelium can grow vegetatively in an asexual manner or generate a sporocarp where the sexual cycle occurs; the parental nuclei attach together through karyogamy and divide meiotically to generate haploid spores that produce a new monokaryon mycelium which continues the life upon germination [27]. From the dikaryon, two parental nuclei can be recovered *in vitro*, and these new monokaryotic mycelia can originate new haploid cell lines [28]. The most widely studied cell lines mkPC15 and mkPC19 were obtained from the dikaryotic parental dkN001, a commercial strain of *P. ostreatus* whose genome can be found in http://jgi.doe.gov/fungi [29], [30]. It was determined that the genome of *P. ostreatus* contains 11 pairs of chromosomes with varying sizes between 1.4-4.7 Mbp [31].

P. ostreatus is used in the biological pretreatment of lignocellulosic biomass due to its ability to produce enzymes capable of modifying the lignin present in the lignocellulosic complex. This ability to modify lignin is attributed to an enzyme system from the superfamily of oxidases, in which only laccases, heme peroxidases from the lignin peroxidase (LiP) family, manganese peroxidase (MnP), and versatile peroxidase (VP) are recognized as lignin-modifying enzymes (LMEs) [30]. It has been found that the genome of *P. ostreatus* contains nine genes that code for ligninolytic peroxidases; these genes code for six MnP and three VP isoenzymes but no LiP [32], [33], whereas the genome of *P. ostreatus* codifies 12 laccases genes [28]. Additionally, these genes, transcripts, and the resulting isoenzymes from these genes have been identified for their effect in diverse culture conditions and under the influence of varying substrates [19], [30]. Although the MnP and VPs have been detected, laccases are the main enzymes secreted by *P. ostreatus*[30]; therefore, lignin oxidation in the fungal pretreatment using *P. ostreatus* is mainly attributed to laccases.

Fungal laccases are glycosylated, multicopper oxidase enzymes that catalyze the hydroxyl functional group oxidation on various substrates and the molecular oxygen reduction to water [34]–[36]. This reaction is catalyzed in four copper atoms arranged in T1, T2, and T3 copper centers. The T1 center has a single copper coordinated by His,

His, Cys, and Met residues. Usually, T2 and T3 centers form a trinuclear copper cluster. The T2 center has a single copper coordinated His and Asp or Tyr residues, and the T3 centers are formed by two copper atoms, each coordinated by three His residues [37]. In this multicopper catalytic center in laccases, T1 copper is involved in substrate oxidation, while in T2 and T3 are arranged in a trinuclear cluster, they catalyze the reduction in molecular oxygen [38].

The exact mechanisms by which laccases work in modifying lignin in lignocellulosic biomass are still unknown, and some controversies exist on whether laccases alone can actually modify lignin [39]. Since lignin is formed by the ether bonds between carbon-carbon ($C-C$) in the structural units of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), which include bonds type β−O−4, β−5, β−β, 5−5, 4−O−5, and β−1; these bonds results in a heterogeneous polymer in which the abundance of one type of structural unit relates to the high, medium, and low wood-like nature of the plant material [40]. Based on the diphenolic substrates, three types of reactions have been associated with laccases in lignin: 1) bond rupture, 2) modification and 3) coupling, which can cause different changes in this polymer. As far as modification reactions, oxidation reactions of the alpha carbon (Cα) are the main ones in which the hydroxyl group is changed for a ketone; however, coupling reactions can result in two types of modifications: 1) polymerization reactions in which new structural units (H, G or S) incorporate into the polymer, which results in increased molecular weight, and 2) reactions that insert low molecular weight sulfonated molecules [39], [40].

Besides the above, there is a model according to which in which laccases could begin the cleavage of the side chain and aromatic ring of lignin; in this model, laccase catalyzes the oxidation of phenolic substrates forming phenoxyl free radical as an unstable intermediate, which then promotes Cα oxidation, alkyl-aryl cleavage, and Cα-Cβ cleavage [41]. However, to enhance the oxidation capabilities of laccase and help overcome the steric hindrance existing between laccase and non-phenolic substrates in lignin during delignification [42], laccase-mediator systems have been found to increase the delignification [43], [44]. The most common mediators are non-phenolic substrates such as 1-hydroxybenzotriazole (HBT), 3-hydroxyanthranilic acid (HAA), and 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonate) (ABTS) [45]. The oxidized non-phenolic compounds coupled with mediators can promote the aromatic ring cleavage, Cα-Cβ cleavage, Cα oxidation, and β ether cleavage [42], [46].

The last consideration in this research was about types of culture. Biological pretreatment of lignocellulose can be carried out in different types of cultures such as solid culture (SSF), static liquid (SLF), or submerged liquid (SmF) which can affect the pretreatment results [47]. SSF mimic the natural conditions in which these organisms grow; however, the fungus grows attached to the substrate impeding the effective recovery of biomass and the valuable metabolites [48]. In SLF, the fungus grows on the surface of the culture broth. It spreads towards the boundaries of the culture flask or bioreactor, which facilitates the separation of the fungal biomass from lignocellulose biomass but limits the fungal growth to the ratio surface volume of the container. In this type of culture, fungal biomass can grow with heterogeneous morphology. Finally, in

SmF, the fungus usually grows with an uniform pellet-like morphology [49], and three phases are generated in the culture; the solid phase with the remains of the pretreated lignocellulose biomass, the fungal biomass (the pellets), and the liquid phase rich in enzymes and metabolites product of fungal growth. Therefore, for this work, submerged fermentation was selected for all culture conditions.

In this thesis, the focus is directed to two different aspects of laccase production. First, how the differences in the culture medium and the presence of inducers influence the laccase activity recovered from submerged cultures of *P. ostreatus.* And, second, since laccases are the enzymes most secreted by *P. ostreatus* when it grows on lignocellulose, we hypothesize that an increase in the induction of these enzymes will increase the degradation of lignin. Under this consideration, it was of interest to determine to which extent the laccases secreted during the growth of *P. ostreatus* are effective for the pretreatment of rice husks accomplish delignification, thereby diminishing the recalcitrance of this biomass.

1.1 Research objectives

We aimed to determine the initial composition of the culture medium that results in the highest laccase activity when *P. ostreatus* is grown in submerged fermentation and compare the modifications observed on the lignocellulosic substrate for several rice husks due to the action of these laccases using various biological pretreatment conditions. All this is to gain a deeper understanding of the pretreatment of biomass by white-rot fungus *P. ostreatus* to apply this knowledge to develop a strategy to improve the pretreatment of rice husks.

In order to achieve this general research objective, the following specific objectives are considered:

- Determine the nutritional carbon and nitrogen requirements to increase fungal growth and laccase activity by *P. ostreatus* in SmF.
- Evaluate the effect of laccase copper sulfate inducer and lignocellulose-derived compounds in optimal nutritional conditions for *P. ostreatus*.
- Evaluate the influence of rice husks as lignocellulosic substrate in the production of laccases by *P. ostreatus* in submerged cultures.
- Compare the transcriptome of *P. ostreatus* under high and low laccase activity conditions, focusing on genes associated with laccase regulation and other lignocellulolytic enzymes.
- Determine the compositional and structural changes of the rice husks recovered from different biological pretreatment systems that use enzymes secreted by *P. ostreatus* in selected SmF.

The methodological approach to achieve these goals is both experimental and analytic. The intention is to use a statistical experimental design to determine the significance of nutritional factors and composition on the response of *P. ostreatus* and the extent of delignification.

Chapter 2
Effect of Nutritional Factors and Copper on the Regulation of Laccase Enzymes Production in *Pleurotus ostreatus*

Abstract: Laccase enzyme production by *Pleurotus ostreatus* depends on multiple factors. This research aimed to establish the relationship between carbon-nitrogen nutritional factors and the laccase inducer copper sulfate on laccase production. Initially, the effect of two nitrogen sources, ammonium sulfate and yeast extract, on biomass production and laccase activity was evaluated. Yeast extract was selected as a better nitrogen source than ammonium sulfate because this organic nitrogen source increased 2-fold biomass production and 2-orders of magnitude the laccase activity at the same glucose concentration. Then, the effect of glucose and yeast extract concentrations for biomass production and laccase activity as response variables were evaluated. This evaluation was done using central composite experimental designs with and without 1mM copper sulfate. The results showed that to optimize these two response variables simultaneously, the culture medium composition was glucose 45 and yeast extract 15 gL^{-1}. In this optimal medium with or without copper sulfate, the *P. ostreatus* transcriptome was obtained by RNAseq analysis, and the differentially expressed genes (DEG) were found. The top ten up-regulated transcripts in the DEG showed three laccase genes, *lacc2*, *lacc6,* and *lacc10,* positively regulated by copper sulfate. The top ten down-regulated transcripts included a copper transporter (*ctr1*) and a regulator of nitrogen metabolism (*nmr1*). These results suggest that Ctr1, which facilitates the entry of copper into the fungal cell, is regulated by glucose and nitrogen sufficiency conditions. Once inside, copper induces transcription of laccase genes. This finding could explain why a 10 to 20-fold increase in laccase activity occurs with 1 mM copper sulfate as opposed to cultures without copper sulfate, with the optimal concentration of yeast extract as nitrogen sources.[1]

2.1 Introduction

Fungal laccases are glycosylated, multi-copper oxidase enzymes that catalyze the hydroxyl functional group oxidation on various substrates and the molecular oxygen reduction to water [34]–[36]. Laccases can oxidize phenolic and non-phenolic compounds; therefore, these enzymes are attractive in many processes or biotechnological applications, such as

[1] To be submitted: Journal of Fungi

Dinary Durán-Sequeda[1, 2] *, Daniela Suspes[1], Estibenson Maestre[1], Manuel Alfaro[2], Gúmer Perez[2], Lucía Ramírez[2], Antonio G. Pisabrarro[2], and Rocío Sierra[1]

[1] Product and Process Design Group (GDPP), Department of Chemical and Food Engineering, Universidad de los Andes, Colombia; e-mail@e-mail.com

[2] Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre (UPNA), 31006 Pamplona, Navarre, Spain

* Correspondence: de.duran@uniandes.edu.co

bioremediation, wastewater treatment, nanobiotechnology, biofuel production, pharmaceutical, and food industry [50], [51].

Pleurotus ostreatus is a white-rot fungus considered a biotechnological model for studying and producing fungal laccases[30]. This fungus is easily cultivable on several synthetic or natural media [52], its genome has been decoded [53], and it contains a laccase multi-gene family [54]. Despite these advantages, laccase production in the *P. ostreatus* cultures is affected by complex, not fully understood laccase gene expression regulatory mechanisms at multiple levels.

At the genome level, 12 laccase genes have been identified in the genomes of the *P. ostreatus* monokaryotic strains, mkPC15 and mkPC9 [28]. These genes are distributed on several chromosomes of the fungus: seven on chromosome VI (*lacc1*, *lacc4*, *lacc6*, *lacc7*, *lacc9*, *lacc10*, and *lacc11*), two on chromosome XI (*lacc5* and *lacc12*), and only one among *lacc3*, *lacc8*, or *lacc2* on the chromosomes IV, VII, and VIII, respectively[55]. However, the physiological and functional roles of each gene and the consequences of their clustering are still under study.

At the transcriptional level, each laccase gene expression regulation is mainly controlled by the *cis*-acting elements localized upstream of the transcriptional start site in the promoter [56]. In addition to the TATA and CAAT putative boxes, other *cis*-acting elements in these promoter regions differ for each laccase gene on *P. ostreatus*. These additional cis-acting elements can be divided into three groups *i*) response to nutrient-sufficient conditions (carbon and nitrogen sufficient) including catabolic responsive elements (CRE) and nitrogen binding site (NIT), *ii*) response to inducers such as metal responsive (MRE), xenobiotic responsive (XRE), and antioxidant responsive elements (ARE); and *iii*) response to stress including heat shock sequence (HSE), and stress-responsive elements (STRE) [57], [58]. The first and second groups, response to nutritional status and inducers, could be stimulated by changing the culture medium composition; however, these changes have been insufficient to explain the transcriptional profile of laccase in different culture conditions [59], [60]. Moreover, there is a lack of knowledge about how other *trans*-regulatory factors can interact and intervene at this regulation level.

Other laccase regulation mechanisms occur at the post-transcriptional and post-translational levels. These regulations could explain changes in the molecular weight of laccase isoenzymes or the heterogeneity observed in the electrophoretic migration patterns in laccase isoforms [61]. Most isolated and characterized laccases have an apparent molecular weight between approximately 60 and 85 kDa. Although almost all are monomers, Lacc2 isoenzymes can be heterodimers [56], [57]. The large subunits are at least two transcriptional variants by alternative splicing of the *lacc2* gen, whereas the small subunits are produced by translating the two different genes [61], [62]. Concerning the post-translational regulation level, N-glycosylation is the main post-translational modification in fungal laccases [63]. N-glycosylation is critical in several biochemical aspects, such as the folding, location, and catalytic activity of these enzymes [64], [65]. On the one hand, N-glycosylation is the main challenge to produce laccases in the heterologous system [66]. On the other hand, these modifications are also affected by fungal growth conditions and culture medium composition [67].

Since laccase regulation responds to several factors in the composition of the culture medium, a wide range of maximum laccase activity from *P. ostreatus* has been reported for

submerged fermentation, as shown in Table 2-1. Even though several culture conditions were considered in all of the selected studies, in this Table 2-1, only nutritional factors as carbon, nitrogen source, and laccase inducer are shown. Most media had other compounds. These media contained different glucose concentrations as the primary carbon source, various organic, inorganic, single, or mixed nitrogen sources, and varying concentrations of copper sulfate, a known laccase inducer, and various other inducers derived from lignocellulose. This work focuses only on the following nutritional factors: glucose and nitrogen sources and concentration with and without copper sulfate inducer to keep experimentation and analysis viable.

Table 2-1. Carbon, nitrogen, and inducer of laccase source and concentration in culture media with the maximum laccase activity by *P. ostreatus* from literature in SmF

P. ostreatus **strain**	**Carbon (gL^{-1})**	**Nitrogen (gL^{-1})**	**Inducer of Laccase (gL^{-1})**	**Maximum laccase activity (UL^{-1})**	**Day**	**Ref.**
ATCC 32783	Glucose (10)	Yeast extract (5)	$CuSO_4$ (0.25)	37490 *	20	[68]
ATCC 32783	Glucose (10)	Yeast extract (5)	$CuSO_4$ (0.25)	37000 *	18	[69]
ATCC 32783	Glucose (10)	Yeast extract (10)	$CuSO_4$ (0.25)	13000 *	13	[70]
ATCC 32783	Glucose (10.5)	Yeast extract (5)	$CuSO_4$ (0.25)	12000 *	17	[71]
N001	Glucose (20)	Potato extract (4)	$CuSO_4$ (0.125)	80000 **	9	[72]
CP-50	Glucose (20)	Yeast extract (10) Malt extract (20)	$CuSO_4$ (0.125)	3780 **	3.5	[73]
ATCC MYA-2306	Glucose (20)	Potato extract (4)	$CuSO_4$ (0.125) Feluric acid (0.39)	40000 **	10	[60]
N001	Glucose (10)	Yeast extract (4) Malt extract (10)	$CuSO_4$ (0.01) Wheat Straw Water Extract	600 *	24	[74]
PO 108	Glucose (10)	Yeast extract (2) Peptone (0.42) $(NH_4)NO_3$ (2)	$CuSO_4$ (0.005)	490 ***	8	[75]
CP-50	Glucose (10)	Yeast extract (10) Peptone (10) Tryptone (5)	$CuSO_4$ Lignin	11000**	4.5	[76]
(Jacq.:Fr.) Kumm. 494	Glucose (10)	Yeast extract (5) NH_4Cl (2)	Dry ground mandarine peels (40)	501	10	[77]

*Laccase activity was measured using 2, 6-dimethoxyphenol (DMP) as substrate.
** Laccase activity was measured using 2, 2–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate.
*** Laccase activity was measured using syringaldazine as substrate.

P. ostreatus, like other fungi, senses carbon and nitrogen sources and their concentrations in the cellular environment through nutrient detection mechanisms and pathways [78]. Usually, these mechanisms distinguish and respond to two nutrient conditions:

nutrient-sufficient and nutrient-limited, which are different for each organism [79]. If the fungus most easily assimilates the carbon or the nitrogen source, and its concentration in the culture medium is high, catabolic repression mechanisms are activated specifically for nutrient-sufficient conditions. For carbon, the mechanism is called Carbon Catabolite Repression (CCR) [80]. In this case, since glucose is the most assimilated sugar by lignocellulolytic fungi, CCR turns off certain enzymes available only in the absence or scarcity of this sugar [81]. CCR is regulated primarily by the transcription factor Cre [82]. On the other hand, for nitrogen sources, the mechanism is called Nitrogen Metabolite Repression (NMR), and it guarantees the preferential use of ammonium (NH_4^+) or L-glutamine as nitrogen sources [83], [84]. In filamentous fungus, this mechanism is controlled by the transcription factor Nmr [85]. Both catabolic processes, CCR and NMR, have been described in other fungi, are redundant, and have multiple activations or inhibition checkpoints [86]–[88].

Copper is a metal cofactor in several oxidoreductase enzymes involved in cellular redox processes such as cellular respiration, free radical detoxification, pigmentation, collagen maturation, and iron acquisition [89]–[91]. This metal is present in the environment in two oxidation forms, Cu^+ and Cu^{2+} [91], of which the former is recognized as a substrate for the high-affinity copper transporters of the CTR family [91]; hence, environmental Cu^{2+} should be reduced to Cu^+ before being up-taken into the cell [92]. In the cell, these copper ionic states can vary in the functional copper-binding sites in proteins classified as type 1 (T1), 2 (T2), or 3(T3) [93]. In T1, a single copper atom is coordinated by two nitrogen from two His residues, one sulfur from a Cys residue, and typically one sulfur from a Met residue. In T2, copper is coordinated by nitrogen ligands typically provided by His and oxygen ligands provided by Asp or Tyr residues; and the last one. T3, copper centers are binuclear because they bind two copper atoms, coordinated by three nitrogen from three His residues [93], [94]. As laccases, fungal multicopper oxidases are characterized by the presence of at least one T1 copper center, together with at least three additional copper atoms: one T2 and two T3 copper atoms, arranged in a trinuclear cluster [37]. In this multicopper catalytic center, T1 copper is involved in substrate oxidation, while T2 and T3 coppers catalyze the reduction into molecular oxygen [38]. Therefore, copper has two roles in regulating the laccase activity of *P. ostreatus*: as a catalytic site cofactor and as an inducer of the transcription of some laccase genes.

Systematic research on the interactions between nutritional factors on the laccase activity of *P. ostreatus* is currently lacking; therefore, this work is aimed to consider the interaction between nutritional factors (carbon and nitrogen concentration) and copper sulfate at 1mM to determine how the medium composition affects laccase induction by copper. First two nitrogen sources (ammonium sulfate and yeast extract were chosen to establish which one best increases fungal biomass production and laccase activity in submerged fermentation. Yeast extract was found better than ammonium sulfate to stimulate biomass and laccase activity; therefore, in subsequent experimentation, glucose and yeast extract concentrations were varied using a central composite experimental design, which applies in the surface response methodology allowing for optimization. It was found that glucose and yeast extract concentrations do not sufficiently explain the laccase activity in cultures without copper inducer; however, when copper sulfate is in the culture media, both glucose and yeast extract concentrations influence this enzymatic activity. Moreover, glucose and nitrogen concentrations for optimal laccase activity were identified. Furthermore, the presence of copper-induced around a 20 fold increase in the laccase activity. To better understand these results, a transcriptome study of culture from glucose-sufficient and nitrogen-sufficient conditions was performed. The transcripts of transcription factor Nmr1, and genes coding for copper transporters, were found among the more up-regulated genes.

2.2 Materials and Methods

2.2.1 Fungal strain and culture conditions

The *Pleurotus ostreatus* strain ANDES-F515, provided by the Laboratory of Mycology and Phytopathology of the Universidad de Los Andes-LAMFU and deposited in the ANDES Natural History Museum (MHN ANDES), was isolated in the Bosque de la Merced, Santa Bárbara village, Bojacá, Cundinamarca, Colombia. The dikaryotic mycelium was maintained and conserved on Malt Extract Agar at 4 °C, with periodic replication of the growth zone for eight days of incubation at 25 ° C in the dark.

The submerged fermentation (SmF) cultures were performed in 250 mL flasks containing 100 mL of culture medium. For the experiments made using an inorganic nitrogen source 1.0 or 10.0 gL^{-1} of ammonium sulfate $(NH_4)_2SO_4$ were added in a minimum salt medium (MSM) composed of 0.5 gL^{-1} K_2HPO_4, 0.25 gL^{-1} $MgSO_4 \bullet 7H_2O$, 0.1 gL^{-1} $CaCl_2$, 0.5 gL^{-1} KCl, 0.5 gL^{-1} thiamine, 2.1 gL^{-1} citric acids, 2.94 gL^{-1} sodium citrate, 6.5 final pH adjusted with 0.1 M NaOH, was used. For the experiments made using an organic nitrogen source, yeast extract 5 or 10 gL^{-1} and glucose were the only medium components. Glucose (0.5, 1.0, 10, or 20 gL^{-1}) was added to achieve different C:N ratios in both experimental conditions.

The elemental compositional analysis of yeast extract used in this study was performed and revealed a C:N ratio estimated at 4:1.

Only glucose and yeast extract were used in culture media to evaluate models of surface response optimization using the central composite experimental design. The composition of these media resulted from pairwise combinations of glucose (23.8, 30.0, 45.0, 60.0, or 66.2 gL^{-1}) and yeast extract (4.4, 7.5, 15.0, 22.5, or 25.6 gL^{-1}) according to the results front the experimental design shown in section 2.3.2 (Table 2-4). All combinations were tested in the absence or presence of copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$) at 0.25 gL^{-1} (1mM).

All SmF cultures were inoculated with five 4 mm diameter plugs taken from the growth zone of eight-days Malt Agar plate cultures and incubated at 25°C, 150 rpm, in the dark for 21 days. All experiments were performed in duplicate.

2.2.2 Biomass production

Fungal biomass production was gravimetrically determined at 21 days of culture. The culture was filtered through a previously dried and weighed filter paper using a vacuum filter system. The collected, filtered mycelium was dried at 45°C for 72 h and weighed [73].

2.2.3 Biochemical analyses

Spectrometric analyses

Laccase activity was determined with ABTS **(**2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)**)** as the substrate [95]. The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 5.0), and 10 µL aliquots of an appropriately diluted enzyme sample. Oxidation of ABTS was monitored by following the increase in A_{436} (ε 29.3 $mM^{-1}cm^{-1}$). A

laccase activity unit was defined as the enzyme required to oxidize 1 µmol ABTS per minute at 25 °C. Reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) reagent by DNS method at 540 nm [96] using glucose as a reference for the calibration curve. Bradford protein assays (Bio-Rad) were used for total protein quantification. Standards and samples were mixed with Coomassie Blue Assay Reagent G-250 according to the manufacturing instructions. Each reaction was measured at 595 nm [97].

Zymogram

Non-denaturing electrophoresis conditions (native-PAGE) were used to visualize the isoenzymes present in samples [98]. The stacking and running gels contained 4% and 9% acrylamide and were adjusted to pH 6.8 and 8.8 with Tris-HCl buffer as needed. The running buffer was Tris-Glycine pH 8.3. The isozymes were revealed through gel staining after the run with ABTS 2mM.

2.2.4 RNA isolation and transcriptional analyses

The fungal biomass produced in the cultures containing 45 gL^{-1} glucose and 15 gL^{-1} yeast extract with or without 1 mM $CuSO_4$ were harvested on day 12 of culture for RNA isolation. The mycelium was collected by filtration, immediately frozen in liquid nitrogen, and ground to a fine powder in a mortar. Then, 100 ng of the powder was transferred to a 1.5 mL microcentrifuge tube for total RNA extraction using a Fungal RNA EZNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer guidelines. Finally, the integrity and quantity of RNA were validated using Bioanalyzer (version 2100) and Qubit 2.0 fluorometer.

Real-time qPCR

Reverse transcription (RT) was performed using 800 ng per sample of the total RNA to obtain cDNA in a 20 µL volume using an iScript cDNA synthesis kit (Bio-Rad, Alcobendas, Spain). The complete reaction mix was incubated according to manufacturing instructions in a thermal cycler (MJ Research, Inc). RT products were diluted 1:20 and kept at -20 °C until used Real-time qPCRs were performed in a CFX96 real-time system (Bio-Rad Laboratories, SA) using SYBR green dye to detect the product amplification [59]. Each reaction mixture was set to a final volume of 20 µL containing 10 µL iQ SYBR green supermix (Bio-Rad Laboratories, SA), 2 µL of 5 µM stock forward and reverse primers (Table 2-2), 1 µL of RT product diluted, and 5 µL of sterile water. Cycling conditions were as follows: denaturation 5 min at 95 °C, 40 cycles for 15 s at 95 °C, 30 s at 63 °C, 15 s at 72 °C, and a final step using a linear gradient increase of 0.5°C every 5 s from 65 to 95°C. Each reaction was performed in triplicate, and nontemplate controls (NTCs) were included for each primer set. An experimentally validated inter-plate calibrator (IPC) was used to compensate for inter-plate variations. Crossing-point (Cp) values and relative fluorescence units were recorded, and the latter was used to calculate amplification efficiencies by linear regression using the LinReg program [99]. *Sar1*, *gapdh1*, *actin1*, and *pep* were used as reference genes for normalization (Table 2-2).

Table 2-2. Forward (Fw) and reverse (Rv) primers sequence to for the laccase and reference genes [59], [99], [100].

Gene name[1]	Primer sequence (Fw)	Primer sequence (Rv)
lacc1	GGTACATCCTAGCACCCAATG	GACGAGATCAGTTTCCAAGAGG
lacc2	CCCTGGCAGATTGGTATCATG	ATGACAGCGTAAGGGACAAG
lacc3	TCGTTTCCGTCTCGTTTCTC	CTGCGAAGATTTGGATGCTGG
lacc4	CCCCATCCTTTCCATCTTCAC	TAGTTATACACCGAGCTTCCG
lacc5	CGCATTTGCCGCTTTCTT	GGTGACTAGGACTGAGTATCTC
lacc6	GTACAACTACGAAAACCCCG	CAAGGTCAAGATGCCAGT
lacc7	GTTGATAGCCTCCAGATCTTCG	GTAGGATGGCGGAGTTGATG
lacc8	CATTGGCTGTGACTCGAA	GGATCAGAGAATAGCGTTGG
lacc9	CTATCCTTCGGTATGCTGGTG	ATATTGATGTCTGCGCCTCC
lacc10	CCTACTTCCCCTTTGGCTATC	ATGACGAGCAAAGAGTGACC
lacc11	CCTGAATGGTCTGATCTCTGC	CCTATGACTTGGGCTCTTCG
lacc12	GTACTCATTTTCGGCTCCTG	CCACGTAGTCCATCGCAATA
sar1	GGATAGTCTTCCTCGTCGATAG	GGGTGCGTCAATCTTGTTAC
gapdh1	TGGTCCATCGCATAAGGA	ACACGGAAGGACAAACCA
actin1	AGTCGGTGCCTTGGTTAT	ATACCGACCATCACACCT
Pep	GATTCCAGAGGACAAGGACGCAA	AAATCTTCCGCGATACGGGTCACT

mRNA-seq analysis

Illumina compatible libraries were prepared to be sequenced using the Illumina Nova Seq 6000 system from an mRNA isolate originating from RNA total. Following sequencing, RNA-seq data were filtered for assurance quality using FastQC and trimmed with BBDuk to remove adapters and low-quality reads (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). www.genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html was used as a *P. ostreatus* genome reference for aligning the resulting reads using STAR v2.3.16 [101]. The parameters used to achieve a single hit mapping were: --outReadsUnmapped Fastx --outFilterMismatchNoverLmax 0.04 --outFilterMultimapNmax 1. The mkPC15 v2.0 reference genome was assembled entirely in twelve scaffolds (34.3 Mb genome size)[102]. In total, 12,330 genes were annotated in this genome [103]. The expression levels were quantified using Python script rpkmforgenes.py (www.sandberg.cmb.ki.se/media/data/rnaseq/rpkmforgenes.py) to calculate values of reads per kilobase of transcript per million mapped reads (RPKM).

2.2.5 Differential gene expression analysis and gene annotation

Differentially expressed gene (DEG) analyses were performed using the EdgeR Bioconductor package and a dispersion parameter of 0.1. These analyses determined the transcriptional changes in the two culture conditions by comparing the gene expression values based on read counts. The gene expression values with Log_2 fold changes of read counts with a p-value < 0.01 and an FDR (False Discovery Rate) < 0.05 as the cut off for statistical significance were used. A DEG with log_2 Fold Change ≥ -2 was established as an upregulated gene, and log_2 Fold Change ≤-2 was established as a downregulated gene

The genes annotations were based on the Joint Genome Institute (JGI) automated annotation to transcript identifications of the mkPC15 v2.0 reference genome. JGI automated annotation uses the following databases: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), InterPro (IPR), Eukaryotic Orthologous Groups (KOG) and Enzyme Commission numbers (EC number), Transporter Classification Database (TCDB), Carbohydrate-Active EnZymes (CAZymes) and MEROPS database (proteolytic enzymes)

[104]. In the case of unannotated genes, the Basic Local Alignment Search Tool (BLAST) was used to find local similarity between JGI sequences unannotated and The National Center for Biotechnology Information (NCBI) standards database sequence [105], [106].

2.2.6 Statistical analyses

Minitab® version 18 software was used to construct the statistical design, evaluate statistical significance, obtain the regression models, and find the simultaneous local optimum of one or more response variables.

2.2.7 Principal component analysis (PCA)

A principal component analysis (PCA) was performed to explore the correlations between nutritional variables, glucose, ammonium sulfate, and yeast extract with the production of fungal biomass and laccase activity with and without copper sulfate, and was done using the data shown in in the results section 2.3.1 (Table 2-3)

2.2.8 Central Composite Design (CCD)

The central composite experimental design allows for the estimation of the curvature of a response surface for a chosen response variable (y) and the estimation of the terms (β y ε) of a first degree (1) or second degree (2) regression model that allows for the calculation of an optimal point

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon \quad (1)$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{11}^2 + \beta_{22} x_{22}^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (2)$$

Factorial ANOVAs were performed to determine the main effect and interaction effects on chosen variables on the response variable laccase activity and biomass production.

2.3. Results

2.3.1. Effects of inorganic and organic nitrogen source on laccase activity

The effect of nitrogen source and carbon-nitrogen ratio (C:N) were evaluated in submerged fermentation cultures of *P. ostreatus*. The composition of these culture media, fungal biomass production, final glucose concentration, and maximum laccase activity at 21 days of fungal growth are shown in Table 2-3. These results showed that the highest biomass production and laccase activity were obtained in cultures with yeast extract as the nitrogen source. In comparison, the highest final glucose concentration, lower biomass, and all laccase activities inferior to 10 UL^{-1} were obtained when the nitrogen source was ammonium sulfate. Therefore, when the nitrogen source was yeast extract, the maximum laccase activity increased an average of 16-fold compared to the laccase activity observed in similar cultures with ammonium sulfate as a nitrogen source. Using yeast extract as a nitrogen source, the magnitude of laccase activity seems to depend on glucose concentration and C:N ratio.

On the one hand, the highest laccase activity was produced in the cultures with the highest glucose concentration. On the other hand, higher laccase activity was obtained in

cultures with the lowest C: N ratio concerning cultures with the same yeast extract or glucose initial concentrations in their composition. Because the lower C: N ratio indicates more nitrogen disponibility than higher C:N ratios in the culture, both results suggest that laccase activity is dependent on carbon-sufficient and nitrogen-sufficient conditions.

Table 2-3. Biological growth parameters of *P. ostreatus* in culture media with ammonium sulfate or yeast extract in SmF

Ammonium sulfate (gL^{-1})	Yeast Extract (gL^{-1})	.Glucose (gL^{-1})	C:N	Biomass (gL^{-1})	Final glucose (gL^{-1})	Maximum laccase activity (UL^{-1})
1.0	0	0.5	1:1	0.26 ± 0.02	0.18 ±0.03	2.85 ± 2.31
1.0	0	1.0	2:1	0.39 ± 0.02	0.58 ±0.02	3.40 ± 0.58
1.0	0	10.0	22:1	1.30 ± 0.18	6.86 ±0.97	6.35 ± 3.07
10.0	0	10.0	2:1	1.18 ± 0.10	6.47 ±0.51	1.74 ± 0.15
0	5.0	1.0	5:1	0.91 ± 0.04	0.06 ±0.02	944 ± 71.6
0	5.0	10.0	14:1	3.62 ± 0.01	0.12 ±0.04	489 ± 63.2
0	5.0	20.0	23:1	7.65 ± 0.08	1.16 ±0.89	104 ± 10.1
0	10.0	20.0	14:1	7.04 ± 0.18	0.49 ±0.13	2317 ± 787.7

The results from the principal components analysis (PCA) are shown in Figure 2-1. In this figure, most data were grouped into three principal components. These three components explain 93.3 % of the variation in the data, with the first two dimensions (PC1 and PC2) accounting for most of the correlation of all measured variables (53.7 % and 26.3 %, respectively). In the first component, this analysis shows that the maximum laccase activity correlates with the highest values of yeast extract in the culture medium. On the other hand, high initial glucose concentrations positively correlated with C: N ratios and fungal biomass production. In the second component, the ammonium sulfate correlates with the highest undigested glucose left in the culture media, suggesting low fungal assimilation of glucose when ammonia sulfate is the nitrogen source. These results support that yeast extract is a more assimilable nitrogen source than ammonium sulfate for the evaluated culture media conditions. Furthermore, from this analysis, it is possible to infer that the organic nitrogen-sufficient conditions result in an increment of laccase production.

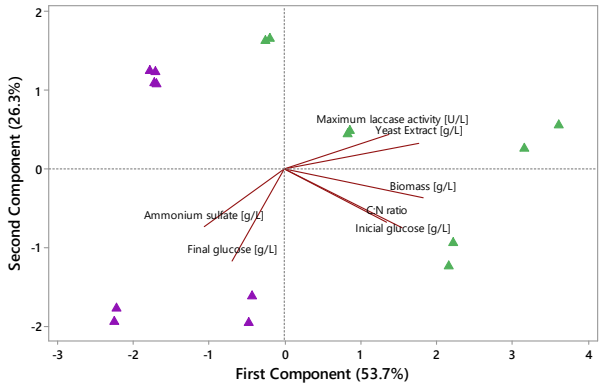


Figure 2-1. PCA of biological parameters of growth of *P. ostreatus* in culture media with ammonium sulfate or yeast extract in SmF. The purple triangles represent culture media with ammonium sulfate, and the green triangles media with yeast extract.

Sometimes nutrient-sufficient and nutrient-limited conditions in the culture medium can induce morphological changes in the hyphae of some filamentous fungi. Therefore, these morphological changes were explored and shown in Figure 2-2, which shows some representative pictures of fungal hyphae found in these cultures after seven days. Because *P. ostreatus* F515 is a dikaryon strain, clamp connections were searched on terminal hyphal pellets. Clamp connections in different formation states were found using ammonium sulfate (Figure 2-2, a-d) or yeast extract (Figure 2-2, e-h. The main differences observed in the fungal hyphae were the shape and frequency of vacuoles found in both apical and sub-apical cells. Interestingly, when yeast extract was used, the highest nitrogen-limited condition (Figure 2, g) produced fungal hyphae with the highest vacuolated pattern. Moreover (as shown in Table 2-3), this nutrient-limited condition showed the lowest laccase activity among yeast extract cultures.

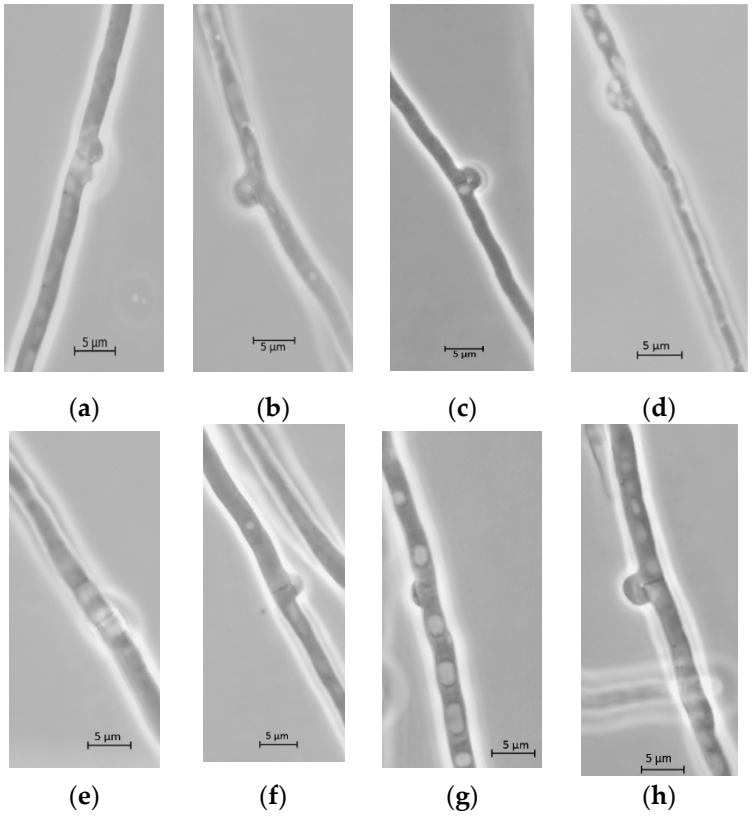


Figure 2-2. Clamp connection in terminal hyphae from *P. ostreatus* cultures at seven day of culture using different nutrient conditions represented as C:N ratio: (**a**) 1:1; (**b**) 2:1; (**c**) 22:1; (**d**) 2:1; (**e**) 5:1; (**f**) 14:1; (**g**) 23:1; and (**h**) 14:1.Nitrogen source used was ammonium sulfate (**a**-**d**) and yeast extract (**e**-**h**).

Although vacuoles were observed in the hyphae when ammonium sulfate was used, the vacuolated patterns are unclear. Fewer vacuoles occurred in the medium with the highest glucose concentration and the lowest ratio C:N (Figure 2-2, c), compared to those observed in a medium with the same glucose concentration and a higher C: N ratio (Figure 2-2, d). However, laccase activity (Table 2-3) in both conditions was similar.

Finally, when vacuolated pattern fungal hyphae from two yeast extract nutrient-sufficient conditions were compared to each other, the results suggest that nutrient-sufficient conditions, both for nitrogen and for glucose, are needed to increase laccase activity by *P. ostreatus*. Few vacuolated patterns in fungal hyphae were observed from the culture with the

nitrogen-sufficient conditions (Figure 2-2, e). This condition showed the highest laccase activity production per gram of biomass in the culture, while some vacuolated patterns were observed in fungal hyphae with the highest laccase activity grown in carbon-sufficient conditions (Figure 2-2, g).

2.3.2. Effect of glucose and yeast extract concentrations on laccase activity

Increased fungal biomass production is usually a sign of sufficient nutrient conditions when carbon and nitrogen sources are assimilable in the culture media in the presence of sufficient concentration of other growth factors. Therefore, different yeast extract and glucose concentrations were tested to find which culture medium composition maximizes *P. ostreatus* biomass production in SmF. The results from the central composite experimental design (CCD) are shown in Table 2-4. In all of these experiments, the measured maximum laccase activity was correlated with yeast extract and glucose concentrations to determine how this composition affected laccase activity. All CCD culture media were tested with 1 mM laccase-inducing copper sulfate, as well. These results are shown in Table 2-4 and Figure 2-3.

Table 2-4. Biomass and maximum laccase activity (MLA) in culture media of *P. ostreatus* with different concentrations of yeast extract and glucose from a central compound design (DCC).

Culture Name*	Factors		Biomass (gL^{-1})	Log10 MLA* (UL^{-1})	Biomass (gL^{-1})	Log10 MLA ** (UL^{-1})
	Glucose (gL^{-1})	Yeast Extract (gL^{-1})			Copper sulfate (Cu^{2+}) 1mM	
GY3008	30.0	7.50	9.25	2.69	10.4	3.82
GY6008	60.0	7.50	15.3	3.09	15.6	2.65
GY3023	30.0	22.5	11.2	2.76	9.71	4.22
GY6023	60.0	22.5	8.68	3.12	11.0	4.02
GY2415	23.8	15.0	12.2	3.18	10.9	4.17
GY6215	66.2	15.0	10.9	2.88	12.9	4.13
GY4504	45.0	4.39	5.29	1.99	6.13	2.22
GY4526	45.0	25.6	10.7	3.17	15.5	3.80
GY4515	45.0	15.0	16.6	3.11	22.4	4.50
GY4515	45.0	15.0	14.5	3.06	19.8	4.47
GY4515	45.0	15.0	16.9	3.12	19.4	4.48
GY4515	45.0	15.0	19.5	3.03	19.1	4.40
GY4515	45.0	15.0	16.5	2.99	19.0	4.41

* In the sample name G and Y stand for glucose and yeast-extract, respectively, while the first two digits refer to the initially added glucose and the last two digits refer to the initially added Yeast-extract.
** MLA refers to the maximum laccase activity to the 21st day of culture.

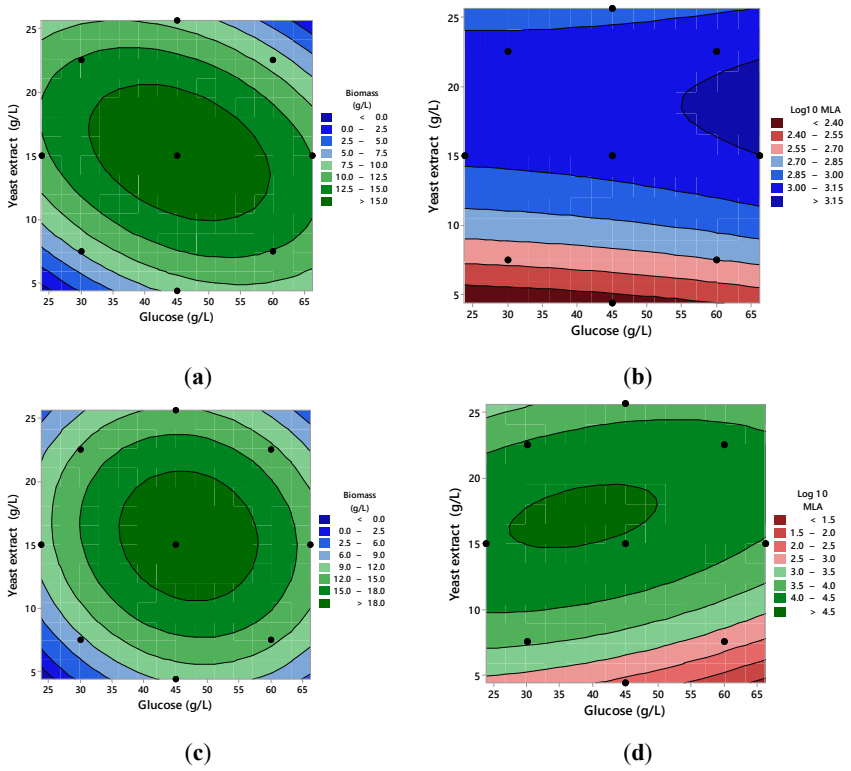


Figure 2-3. Contour plots of yeast extract and glucose concentration *vs*. (a) Biomass production in cultures without copper sulfate; (b) Maximum laccase activity (Log_{10} MLA) production in cultures without copper sulfate; (c) Biomass production in cultures with copper sulfate; (d) Maximum laccase activity (Log_{10} MLA) production in cultures with copper sulfate.

The CCD model fit to a quadratic model was adequate (p-value <0.05) to explain changes in the biomass production as a function of glucose and yeast extract concentrations with R^2 0.8231 and 0.8035 for fungal biomass production with and without copper sulfate, respectively. On the other hand, the laccase activity induced by copper sulfate was adequately explained by a quadratic model (p-value <0.05) with glucose and yeast extract concentration as varying factors (R^2 0.9560); however, in the absence of a laccase inducer (i.e., the copper salt) in the culture, the media composition was statistically insignificant (p-value >0.05) and insufficient (R^2 0.4039) to explain this response variable.

According to the CCD models, the contour plots (Figure 2-3, a, c) showed that biomass production was affected by the main effects squared both for glucose and yeast extract concentrations and both in the absence and presence of copper sulfate. The optimum point for biomass production contained 45 and 15 gL^{-1} glucose and yeast extract, respectively (experiment noted as GY4515) for cultures with and without copper. Optimum biomass production in the culture medium with copper was experimentally slightly better, and the model successfully predicted this result.

In the case of maximum laccase activity (Figure 2-3, d), the CCD model showed that this response variable was affected by the main effect of yeast extract concentration and the interaction between the glucose and yeast extract concentrations. The experimental maximum laccase activity from the culture media GY4515 was close to where the model predicted the maximum laccase activity. The laccase activities from GY4515 with copper sulfate were 1.4 to 1.5 logarithmic units greater than GY4515 without copper sulfate (Figure 2-3, b, d).

2.3.3. Characterization of the growth and laccase activity of P. ostreatus under nutrient-sufficient conditions with and without copper sulfate.

The GY4515 composition medium was selected to characterize the growth of *P. ostreatus* in the presence and absence of 1 mM copper sulfate. For this characterization, biomass production, glucose consumption, total protein, and laccase activity were measured at different culture times until day 21 (Figure 2-4). The fungal growth profiles in both cultures (Figure 2-4, a) showed two phases characteristic of diauxic growth. The maximum specific growth rates (μ_{max}) were 0.52 and 0.69 d^{-1} for the copper-free and copper sulfate-containing media. Although μ_{max} was 32% higher in the presence of copper, the total biomass produced at the end of the culture time (21 d) was similar in both systems (17 and 20 gL^{-1}, respectively) (Figure 2-4, a).

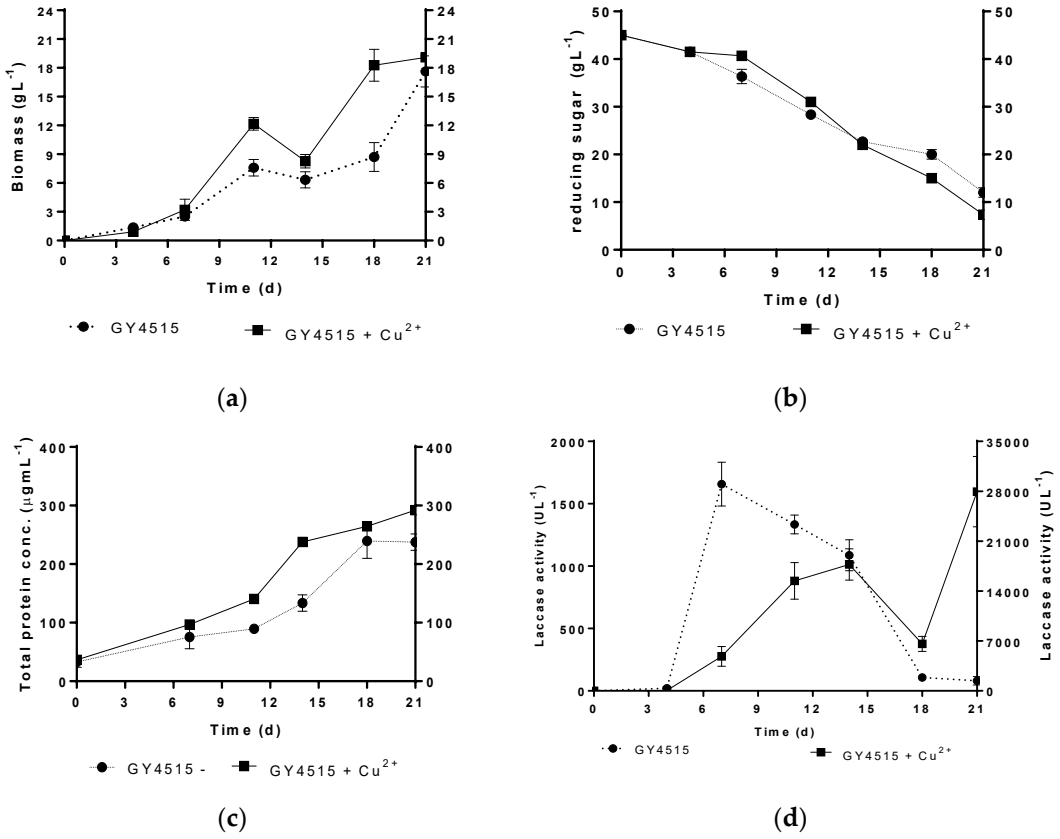


Figure 2-4. Growth profile of *P. ostreatus* in GY4515 culture media: (a) Biomass production; (b) glucose consumption; (c) total protein in culture extract; and (d) laccase activity. Black circle- discontinuous line (no copper sulfate), scale on the right. Black square -continuous line (copper sulfate 1mM), left scale.

The glucose consumption profile was similar in both systems. Glucose concentration decreased with the culture time to a final glucose concentration equivalent to 20% of the initial concentration (Figure 2-4, b). At the same time, the total protein concentration increased in both systems. In the medium with copper sulfate, the total protein was 20% higher than in the culture media without copper (Figure 2-4, c).

The most significant difference between both cultures was the laccase activity profile. While the highest laccase activity values in the medium without copper sulfate were detected before day 12th, the highest activity values occurred beyond this day in the media with the laccase inducer. At the end of the culture, the laccase activity decreased in the media without copper, while it tended to continue increasing when the inducer was present. A minimum 10-fold higher laccase activity was obtained by adding 1 mM copper sulfate to the culture media. These results suggest that the regulatory mechanisms of laccase activity are different in both systems.

2.3.4. Transcriptome analysis of P. ostreatus under nutrient-sufficient conditions with and without copper sulfate.

The top 10 more regulated and differentially expressed genes revealed by the analysis of the P. ostreatus transcriptome in the GY4515 culture medium with o without copper sulfate are shown in Figure 2-5 and Table 2-5. These results show that laccase gene transcripts *lacc10*, *lacc6*, and *lacc2* are among the top 10 differentially overexpressed transcripts with copper in the media. Furthermore, the transcripts of the small subunit of laccase POXA3a, two cupredoxin, the unknown gen similar to *pox2* gene, and a transcript with copper-binding domains showed a metabolism adapted to the presence of this compound. Interestingly, five gene transcripts of unknown function were among the 10 most overexpressed genes in the absence of copper sulfate. Of the five remaining transcripts identified, three suspected copper transporters made it to the top-10-list. Also, lipid transporters and the regulator of nitrogen metabolism, Nmr, were identified. Last but not least, these results showed that in the GY4515 medium, the yeast extract and the concentration used could induce the metabolic repression of nitrogen. This result is corroborated by the overexpression of a transcript with an NMR-like domain. These results suggest repression by nitrogen could be related to the regulation of copper transporters that, once present in the culture medium, facilitate the entry of this metal into the fungal cell and induce the transcription of genes *lacc2*, *lacc6,* and *lacc10*. Furthermore, the *P. ostreatus* transcriptome from the GY4515 culture medium with copper sulfate revealed that the *lacc2*, *lacc6*, and *lacc10* genes responded to the addition of this laccase inducer under nutrient-sufficient conditions.

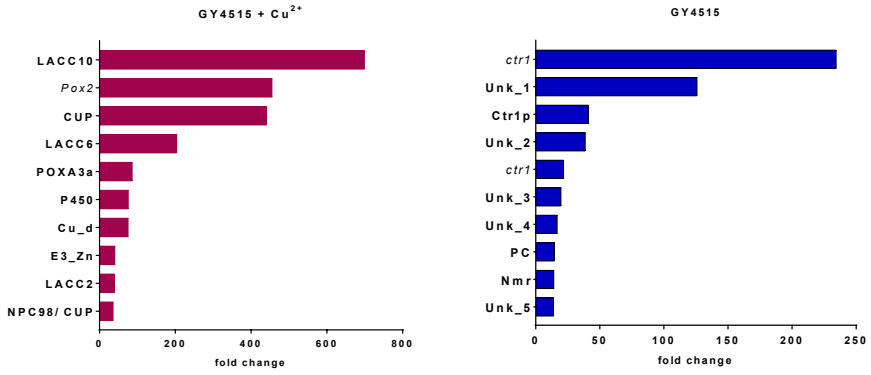


Figure 2-5. Differential gene expression (DGE) analysis of *P. ostreatus* in culture GY4515 vs. GY4515 + Cu^{2+}: (a) The top 10 of upregulated genes and (b) The top 10 of downregulated genes

Table 2-5. Transcript identification (Id.) and annotations for 10 DEG transcripts in *P. ostreatus* from cultures in GY4515 medium with or without copper sulfate.

JGI PC15 V2.0 Transcript Id.	JGI Annotations	Short name	JGI PC15 V2.0 Transcript Id.	JGI Annotations	Short name
GY4515 + Cu^{2+}			**GY4515**		
1089723	Multi-copper oxidase Laccase Lacc10 (PoxC)	Lacc10	1095975*	(unannotated gen) Similar to *Pleurotus* sp. 'Florida ctr1 gene for copper transporter, exons 1-3	*ctr1*
1105204*	(unannotated gen) Similar to Phenol oxidase (pox2) gene	*Pox2*	159791	Fungal uncharacterized conserved protein	Unk_1
1097654	Cupredoxin domin	Cup	1092022	Copper transporter, Ctr1p	Ctr1p
1113032	Multi-copper oxidase Laccase Lacc6 (PoxA1b)	Lacc6	1086646	*P. ostreatus* uncharacterized conserved protein	Unk_2
1067572	Small subunit of laccase PoxA3a	PoxA3a	1088435*	(unannotated gen) Similar to *Pleurotus* sp. 'Florida' ctr1 gene for copper transporter, exons 1-3	*ctr1*
1063469	Cytochrome P450 CYP2 subfamily	P450	1090041	*P. ostreatus* uncharacterized conserved protein 1	Unk_3
1087630	Blue (type 1) copper domain	Cu-d	1090781	No significant similarity found1	Unk_4
1105457	Predicted E3 ubiquitin ligase/Zinc finger, C3HC4 type	E3_Zn	1099858	Polyketide cyclase	PC
1067328	Multi-copper oxidases Lacc2	Lacc2	171939	NmrA-like family	Nmr
1062660	Nuclear pore complex, Nup98 component (sc Nup145/Nup100/Nup116) Cupredoxin	NPC98/Cup	1077411	*P. ostreatus* uncharacterized conserved protein1	Unk_5

*The Basic Local Alignment Search Tool (BLAST) was used to find local similarity between JGI sequences unannotated and The National Center for Biotechnology Information (NCBI) standards database sequence [105], [106].

2.3.5 Analysis of laccase gene transcripts

The relative quantification of the transcripts of the 12 laccase genes annotated in the *P. ostreatus* genome was carried out to determine the genes responsible for the activity in the GY4515 medium with and without the laccase inducer copper sulfate. In Figure 2-6, the results show that in the absence of copper sulfate, the *lacc2* gene transcript was the only one significantly up-regulated, while in the presence of this laccase inducer, the *lacc2*, *lacc6*, and lacc10 gene transcripts were the most highly expressed. To a lesser extent, the transcripts of *lacc3* and *lacc5* genes were also overexpressed in the presence of copper sulfate. The *lacc8* gene could not be amplified.

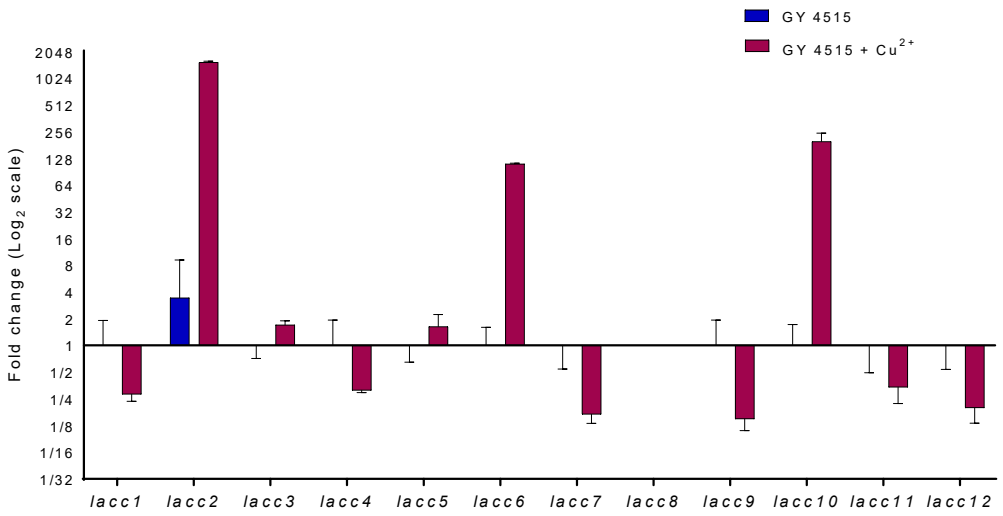


Figure 2-6. Relative quantification to the twelve P. *ostreatus* laccase genes in GY4515 medium, on day 12 of culture. Blue bar: GY4515 without copper sulfate. Red bar: GY4515 with copper sulfate, 1mM

2.3.6 Effect limited-nutrition conditions on laccase production

As copper-induced laccase activity depended on both glucose and yeast extract concentration, the effects of copper in nutrient-limited conditions were tested. For this, two cultures in nutrient-limited conditions, carbon- and nitrogen-limited conditions, were chosen as follows: The carbon-limited medium was glucose, 5.0 gL^{-1}, and yeast extract, 15 gL^{-1} (GY0515); the nitrogen-limited medium was glucose, 45 gL^{-1}, and yeast extract, 4.0 gL^{-1} (GY4504). The electrophoretic migration patterns of the native proteins of crude extracts of the GY4515, GY0515, and GY4504 media and laccase activity are shown in Figure 2-7 for both the 12th and 21st culture days in the absence and presence of copper.

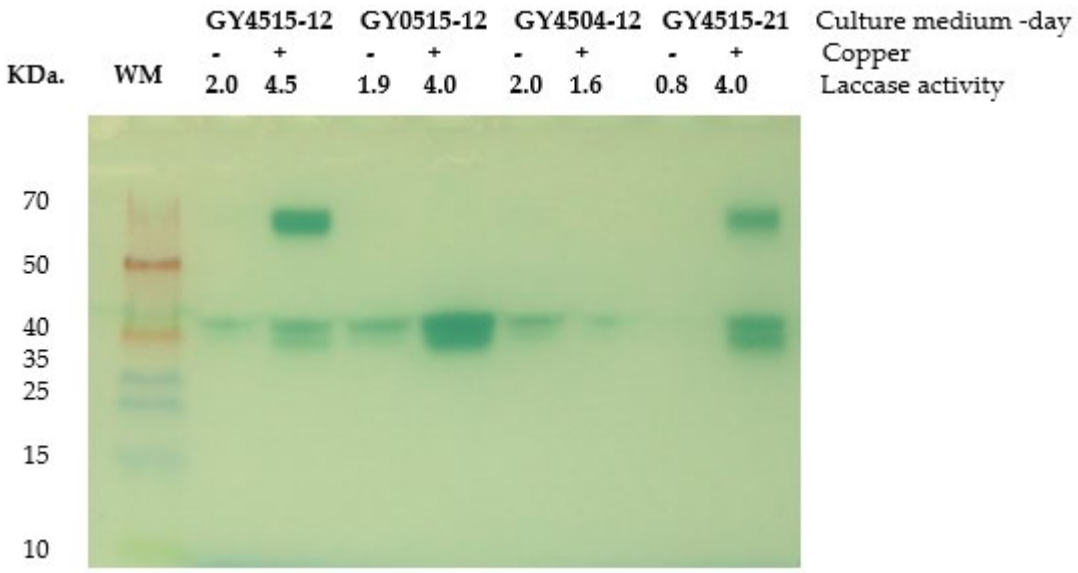


Figure 2-7. Zymogram of laccases in extracts crude in culture media with different nutrient conditions at 12th or 21st days of culture. Lane 1. Molecular weight marker (WM). Left to right. Lane 2-3 nutrient-sufficient condition GY4515 day 12 (GY4515). Lane 4-5 carbon-limited condition GY0515 day 12 (GY515). Lane 6-7 nitrogen-limited condition GY4504 day 12 (GY4504). Lane 8-9 nutrient-sufficient condition GY4515 day 21 (GY4515-21). Medium without copper (-), with copper (+). Laccase activity is presented as $\log_{10}$ (UL^{-1})

In the zymogram (Figure 2-7), the migration patterns and intensity of bands changed depending on the presence or absence of copper sulfate, the composition of the culture medium and the culture day. In the absence of copper sulfate, only two weak bands sized around 40 KDa are present, which coincide with the laccase activity for all these extracts cultures on day 12th of culture around two logarithmic units (Figure 2-7, lane 2, 4, and 6). The addition of copper sulfate to the nutrient-sufficient conditions medium, GY4515, revealed four bands, two at the size of 70 kDa, and other two at 40 kDa. The laccase activity in the extracts evaluated on days 12^{th} and 21^{st} were of more than four logarithmic units UL^{-1} (Figure 2-7, lane 3 and 9). In the nutrient-limited conditions medium, the pattern of bands was such that in the carbon-limited conditions medium (GY0515), where the laccase activity was around two logarithmic units higher in the copper sulfate medium than in the similar culture without copper, only two stronger intensity bands appeared at 40 kDa. In contrast, in the nitrogen-limited cultures GY4504, the addition of copper sulfate was insufficient to increase laccase activity. For these cultures, only one weak band appeared at 40 kDa.

In conclusion, the isoenzymes induced by copper are affected by different nutrient-conditions. In nitrogen-sufficient conditions, that are also carbon-limited, the expression of isoenzymes around 70KDa (Presumably lacc6) is negatively affected, while the isoenzymes Lacc2 and Lacc10 (around 40 KDa) are positively affected. All isoenzymes induced by copper were negatively affected in the inverse nutrients conditions (i.e., in carbon-sufficient conditions and nitrogen-limited). These results show an important effect of nitrogen-limited laccase activity induced by copper, suggesting that processes associated with organic nitrogen metabolisms influence copper uptake. In contrast, in nitrogen-sufficient that are simultaneously, the carbon-limited conditions, only some specific laccase isoenzymes induced by copper are affected. In these carbon-limited conditions, laccase activity is still influenced by copper because there is probably copper uptake.

2.3.7. Hyphal morphology of P. ostreatus under nutrient different condition with copper

In the absence of copper, the nitrogen-limited conditions culture produced evident vacuolated patterns in the hyphal morphology (Figure 2-2, g; section 2.3.1). In this section, the hyphal morphology was observed in three selected nutrient-conditions with copper, these were: nutrient-sufficient (GY4515), carbon-limited (GY0515), and nitrogen-limited (GY4504). The micrographs of the clamp connections in terminal fungal hyphae at seven days of culture are shown in Figure 2-8. The results showed that fungal hyphae from all nutrient conditions medium had clamp connections. Fungal hyphae in nutrient-sufficient condition, GY4515, had a no-vacuolated pattern. However, in both nutrient-limited conditions, some level of vacuolation was observed. In hyphae from the carbon-limited medium, GY0515, this level vacuolated pattern was barely observed, while it was clear in hyphae from the nitrogen-limited medium, GY4504. These results showed that nitrogen-limited conditions had a stronger relationship with the vacuolated patterns. Moreover, nitrogen-limited conditions affected laccase induction by copper. All these results again suggest a likely relationship between nitrogen metabolisms and copper uptake. In this relationship, *P. ostreatus* in initial nitrogen-limited conditions medium produce hyphal with vacuolated morphology. From the transcriptomic analysis results, these hyphal could have a lower expression level of copper transporters such as ctr1 than hyphal obtained in the nitrogen- sufficient condition. Consequently, copper uptake mechanisms are less prevalent in nitrogen-limited than in nitrogen-sufficient conditions, affecting laccase genes regulation by transcriptional mechanisms in the cis-acting elements promoter sequences as metal responsive elements (MRE) [56].

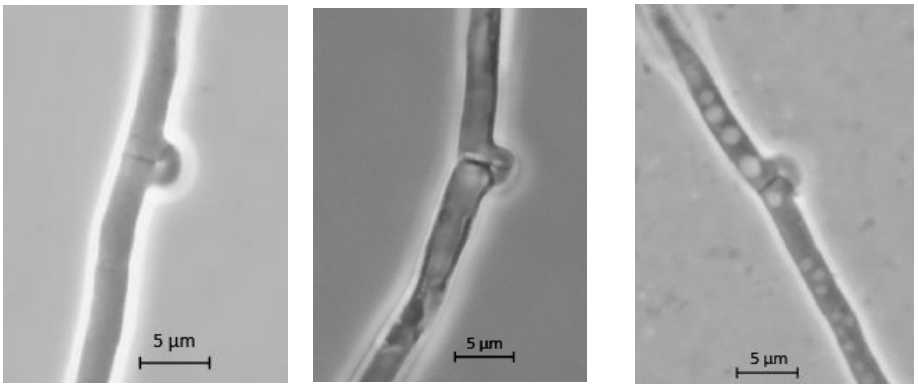


Figure 2-8. Clamps connections in the terminal hyphae of *P. ostreatus* in culture media with different nutrient conditions and copper sulfate 1mM at seven culture day: (a) nutrient-sufficient condition, GY4515 + Cu^{2+}; (b) carbon-limited condition, GY0515 + Cu^{2+}; (c) nitrogen-limited condition GY4504 + Cu^{2+}.

2.4. Discussion

In this study, the search for regulatory factors of laccase secretion by *P. ostreatus* was approached through selected the changes in the composition of the culture medium in submerged fermentation. This approach focused on achieving nutrient-sufficient conditions, only carbon-sufficient conditions, only nitrogen-sufficient conditions, and assessing laccase activity in the culture media in the absence or presence of laccase-inducing copper sulfate.

When the culture medium composition with sufficient carbon and sufficient nitrogen was found, profiles of fungal growth were compared, and laccases gene family transcriptional local and global analyses were performed through high-resolution techniques such as Real-time qPCR and RNA-seq. These comparisons showed the main change in *P. ostreatus* growth with and without copper was laccase activity by the copper-induction of three laccases genes, *lacc2*, *lacc6*, and *lacc10*. At the transcriptional level, fungal growth without copper overexpressed copper transporter of high affinity (*ctr1*) and the transcriptional factor, Nitrogen Metabolic Repression (*nmr*), a signal of nitrogen sufficient condition. Nitrogen-sufficient conditions could mainly regulate the *crt1* expression because in nitrogen-limited conditions, laccase inducing by copper sulfate was lower than carbon-limited conditions.

Laccase activity in *P. ostreatus* and other white-rot fungi has been frequently reported to be affected by nitrogen sources [107]. Therefore, we tested the effect of ammonium sulfate and yeast extract on laccase activity. Ammonium sulfate was insufficient to increase laccase activity at different nutrient concentrations (C: N ratio). Although in other studies, the increase of ammonium sulfate concentration increased laccase activity in the culture [108], the positive effect of a lower C: N ratio (nitrogen - sufficient conditions) seems to depend on the complex carbon source and the culture in solid-state fermentation (SSF) [109]. On the contrary, we found a positive effect of yeast extract on laccase activity. This positive effect was also reported in both SmF [110] and SSF [111].

Because we explored the fungal microscopic-morphological changes in terminal hyphae with clamps connections in culture media using ammonium sulfate or yeast extract, we observed interesting microscopic-morphological changes according to the sources of nitrogen and the C:N ratios. A large pattern of vacuoles was found using yeast extract. This behavior was studied in hyphae of *C. albicans*, which were cultivated in nutrient-rich media showing fewer vacuolated compartments than hyphae grown in low nitrogen media. Compared to that reported for this pleomorphic fungus, our results are similar[112]. The *P. ostreatus* hyphae grown in nutrient-rich media had fewer vacuolated compartments similar to those reported for *C. albicans*. Our study showed more vacuolated compartments grown in media with low nitrogen content in the culture media with higher C:N ratios. In another study, *P. ostreatus* was cultured in Potato-Dextrose-Yeast-extract (PDY). The pellets from the culture media showed a young mycelium barely vacuolated (at the 5th culture day); however, when glucose concentration decreased for the culture time, the old mycelium was vacuolated (at the 12th culture day) [72]. Therefore, those vacuolated patterns in young mycelium of *P. ostreatus* in SmF seem to signal nutrient-limitation in this fungus that could affect laccase activity.

Another sign of nutrient-sufficient conditions for carbon and nitrogen was tested, a culture medium whose composition increased biomass production using only glucose and yeast extract in the culture medium. At the same time, the effect of glucose and yeast extract concentrations on laccase activity in the absence and presence of 1 mM copper sulfate was also evaluated. The GY4515 medium was the composition closest to the maximum biomass production predicted by the statistical model. Surprisingly, the effect of the nutrient conditions in laccase activity was strongly dependent on copper sulfate in the culture medium.

Without copper sulfate, the glucose and yeast extract concentrations were insufficient to explain the changes in laccase activity in the different culture media evaluated in this study. However, increasing the glucose concentration from 5 to 20 gL^{-1} resulted in a more than five-fold increase in laccase activity. An additional increase of up to 40 gL^{-1} in glucose

concentration did not improve the laccase activity. On the contrary, lower activities were obtained [113]. In reports for another fungus, *Cerrena* sp., high carbon and nitrogen concentrations in the fermentation medium were beneficial for laccase production. In contrast, regardless of carbon concentration, low nitrogen concentrations drastically reduced laccase production [114]. However, the composition of the culture medium had little influence on laccase activity in *P. ostreatus* nutrient-sufficient conditions when the fungus grew in a culture medium with a lot of glucose 102.68 gL^{-1} and yeast extract 43.65 gL^{-1} concentrations, where the lignocellulosic biomass pretreatment and laccase activity improved the pretreatment system [115].

With copper sulfate, the changes in laccase activity in the different culture media were statistically explained by the glucose and yeast extract concentrations evaluated using a CCD. Here we only tested a fixed concentration of copper sulfate (1 mM) because this was the concentration that had the highest increase in laccase activity when different copper sulfate concentrations (0.5 to 5 mM) were tested in *P. ostreatus* in SmF [116]. In other studies in *P. ostreatus* and other fungi, the design of experiments (DOE) of response surface methodology was also used; however, copper sulfate and nutritional factors were tested at various concentrations[111], [113], [117]–[120]. Despite this, the main finding was similar to the finding in this study: nutritional factors, mainly organic nitrogen source and copper concentrations, showed an interaction effect. This interaction effect increased laccase activity considerably in the culture media. Therefore, evidence was collected to infer that fungal biomass in nutrient-sufficient conditions is more susceptible to laccases inducers than in nutrient-limited culture conditions. However, when the profile of *P. ostreatus* growth in GY4515 was characterized with and without copper, the main difference was laccase activity. Furthermore, the local transcriptional analysis of the laccase gene family showed that *lacc2*, *lacc6*, and *lacc10* were overexpressed in the culture with copper.

Copper-induced laccase isoforms enzymes were previously identified in SmF [121]. The most abundantly secreted with the highest migration pattern Lacc10 (PoxC), then Lacc2 (PoxA3) showed an intermediate migration pattern [61], and Lacc6 (PoxA1b) was the one that presented the lowest migration pattern [98]. However, the zymogram analysis in this study showed four laccase isoenzymes. The transcriptome analysis found *lacc2*, *lacc6*, *lacc10*, *pox2*, and *PoxA3a* among the top 10 overexpressed transcripts. This last transcript does not code for a laccase enzyme but for a protein that can form heterodimers with Lacc2 (PoxA3) [61], [122]. We do not know if any laccase heterodimeric was present in the analyzed enzymatic crude extract; however, four bands in the zymogram analysis suggest Lacc6, Lacc2, Lacc10, and another isoform.

It is noteworthy to observe how these isoforms changed in the nutrient-limited conditions in the culture medium. In carbon-limited conditions, the two bands at 70 KDa disappeared, while in nitrogen-limited conditions, two bands at 70 KDa and two bands at 40 KDa were drastically affected. These results may support the idea that nutrient-sufficient conditions confer the fungus more susceptibility to laccase inducers. A marker for nutrient-sufficient conditions was found on the *nmr1*-like transcript listed in the top ten transcripts in culture without copper. In these nutrient-sufficient conditions, three *ctr1* copper transporters were also found. This copper transporter has already been shown to negatively regulate CTR1 transcription in the dikaryon strain dkN001 and its monokaryon parents mkPC9 and mkPC15 of *P. ostreatus* [123].

2.5. Conclusions

This study describes a systematic analysis of the composition of the culture medium based on two nutritional factors, glucose as a carbon source and ammonium sulfate or yeast extract as nitrogen sources. This analysis determined what type of nitrogen source and what combination of glucose and yeast extract concentrations favored the production of fungal biomass and laccase activity induced by copper. The nutrient-sufficient conditions for both carbon and nitrogen in *P. ostreatus* cultures favored the expression of a transporters family of genes with a high affinity to copper: the CTRs, which were able to incorporate copper from the environment. This effect was evidenced by an increase in copper-dependent laccase activity by 20 fold, the presence of four laccase isoforms that were detectable in zymogram, and by an increase of three laccase transcripts *lacc2, lacc6* and *lacc10* determined by real-time qPCR and RNAseq.

Chapter 3
Role of copper-induced laccases and lignocellulose derived compounds on the rice husks modifications

ABSTRACT: In this study, rice husks were pretreated with enzymatic extracts produced by Pleurotus ostreatus in different culture media. Six media compositions were selected to produce the crude enzymatic extracts used for rice husks enzymatic treatments. This selection was done after establishing the change in laccase activity in 16 glucose, and yeast-extract (GY) based culture media supplemented with lignocellulose derived compounds (LDC) (such as carboxymethylcellulose (CMC), xylose, and lignin), and copper sulfate; using a complete factorial design aimed to determine the statistical significance of the controlled variable. The extracts produced in the selected culture media differed in laccase activity, total protein concentration, and isoenzymes of laccase found in the extract. The compositional characterization of the rice husks treated with these extracts showed a lignin loss between 24 and 37%. The enzymatic treatments also modified cellulose and hemicellulose fractions with removal percentages around 1 to 23% and 13 to 47%, respectively. Despite the decrease laccase activity and the induction of additional laccase isomorphs, in the extracts produced with a high concentration of lignin with copper, rice husks from this treatments showed the largest deslignification (37%), functional group modification, and microstructure differences which suggest that previous lignin metabolisms in the culture of *P. ostreatus* could produce molecules that improve delignification. An attenuation of bands associated with carbonyl functional groups and aromatic skeletal was evident in the FTRI spectra of the treatment of rice husks with lignin and copper, and a greater degree of degradation of the microstructure of the husks with this treatment was also observed (as seen through Scanning Electronic Microscopic).[2]

3.1 Introduction

The abundant (750 MT in 2019 worldwide [4]) and speeded production of the staple food rice, with the subsequent byproduct rice-husk (between 20 and 30% of the weight of paddle rice [3]) together with the composition and morphological characteristics of the latter establish this biomass as an attractive renewable feedstock for fuels and chemicals but a recalcitrant one [124].

The rice husks biomass is a lignocellulosic material, rich in cellulose, hemicellulose, lignin, ash, and other extractable substances, with varied compositions [7]. Cellulose content varies between 38-50%, hemicellulose between 23-32%, and lignin ranges between 15-25% [8]. Ash content in rice husks can reach around 20% of the total weight [8]. These ashes are characterized by being especially rich in silicon

[2] Full author list

Dinary Durán-Sequeda[1,2], Kimberly Lozano[1], Luis Cruz[1], Daniel Durán[1], Lucía Ramírez[2], Antonio G. Pisabrarro[2], and Rocío Sierra[1]

[1] Product and Process Design Group (GDPP), Department of Chemical and Food Engineering, Universidad de los Andes, Colombia; e-mail@e-mail.com

[2] Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre (UPNA), 31006 Pamplona, Navarre, Spain

dioxide or silica [7], also containing lesser amounts of other oxides such as aluminum, iron, magnesium, sodium, calcium, phosphorous, sulfur, and titanium [10]. The amount of nitrogen in the biomass is low; hence, the C:N ratio ranges between 80:1 to 200:2 depending on the variety of rice it originates from [10], [125].

In rice husks, the reducing sugars feedstock, such as hexoses, pentoses, or methyl pentoses, is the holocellulose fraction in lignocellulose [126], [127]. It is composed of cellulose and hemicellulose polymers. Chemically, cellulose is formed by D-glucose residues linked by β-1─4-glycosidic bonds that form linear polymeric chains of up to 10,000 glucose residues [128]. It contains highly crystalline regions joined by individual chains and other less organized amorphous regions [128]. Hemicelluloses are polymeric carbohydrates based on xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-xylose and D- mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose, and D-mannose), and arabinogalactan (heteropolymer of D-galactose and arabinose), building blocks [18]. Both polymers cellulose and hemicellulose could be up to 50% of the weight of rice husks. Despite this significant richness of sugar content, the polymerization degree, crystallization, and interaction of these polymers confer resistance to biodegradation of this lignocellulosic biomass.

Rice husks biomass is additionally hard to degrade due to the constituent polymer lignin. It is a recalcitrant heteropolymer that limits the access of microorganisms to cellulose and hemicellulose in plant cell walls. Lignin gives rigidity and resistance to the structure of the plant [129]. Chemically, lignin has three monolignol monomers with diverse degrees of methoxylation: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These lignols are incorporated with varied frequency and order in lignin as units of phenylpropanoid p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively [40]. Lignin is formed by the ether bonds between carbon-carbon (C-C) in the structural units of H, G, and S, which include bonds type β-O-4, β-5, β-β, 5-5, 4-O-5, and β-1, producing a heterogeneous polymer in which the abundance of one type of structural unit relates to the high, medium and low wood-like nature of the plant material [40].

Rice husk morphological structure has several levels of organization and features. At the macrostructure level, the rice husks can be palea leaves of the spikelet covering the ventral part of the seed or lemma leaves of the spikelet covering the dorsal portion of the seed [130]. Either lemma or palea husks have identifiable upper and lower parts. The upper part is a bristle-like structure called the awn, and the lower part is a short stem called the rachilla [131]. The microstructure is more complex and similar to rice leaves microstructure. The lemma and palea tissue organization consists of the outer epidermis, fiber layers, vascular bundles, parenchymal cells, and the inner epidermis [130]. In the outer layer of the epidermis, epidermal cells are arranged in linear profiles alternating with and without ridges [130]. This cellular arrangement creates a highly striated mosaic, where the undulations and spaces are covered by a layer of silica and a cuticle [132]. Trichomes, hair-like or pubescent, also emerge from the epidermis [133]. The outer epidermal walls are extremely thick, highly convoluted, and lignified [130]. In the intermedia layers, the fiber layers are thick-walled and lignified in the hypodermis, whereas, in the sub-hypodermis, the parenchyma cells are thin-walled un-lignified [130], [131]. The last layer, inner epidermal cells, are not also lignified [130].

Decreasing lignin recalcitrance in the rice husks to release the holocellulose fraction is the main challenge of pretreatment. The pretreatment techniques have been classified in physical, chemical, or biological treatments [18]; however, seldom only one pretreatment is applied, and more often, a combination of pretreatment methods of different nature is necessary [134]. For example, reducing size through milling is usually performed for rice husk physical pretreatment, and this practice is used more than all other pretreatments [135]. As for chemical pretreatments, acid hydrolysis has been evaluated and performed before to enzymatic hydrolysis of rice husks [8], [15], [136]. A combination of biological pretreatment with enzymatic hydrolysis has also been performed [137]. One of the advantages of biological pretreatment is that it reduces operating costs associated with processing, washing, and eliminating inhibitors in biomass during chemical pretreatment [126].

Pleurotus ostreatus is a white-rot fungus that degrades lignin and hemicellulose, preferably over cellulose in rice straw [22], [23]. The breaking down of lignin by *P. ostreatus* is attributed to a system of lignin-modifying enzymes (LMEs) composed of oxidases, laccases, manganese peroxidases (MnP), and versatile peroxidases (VP); however, no members of the lignin peroxidase (LiP) family were found in the genome of this fungus [30]. Nevertheless, the abundance of laccases in the secretome of *P. ostreatus* suggests that laccases could be the LMEs that play the central role in this process [30].

This study compares compositional, chemical, and morphological modifications obtained of rice husks after different enzymatic pretreatments from crude extracts obtained from submerged fermentation cultures of *P. ostreatus*. These cultures were carried out using a base composition of glucose and yeast extract. The first objective was to evaluate the effect of adding different concentrations of the following compounds derived from lignocellulose: CMX, xylose, and lignin, with and without copper sulfate inducer to determine which of these compounds or their interactions increased laccase activity and to have crude enzymatic extract with high rich in laccases activity. In a pretreatment context, we hypothesize that higher enzymatic activity of laccase could favor a more significant loss of lignin in the husks or changes in their chemical or morphological structure. Hence, we selected six extracts that differed in laccase activity, total protein concentration, and laccase isozymes to pretreat rice husks. The lignocellulose compositional analysis of functional groups associated with lignocellulose and microstructure of rice husks treated had differences with husks without treatment and between treatments. From the analysis of results, a strategy to produce enzymatic cocktails for the pretreatment of lignocellulosic biomass was proposed.

3.2 Methods

3.2.1 Fungal strain and culture conditions

The *P. ostreatus* strain ANDES-F515, provided by the Laboratory of Mycology and Phytopathology of the Universidad de Los Andes-LAMFU and deposited in the ANDES Natural History Museum (MHN ANDES), was isolated in the Bosque de la Merced, Santa Bárbara village, Bojacá, Cundinamarca, Colombia. The dikaryotic

mycelium was maintained and conserved on Malt Extract Agar at 4 °C, with periodic replication of the growth zone after eight days of incubation at 25 °C in the dark.

The submerged (SmF) cultures were performed in 250 mL flasks containing 100 mL of culture medium composed of glucose 40 gL^{-1} and yeast extract 20 gL^{-1}. A full factorial experimental design with two levels and four factors (2^4) was used to test the effect of each individual and interaction between lignocellulose derived compounds (LDCs): lignin alkali (Sigma-Aldrich), carboxymethylcellulose sodium (CMC, Sigma-Aldrich), xylose (Sigma-Aldrich), and copper sulfate at two levels of concentration as shown in Table 3-1. All SmF cultures were inoculated with five 4 mm diameter plugs taken from the growth zone of eight-day Malt Agar plate cultures and incubated at 25°C, 150 rpm, in the dark for 21 days. All experiments were performed in duplicate.

Table 3-1. 2^4 full factorial design

Factor	Low level (gL^{-1})	High level (gL^{-1})
CMC	0.5	5.0
Xylose	0.5	5.0
Lignin	0.0	5.0
$CuSO_4 \cdot 5H_2O$	0.0	0.25

3.2.2 Biomass determination

Fungal biomass production was gravimetrically determined on the 21st day of culture. The culture was filtered through a paper filter (previously dried and weighed) using a vacuum filter system, and the filtered mycelium was dried at 45°C for 72 h. The filter paper with the dried biomass was weighed again [73].

3.2.3 Total protein concentration

Bradford protein assays (Bio-Rad) were used for total protein quantification. Standards and samples were mixed with Coomassie Blue Assay Reagent G-250 according to the manufacturing instructions. Each reaction was measured at 595 nm [97].

3.2.3. Laccase activity

Laccase activity was determined with ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) as the substrate [95]. The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 5.0), and 10 µL aliquots of an appropriately diluted enzyme sample. Oxidation of ABTS was monitored by following the increase in *A*436 (ε 29.3 $mM^{-1}cm^{-1}$). A laccase activity unit was defined as the enzyme required to oxidize 1 µmol ABTS per minute at 25 °C.

3.2.4 Zymograms

Non-denaturing electrophoresis conditions (native-PAGE) were used to visualize the isoenzymes present in the experimental samples [98]. The stacking and running gels contained 4% and 9% acrylamide, respectively, and were adjusted to pH

6.8 and 8.8 with Tris-HCl buffer. The running buffer was Tris-Glycine pH 8.3. The isozymes were revealed by staining the gel after running with 2mM of ABTS solution.

3.2.5 Enzymatic treatment of rice husks

For enzymatic treatments of the rice husks, mixtures of 5% (w/v) of rice husks and six different crude extracts enzyme-containing were evaluated. Around 100 mL of the extracts were obtained by biomass separation in the culture medium by filtration of a sample obtained at 15th culture day produced in 500 mL flasks. Previously, rice husks were ground to a particle size of 1 mm, sterilized, and dried at 45 °C for two days. The same treatment was carried out on non-ground rice husks for morphological analysis using scanning electron microscopy (SEM). The mixture was then incubated at 25° C on a rotary shaker at 150 rpm for 72 hours. When the reaction time ended, the aqueous extract and the solid fraction were again separated by filtration. For compositional, chemical, and morphological analyses, the treated rice husks were washed and dried at 45 °C.

3.2.5 Rice husks compositional analysis

After enzymatic treatment, the concentrations of lignin and constitutive sugars (glucose and xylose) in rice husks were determined following the NREL/TP510-42618 protocol [138]. This protocol consisted of biomass separation into the constituting polymers using an aqueous acid phase (H_2SO_4, 72 % w/w) where each of the rice husks samples was hydrolyzed. For this, 300.0±10.0 mg of the rice husks sample were mixed with the acid solution for 1h at 30°C. Next, each sample was diluted by adding 84.00±0.04 mL of deionized water, placed in a 250 mL Schott flask, and put in an autoclave for 1h at 121° C. After the concentrated and diluted hydrolysis, the holocellulose found in the liquid phase was quantified using HPLC fitted with a column for sugars AMINEX HPX-87H (Bio-RAD), with a refractive index detector. The lignin in the solid phase was quantified gravimetrically.

3.2.6 Fourier Transform Infrared Spectrometry (FTIR)

Infrared spectra were recorded with a Nicole 380 FT-IR Spectrometer (Thermo Scientific). The samples were mixed with KBr and pressed as tablets. The absorption spectra were recorded in the region from 4000 to 400 cm^{-1}. OriginPro8 software was used to analyze the results.

3.2.7 Scanning Electron Microscope (SEM)

Non-ground, treated, and untreated rice husks samples were loaded on the carbon belt surface and covered with gold. The metallic samples were observed in a Phenom ProX Desktop scanning electron microscope (SEM) under high-pressure conditions at 15kv.

3.2.8 Statistics analysis

Minitab® version 18 software was used to construct the statistical design, evaluate statistical significance, obtain the regression models, and find the simultaneous local optimum of one or more response variables.

3.3. Results

3.3.1 Characterization of the effect of lignocellulose-derived compounds and their interaction with copper sulfate on the laccase activity of P. ostreatus.

The effect of Lignocellulose Derived Compounds (LDCs) and their interactions with copper sulfate on the production of fungal biomass and laccase activity were evaluated according to the factorial design presented in Table 3.2. This table shows that both biomass production and laccase activity were affected by LDCs and copper in the cultures. On the one hand, copper positively affected biomass production, while high concentrations of LDCs reduced its production. This effect was statistically significant (P-value <0.05) and was fitted to a linear model (R^2 0.9445) in which the interactions between LDCs and copper also affected biomass production. Generally speaking, the average biomass production was around 13 gL^{-1}; this amount increased to an average of 16 gL^{-1} by adding copper sulfate in the cultures and decreased to around 11.1 gL^{-1} by adding 5gL^{-1} of either LDCs evaluated in the cultures without copper inducer. Furthermore, some interactions between LDCs and copper affected the average biomass production by about 50%. Accounting for all samples, maximum biomass production in some culture media was around 20 gL^{-1}, while the minimum was close to 7 gL^{-1}.

Table 3-2. 2^4 full factorial design culture media composition, and variable response biomass and maximum laccase activity

	Culture media or extracts name*	Factors levels				Variable response		
		$CuSO_4$ (Cu^{2+}) (gL^{-1})	Lignocellulose Derived Compounds (LDCs)			Biomass (gL^{-1})	Maximum laccase activity, MLA (UL^{-1})	Log_{10} MLA
			Lignin (L) (gL^{-1})	CMC (C) (gL^{-1})	Xylose (X) (gL^{-1})			
1	LCX + Cu^{2+}	0.25	5.00	5.00	5.00	6.50 ± 0.70	4818 ± 471.4	3.68
2	LCX	0.00	5.00	5.00	5.00	8.62 ± 4.12	1479 ± 161.0	3.17
3	CX + Cu^{2+}	0.25	0.00	5.00	5.00	20.5 ± 0.71	11236 ± 414.50	4.05
4	CX	0.00	0.00	5.00	5.00	6.70 ± 0.05	4251 ± 493.3	3.63
5	LX + Cu^{2+}	0.25	5.00	0.50	5.00	13.4 ± 1.84	3168 ± 649.6	3.50
6	LX	0.00	5.00	0.50	5.00	13.9 ± 0.90	1645 ± 3.803	3.22
7	X + Cu^{2+}	0.25	0.00	0.50	5.00	20.0 ± 2.08	10567 ± 996.63	4.02
8	X	0.00	0.00	0.50	5.00	10.1 ± 0.17	3729 ± 71.81	3.57
9	LC + Cu^{2+}	0.25	5.00	5.00	0.50	10.6 ± 0.42	2814 ± 275.2	3.45
10	LC	0.00	5.00	5.00	0.50	19.8 ± 0.32	2452 ± 75.03	3.39
11	C + Cu^{2+}	0.25	0.00	5.00	0.50	19.5 ± 0.70	11178 ± 25.450	4.05
12	C	0.00	0.00	5.00	0.50	6.90 ± 0.14	4167 ± 50.02	3.62
13	L + Cu^{2+}	0.25	5.00	0.50	0.50	16.2 ± 1.29	3086 ± 240.4	3.49
14	L	0.00	5.00	0.50	0.50	12.5 ± 3.54	1614 ± 412.9	3.20
15	GY+Cu^{2+} ***	0.25	0.00	0.50	0.50	15.2 ± 1.17	8200± 153.4	3.91
16	GY**	0.00	0.00	0.50	0.50	10.9 ± 0.07	2080 ± 118.0	3.32
Average						13.2 ±5.00	4781 ± 3441	3.58

*All culture media contain glucose and yeast extract. The initial letter in the name of the culture medium represents the high level of the factor or LDC.

**The low levels of the factors are not included in the name of the medium; the medium with all low factors was named GY.

***The medium with all low factor LDCs was named GY + Cu2+

The maximum laccase activities achieved in each treatment with and without a logarithmic scale are shown in Table 3-2. Statistical analysis of these results showed that laccase activity was significantly affected (p-value <0.05) by copper sulfate and LDC concentrations and their interactions in the culture medium. A linear regression fit model was obtained (R^2 0.9934). In this case, the main effect of the factors on the laccase activity varied according to the compound evaluated. On the one hand, the high concentrations of copper and lignin had an opposite effect on this enzymatic activity. The high concentration of copper increases the laccase activity, while the high concentration of lignin decreases the laccase activity. For 0.25 gL^{-1} of copper concentration, the average laccase activity increased from 5000 UL^{-1} to 7000 UL^{-1}, whereas this value decreased to 2500 UL^{-1} upon adding 5gL^{-1} lignin. On the other hand, the effect of LDC: CMC and xylose on laccase activity were similar; the high concentrations of both LDCs increased laccase activity by similar magnitudes up to around 5500 UL^{-1}. Therefore, the interactions between one or more factors also had different effects on laccase activity. Thus, the interactions in which higher laccase activity was obtained used high concentrations of copper with CMX or Xylose or both LDCs; meanwhile, those with less laccase activity occurred without and using simultaneously high concentrations of all the LDCs evaluated and in all cultures where lignin was present. All these interactions also can be seen in Figure 3-1.

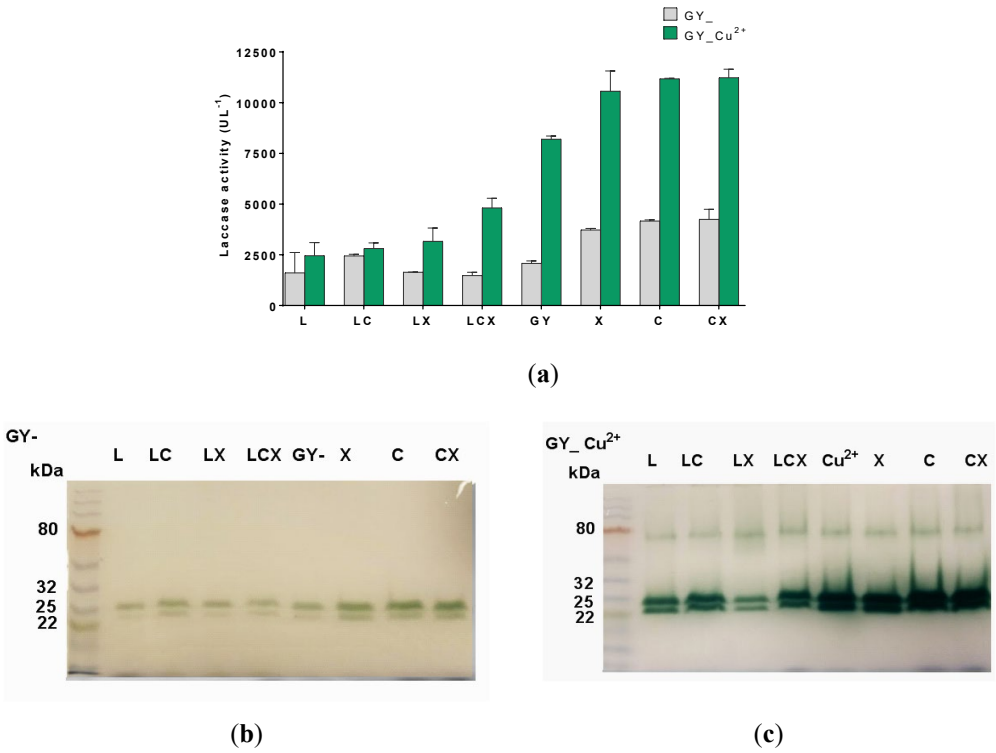
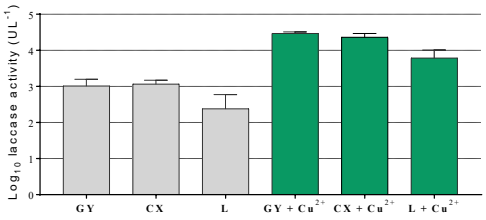


Figure 3-1. Laccase activity and laccase isoenzyme in crude enzymatic extract produce with LDC and copper sulfate: (**a**) Laccase activity in GY culture media without copper sulfate (gray) and GY_Cu^{2+} with copper sulfate (green) (**b**) Zymogram of crude enzymatic extract in GY culture media (**c**) Zymogram of crude enzymatic extract in GY+Cu^{2+} culture media.

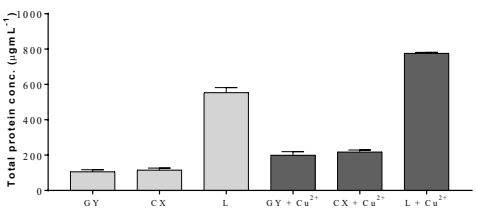
Figure 3-1 shows laccase activity and laccase isozymes in the crude enzymatic extracts with LDC in the absence or presence of copper sulfate. The results showed that

the highest values of laccase activity were obtained in some extracts with copper sulfate, while lignin was in the extracts with lower laccase activity even with copper sulfate (Figure 3-1, a). Two laccase isozymes were observed in all extracts lacking copper sulfate (Figure 3-1, b). These isoenzymes had bands between 32 and 25 kDa. The intensity of these bands varied according to the combination of LDC in the culture medium. The extract with a high lignin concentration (L) had weaker bands than other extracts in the zymogram. In contrast, a high concentration of CMC (C), xylose (X), or both LDCs (CX) extracts had bands with greater intensity in the zymogram. Three isoenzymes were observed in the enzymatic extracts containing copper sulfate (Figure 3-1, c). Two of them were similar to those found in the copper-free extract and ran between 32 and 25 kDa. The third copper-induced isoenzyme was larger and ran about 80 KDa. In this zymogram, the bands with the lowest intensity were obtained in combination with a high concentration of lignin and xylose (LX).

Six culture media compositions were chosen because they either produced poor or laccase-rich enzymatic extracts. The biochemical characterization of these extracts is shown in Figure 3-2. In this characterization, the laccase activity results (Figure 3-2, a) showed a 1.4-unit log difference favoring cultures with copper over the ones without it. This difference means that the laccase activity in the enzymatic extracts with copper sulfate was higher by orders of magnitude than in the enzymatic extracts without copper. Interestingly, adding lignin in the cultures increased the total protein concentration in the extracts more than adding copper sulfate alone (Figure 3-2, b). Thus, the total protein concentration was highest in both extracts with lignin (L and L + Cu^{2+}) than in those from cultures lignin-free. In these last extracts, the effect of CMC and xylose in the total protein concentration was dependent on copper. The total protein concentrations were less than 200 μgmL^{-1} in the extracts from cultures without copper (GY and CX) than in the same extracts of cultures with copper (GY + Cu^{2+} and CX + Cu^{2+}), where the total protein concentrations were almost 400 μgmL^{-1}. Finally, the native electrophoretic profile characterization (Figure 3-2, c) showed a new laccase isozyme, running around 40 and 50 kDa on the zymogram; it was observed in extracts with a high lignin concentration with or without copper. It was observed that this isozyme appeared later than the other laccase isozymes. Furthermore, in these enzyme extracts, the electrophoretic profile of the total native protein was diffuse in both conditions with and without copper sulfate. In contrast, other proteins appeared to be more abundant than laccase proteins in the GY and CX extracts (Figure 3-2, d). In summary, these results showed that the enzymatic extracts produced had differences in laccase activity, laccase isozymes, and total protein concentration.



(**a**)



(**b**)

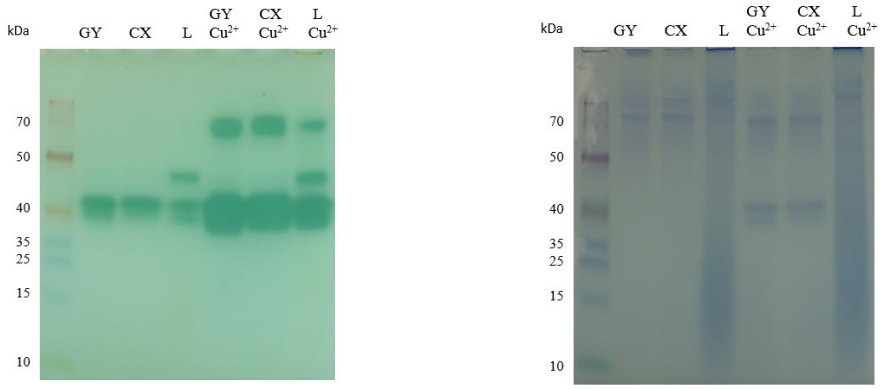


Figure 3-2. Enzymatic crude extracts characterization: (**a**) Laccase activity; (**b**) Total protein; (**c**) zymogram; (**d**) total native protein PAGE

3.3.2 Characterization of rice husks pretreated with enzymatic extracts

In this section, the objective was to evaluate the relationship between different laccase activities and compositional, chemical, or morphological changes of rice husks pretreated with crude enzymatic extracts from cultures where laccase production was stimulated with different compounds. Being aware that the enzymatic composition in the extracts could be different in other lignocellulosic enzymes; this study focused on laccase.

The raw rice husks composition and lignocellulose compositional loss after pretreatment of the husks with the enzyme extracts produced in culture media of *P. ostreatus* with different LDCs and copper are shown in Table 3-3. These results indicated that all rice husks treated with enzymatic extract had lignocellulose composition changes in all fractions. According to these results, a more significant loss of lignin fraction percentage (36.6 ± 2.07 %) occurred with the L+Cu^{2+} enzymatic extract treatment, which means a higher rate of delignification in this treatment condition; however, pretreatment with other extracts was also successfully achieved a rice husk delignification from 24 to 29%. Also, these results suggest that these lignin losses were more dependent on the addition of LDCs to produce the extracts than the laccase activity induced by copper in the extract and showed that lignin-derived metabolites from the presence of lignin in the extract (with a possible role of laccase-mediator) improve the action of laccases induced by copper.

Table 3-3. Rice husks lignocellulose composition and lignocellulose compositional loss

	Extract name	Log_{10} (Laccase activity, UL^{-1})	Lignin loss % (w/w)	Cellulose loss % (w/w)	Hemicellulose loss% (w/w)
	Raw rice husks (RH)	NA	31.2 ± 1.81 *	30.8 ± 1.68 *	13.4 ± 1.19 *
Treatment**	RH + GY	3.01	25.0 ± 5.04 (b)	23.0 ± 3.38 (a)	47.05± 2.62 (a)
	RH + GY + Cu^{2+}	4.47	23.5 ± 6.09 (b)	22.6 ± 5.47 (a)	43.6 ± 2.04 (a)
	RH + CX	3.07	29.4 ± 2.38 (a, b)	16.3 ± 5.51 (a ,b)	36.2 ± 1.64 (c)
	RH + CX + Cu^{2+}	4.36	29.3 ± 2.08 (a ,b)	12.74 ± 3.71 (b)	39.0 ± 1.61 (b, c,)
	RH + L	2.38	24.3 ± 3.95 (b)	19.5 ± 2.10 (a, b)	42.3 ± 0.91 (a, b)
	RH + L + Cu^{2+}	3.79	36.6 ± 2.07 (a)	1.52 ± 1.37 (c)	13.3 ± 3.87 (d)

* Content measured in raw rice husks (compositional percentage). ** Indicate compositional loss referred to raw rice husks.

a,b,c,d Significantly different groups as determined statistically through Tukey-test (P-value <0.05)

The treated rice husks had significant losses of cellulose and hemicellulose. On the one hand, in rice husks treated with enzymatic extracts without and with copper, a significant loss of cellulose, around 13 to 20%, was observed compared to untreated rice husk. This cellulose loss seemed independent of laccase activity in the enzymatic extracts without lignin and suggested possible cellulases activities in those extracts; however, cellulose loss in rice husk treated with $L+Cu^{2+}$ enzymatic extract was the lowest, meaning that in this last extract, there were fewer cellulases enzymes, low cellulases activity or presence of cellulases-inhibitors. On the other hand, hemicellulose composition showed a more significant loss around 13 to 47% (see Table 3-3), suggesting rich xylanase activity in all treatments. Nevertheless, the rice husks treated with $L+Cu^{2+}$ enzymatic extract were the lowest hemicellulose loss showing a possible presence of xylanase inhibitor of this treatment again.

FTIR analyses were performed to explore features and chemical components/bonds in rice husks treated and untreated with an enzymatic extract from cultures of *P. ostreatus* supplemented with different LDCs with and without copper as laccase inducers. Figure 3-3 shows rice husk untreated -FTIR spectrum, and Table 3-4 shows all wavenumbers identified in this spectrum, their functional group, and the possible lignocellulose compound-related.

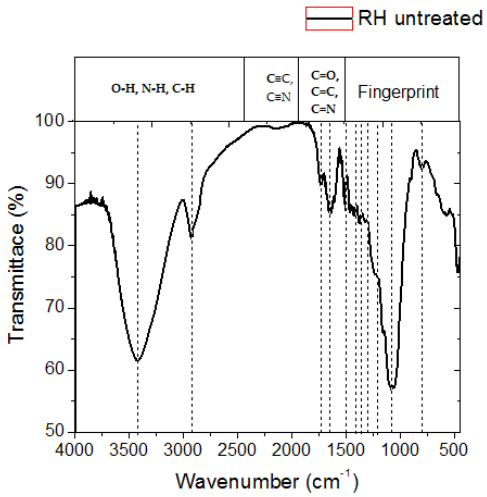


Figure 3-3. Rice husk untreated (RH) -FTIR spectrum. (i) the single bond region (4000-2500 cm-1), (ii) the triple bond region (2500-2000 cm-1), (iii) the double bond region (2000-1500 cm-1), and (iv) the fingerprint region (1500-500 cm-1) [139].

In Figure 3-3 the wavenumbers at four absorption regions are indicated in the RH-FTIR spectrum. These regions are: (i) the single bond region (4000-2500 cm^{-1}), (ii) the triple bond region (2500-2000 cm^{-1}), (iii) the double bond region (2000-1500 cm^{-1}), and (iv) the fingerprint region (1500-500 cm^{-1}) [139]. The RH- FTIR spectrum shows several adsorption bands, two in the first region at 3400 and 2900 cm^{-1} due to O-H and C-H vibrations. In the second region, there were no bands detected. On the third region, the double bond vibrations at least five bands were present between wavenumbers 1740 and 1512 cm^{-1} which are mainly assigned to C=O stretching in hemicelluloses and C=C stretching by aromatic skeletal. In the last region, the fingerprint, the spectrum showed at least eight bands, five between wavenumbers 1460 and 1160 cm^{-1} with the assignment to CH, CH_2, CH_3 bending or C-O stretching, and three with the assignment to silicon at wavenumbers around 1077, 900, and 800 cm^{-1}. (Detail are shown in Table 3-4).

Table 3-4. FTIR bands assignments for the rice husks lignocellulose compounds [140]–[145]

Wavenumber cm^{-1}	Vibration	Functional group or compound	Possible Rice husks – lignocellulosic compounds
3419	-O-H stretching	Water, alcohols, and phenols	Cellulose, hemicellulose, and lignin
2922	C–H stretching	Alkyl, aliphatic, aromatic	Cellulose, hemicellulose, and lignin
1738-1725	C=O stretching	Ketones, aldehydes, and carboxylic acid	Hemicellulose, lignin
1660-1630	C=O stretching	Carbohydrates	Cellulose and Hemicellulose
	Absorbed O–H	water	
	C=C stretching		Water associated with cellulose and lignin
1610	C=C stretching	Aromatic skeletal	Cellulose, hemicellulose, and lignin
	COOH stretching		
1580-1530	C=C stretching	Aromatic skeletal	Cellulose and lignin
1512-1505	C=C stretching	Aromatic skeletal	Lignin
1458-1420	CH bending	OCH_3 (methoxyl) or	Lignin
	CH_2 bending	CH_2 pyran ring	Cellulose or hemicellulose
1377-1375	CH_3 bending	Polysaccharides and lignin	Cellulose or hemicellulose
			Lignin
1322	CH bending or	Cellulose	Cellulose Lignin
	C-O stretching	Syringyl ring	
1230	C–O stretching	syringyl ring	Lignin
1160	C–O stretching	Polysaccharides	Cellulose
			Hemicellulose
1077-1052	Si-O-Si stretching	Organosilicate	Silica
898	C–H deformation	β-pyranose compounds	Cellulose
	Si-H		Silica
800	Si-H	Silica	Silica

As Table 3-4 shows, in the lignocellulose material as rice husks, some of these bands could be due to the vibration of the same functional group in the different lignocellulose polymers at the exact wavenumber, but this is hard to elucidate in the same spectrum using this technique; however, when there is some significant compositional change in the same material by a treatment, these vibrations functional groups could be associated with this change. In Figure 3-4, overlapping of untreated and treated RH-FTIR spectra are shown. The spectra are very close at wavenumbers between 4000 and 1750 cm^{-1}, showing large conservation on the single bond and triple bond regions after the enzymatic treatments. Most areas with little differences in the spectra are at wavenumbers between 1750 cm^{-1} and 500, including the double bond and fingerprint regions. These slight variations in these regions seem associated with the copper in the GY and CX extracts. Moreover, in the spectra from rice husks treated with the L- extracts, the enzymatic treatment seems more affected at wavenumbers around 1077, 900, and 800 cm^{-1}, bands, the assignment to silicon. These results suggest high

chemical conservation after all enzymatic treatments in rice husks, despite lignocellulose compositional changes; however, the changes in transmission intensity in some wavenumbers may be explained by some structural modification in the lignocellulosic material by the enzymatic treatments.

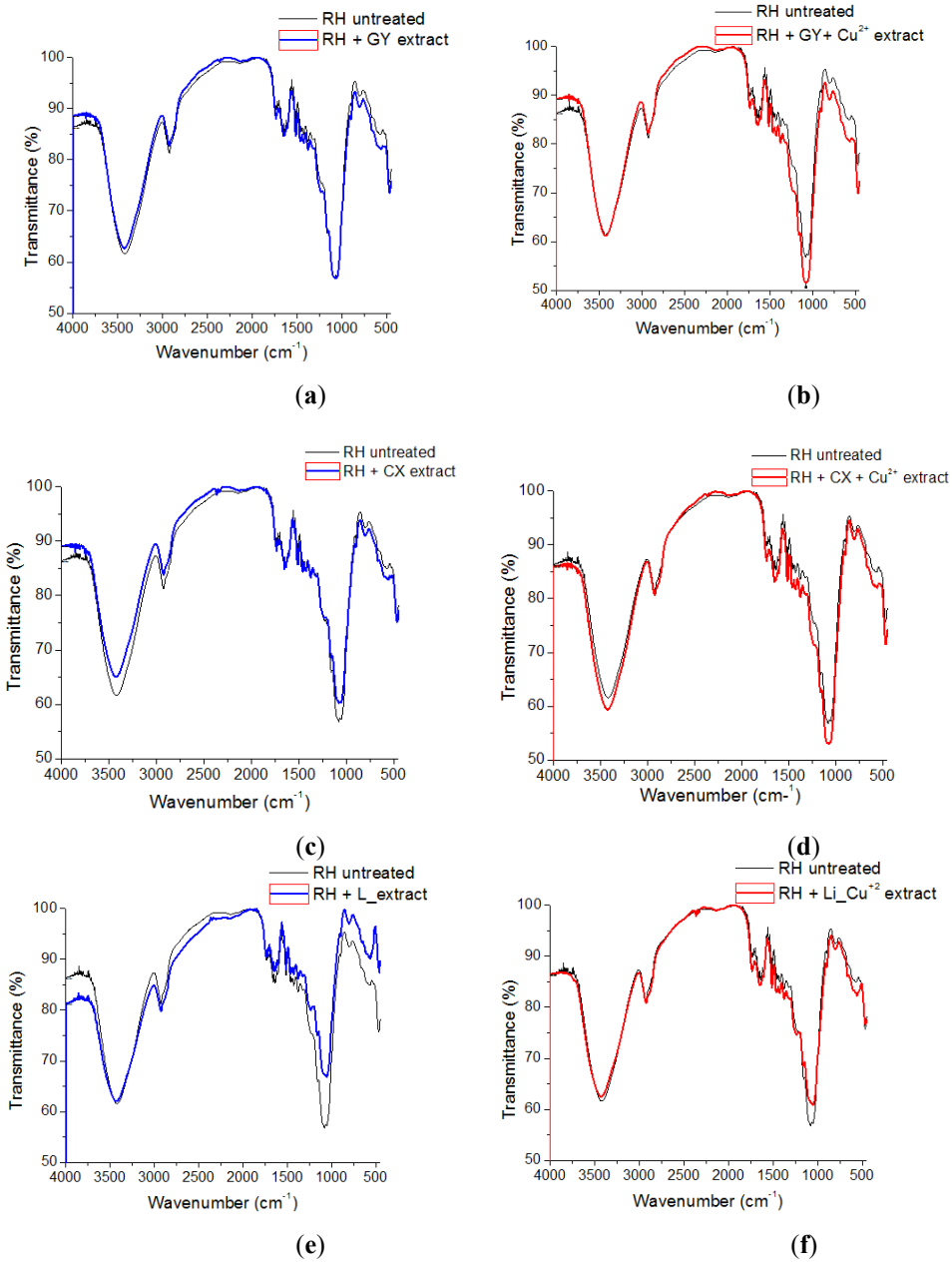


Figure 3-4. Overlapping of untreated and treated RH-FTIR spectra: (**a**) RH and RH + GY-extracts; (**b**) RH and RH + GY + Cu^{2+} extracts; (**c**) RH and RH + CX-extracts; (**d**) RH and RH + CX + Cu^{2+} extracts; (**e**) RH and RH + L-extracts; (**b**) RH and RH + L + Cu^{2+} extracts.

A closer view of the region around wavenumber 1740-1500 cm^{-1}, is shown in Figures 3-5. In this view, most spectra showed that many lower and closer bands are observed. Rice husks treated with GY-extracts without and with copper showed around thirteen bands, and with CX-extracts with and without copper showed around fifteen bands. The main difference in this region was observed in rice husks treated with L-

extracts with and without copper. Both untreated and treated rice husks treated with L-extracts without copper showed around 22 bands, whereas rice husks treated with L-extracts with copper showed a significant reduction to around seven bands in this region. These results suggest that the enzymatic treatments have a specific action in rice husks which probably is correlated with the enzymatic composition induced by the type and amount of LCD in the culture of *P. ostreatus* to produce these extracts. Changes observed in this region could be due to compositional changes in lignocellulose

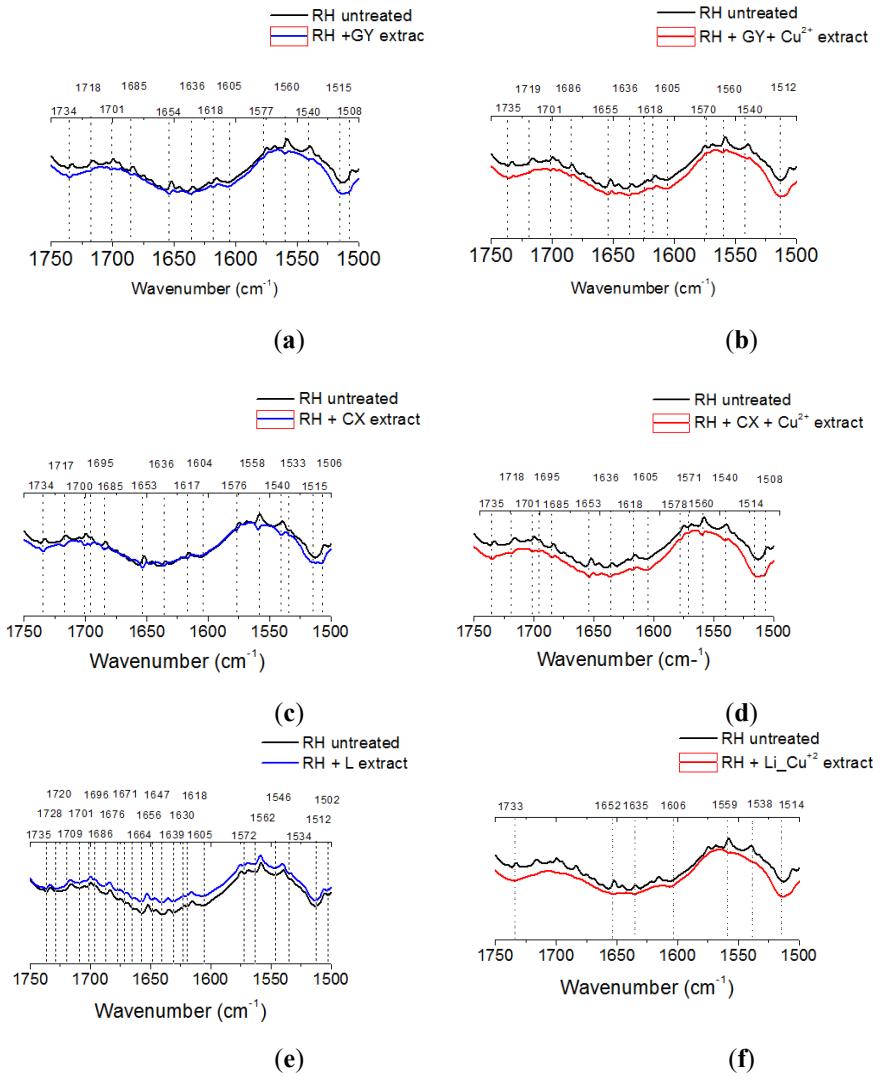


Figure 3-5. Overlapping of untreated and treated RH-FTIR spectra in the functional group regions 1750-1500 cm^{-1}: (**a**) RH and RH + GY-extracts; (**b**) RH and RH + GY + Cu^{2+} extracts; (**c**) RH and RH + CX-extracts; (**d**) RH and RH + CX + Cu^{2+} extracts; (**e**) RH and RH + L-extracts; (**b**) RH and RH + L + Cu^{2+} extracts.

Figure 3-6 shows the microscopic features of rice husks treated with enzymatic extracts. The pictures correspond to the lemma or the palea fractions. The photographed fields show the face of the inner lamina in a ventral view of this structure. Un-milled husks were used for these treatments since the milled husks showed generalized alterations in their structure due to the mechanical effect of grinding (Figure 3-6, b). When comparing these photographs, the

structural alterations of the rice husks with the different enzymatic treatments occur from the upper layer, where the epidermis is placed, towards the lower part where the sub-hypodermis and the parenchymal tissue are organized. Although this degree of alteration is not a quantification, the enzymatic treatments with copper sulfate (Figure 3-6, d, f, and h) seem to produce a more significant modification in the rice husks.

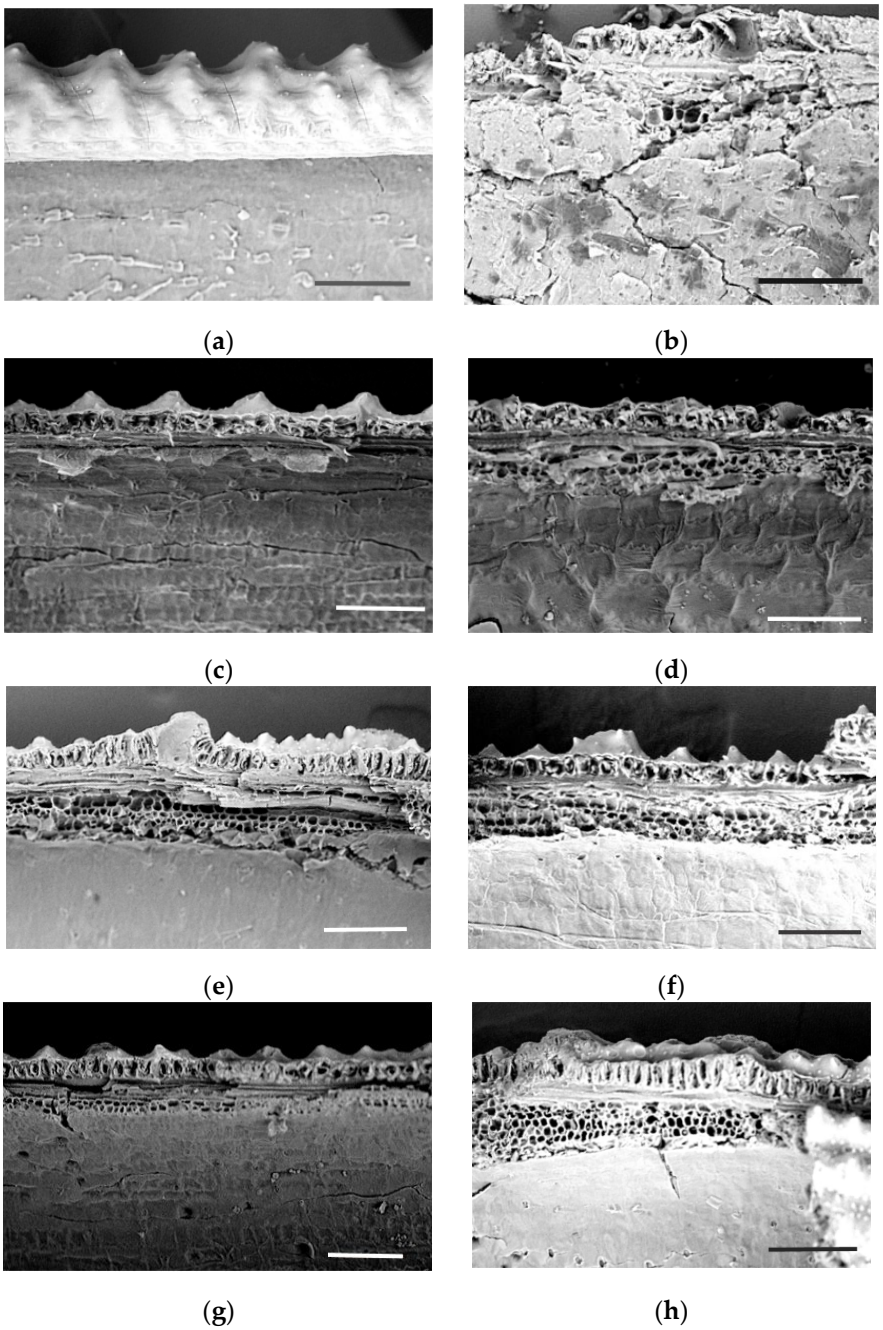


Figure 3-6. Scanning electron micrographs of Rice husks: (**a**) Rice husks un-milled and untreated; (**b**) Rice husks milled and untreated (**c**) Rice husks GY-extract treatment; (**d**) Rice husks GY + Cu^{2+}extract treatment; (**e**) Rice husks CX extract treatment; (**f**) Rice husks CX + Cu^{2+}extract treatment; (**f**) Rice husks L- extract treatment; (**g**) Rice husks L + Cu^{2+} extract treatment. Scale bar 100 μm.

3.4 Discussion

This study focused on the compositional, chemical, and morphological modifications after different enzymatic pretreatments applied on rice husk using crude extracts obtained from submerged fermentation cultures of *P. ostreatus*.

In previous work, the inducing effect of laccase activity by copper sulfate in *P. ostreatus* is mediated by the presence of copper transporters that appear to increase under nutrient-sufficient conditions for the fungus growth in synthetic culture media. However, because the natural substrate of *P. ostreatus* is lignocellulose from decaying trees, the constitutive polymers or lignocellulose-derived compounds (LDCs) could act as enhancers of laccases activity. This study is aimed to determine if that is the case. Evidence that supported this idea was found in previous studies by Our research group, which determined that compounds extracted from *Cordyceps nidu*s affect the laccase activity of *P. ostreatus,* and the inducing effect by these extracts could occur when xylose is present at low concentrations [146]. Therefore, in this study, we evaluated the effect of three lignocellulose-derived compounds (LDC), xylose, CMC, and lignin, on the laccase activity of *P. ostreatus* in a glucose and yeast extract medium base. The aim was to explore which of these components or their combinations act as laccases inducers by increasing laccase activity, whether it is through copper or stimulating other laccase isoenzymes in synthetic culture media. This approach was also proposed as a strategy to have natural enzyme cocktails rich in laccases useful to pretreat rice husks feedstock.

Using a complete factorial design, it was found that the effect of LDCs on the laccase activity of *P. ostreatus* depends on the concentration and interaction of these components in the culture medium (Table 3-2 and Figure 3-1). The results showed that xylose, CMC, or both increased laccase activity interestingly; however, lignin was found to decrease the enzymatic activity. Although the inducing effects of xylose and CMC were synergistic with copper, these two LDCs did not achieve the laccase-inducing power of 1mM copper sulfate, which had been tested as a laccase inducer [116].

On the other hand, although aromatic molecules derived from lignin have shown an inducing effect of laccase activity [147]–[149], few studies have evaluated the inducing effect of the lignin fraction on laccase activity in cultures of *P ostreatus*. Nevertheless, a report was found showing that this effect depends on the glucose and peptone concentration in the culture medium, and laccase activity with lignin (0.5 gL^{-1}) was fivefold higher than in media without lignin [150]. Tinoco *et al*. (2011) also showed a positive effect of lignin in laccase activity and found a synergistic effect with this activity of copper at 0.5 mM and lignin at 0.5 gL^{-1} in cultures of *P. ostreatus* [76]. In contrast, we found an antagonistic effect on the laccase activity by copper at 1mM and lignin at 5 gL^{-1} (Figure 3-1). This discrepancy could be due to the fact that the concentrations of copper and lignin evaluated in each study were different. Despite this, using lignin at 5 gL^{-1} in cultures with and without copper, we found an additional laccase isoform in the enzymatic extracts that was not present in media extracts without lignin (Figure 3-2). Because we did not purify or identify laccase from crude extracts, it is unknown, which of the 12 laccase genes found in the *P. ostreatus* genome this isoform corresponds. Moreover, although a higher concentration of total protein was quantified in the selected enzyme extracts in which lignin was used, these proteins seemed

degraded when stained with Coomassie blue under native PAGE conditions (Figure 3-2, b). Nevertheless, enzymatic activity typically of laccases was observed.

Rice husks treated with enzymatic extracts of *P. ostreatus* cultures supplemented with LDC and copper showed compositional, structural, and morphological changes. The results showed that different compositional of the enzymatic extracts affect rice husks differently. On the other hand, the lignocellulose fractions loss was found influenced by LDC composition more than it was by presence of copper in the extracts. In rice husks treated with GY and CX extracts, cellulose and lignin loss were similar with and without copper. The husks treated with GY extracts showed significant modifications, hemicellulose fraction with and without copper. Although lignin removal in husks was higher using GY and CX extracts with and without copper (23 to 29%). Because laccase activity was not particularly high for L+ Cu^{2+} extract, it was surprising that the highest lignin loss was obtained using the extracts produced with lignin and copper (37%) Table 3-3. These results suggest that the LDCs could induce laccases and other lignocellulosic enzymes that modify lignin and different lignocellulose fractions independent of the magnitude of laccase activity. These results also indicate that other factors in the extracts could contribute to delignification, such as lignin-derived metabolites, which could act synergically with laccases to increase delignification.

Other studies have reported that the action of laccase on lignin in various lignocellulosic biomasses depends on several factors, including the concentration of enzymes, enzymatic activity, the substrate to be delignified, and, mainly, the presence of mediators[3]. In some of these studies, when the concentration of purified laccase from *Trametes villosa* was increased with a fixed concentration of mediator, the percentage of lignin removal increased from 11-32% and 32-48% for elephant grass and *Eucalyptus globulus* wood, respectively; however, the lignin loss using the highest concentration of laccase in the pretreatment system in the absence of the mediator was only 5% [151]. In other cases, in pretreatment of wheat straw using *Pycnoporus cinnabarinus* with high enzymatic activity, lignin removal was 18% in the absence of a mediator and increased to 48% when mediator was used [44]. Up to 50% lignin removal from ground eucalyptus wood was achieved by pretreatment with recombinant *Myceliophthora thermophila* laccase and methyl syringate as a mediator, followed by alkaline peroxide extraction in a multistep sequence. All that indicates that in laccase-mediator systems, notorious improvement in lignocellulose delignification by laccase is achieved.

On the other studies, pretreatment with laccase alone (without mediator) removed up to 20% of lignin from eucalyptus wood [43]. In other cases, the delignification of lignin in the wood of *E. globulus* exploited with steam was 31% in the absence of an enzyme mediator, but using the crude enzyme extract secreted by *Marasmius palmivorus* grown in an optimized culture medium supplemented with copper [152]. This increased delignification using of the whole secretome as in this work suggests as possibly advantageous the use of whole crude enzymatic extracts secreted by the fungus for the enzymatic pretreatment of lignocellulosic biomass. In our case, we were using the whole secretome together with the culture supplementation with copper and LDCs, which could include lignocellulolytic enzymes other than laccases

[3] Usually, mediators are laccase substrates that increase the redox potential of laccases [45].

and metabolites with the role of mediators. Both the diversity of lignocellulolytic enzymes and metabolites in these extracts contributing to the modification of lignocellulose. Depending on the characterization of these extracts should be pursued in future work.

Three types of reactions (bond breaking, modification, and coupling) have been associated with laccases activity on lignin which can cause different changes in this polymer [39], [40]. The main consequence of bond-breaking reactions is depolymerization, which results in the release of lignin substructures and reduced polymer molecular weight [40]. Additionally, chemical modifications in lignin can occur even if there is no weight loss. In sugarcane by-products resulting from laccase-based pretreatment or in lignin alkali treated with laccases, when there was lignin depolymerization significant reductions, were found in the number of aliphatic side chains involved in the major β-O-4′ and β -5' bonds between monomeric units that also increased the content of the hydroxyl group (including hydroxyl aliphatic and phenolic hydroxyl) [153], [154]. Regarding the modification reactions, alpha carbon (Cα) oxidation reactions are the main ones in which the hydroxyl group is changed by a ketone [39]. In addition, demethylation reactions typically transform the methyl group into a hydroxyl group; these modifications were also found in alkaline lignin treated with laccase [154]. The last type of reaction by laccases in lignin is coupling. Coupling reactions can result in two types of modifications: polymerization reactions in which new structural units (H, G, or S) are incorporated into the polymer, increasing molecular weight, and reactions that insert low molecular weight sulfonated molecules [39]. In summary, the possible modifications of lignin by actions mediated by laccases could change the abundance of hydroxyl groups, breaking bonds between monolignol monomers, demethylation reactions, or oxidation of the alpha carbon forming carbonyl groups.

The chemical changes of the rice husks treated with the enzyme extracts by FTIR analysis was evaluated. Although this technique has been used extensively to characterize lignocellulosic biomass, including rice husks, overlapping lignocellulose characteristic bands is common with infrared band assignment [155]. With this in mind, little changes in the profundity around wavenumbers 3400 and 2900 cm^{-1} were observed in most treatments. These suggest high conservation in -O-H stretching and C–H stretching, respectively in rice husk treated with enzymatic extracts. The OH is present in water, alcohols, and phenols, and the C–H is present in the alkyl, aliphatic, aromatic groups. These changes could have occurred in either the cellulose, hemicellulose or lignin polymers and are indistinguishable.

The changes in the bands around 1740 to 1500 cm^{-1} functional group regions were explored (Figure 3-5). The most evident FTIR spectrum changes occurred in the rice husks treated with L+ Cu^{2+} extract in this region, which had a 37% lignin loss (Table 3-3). In this case, a high laccase activity induced by copper sulfate and the presence of lignin in the enzyme extracts could cause in rice husks treated a decrease in the intensity of at least fifteen bands. This attenuation around six bands associated with unconjugated carbonyl groups (1728, 1720, 1709, 1702, 1696, 1686 cm^{-1}) [156] occurred. Other attenuations in the bands associated with hydroxyl groups or water in the region (1656-1619 cm^{-1}) [157] were also present. The other last six bands are associated with aromatic skeletal vibration or C=C stretching (1572, 1562, 1546, 1534, 1521, and 1502 cm^{-1}) (Figure 3-5). In contrast, in the spectrum of rice husks treated with L-extract,

where lignin loss was around 24%, most bands were similar to those in untreated rice husks. These results suggest that in this region of the rice husks spectrum occurred the lignin modifications by actions mediated by laccases similar to that showed by rice husks treated with L+ Cu^{2+} -extract.

All the enzymatic pretreatments were related to changes in the microstructure of the rice husks (Figure 3-6). The internal and external structures of untreated rice husks show ruptures of the inner layer when milled [158] and expose the scaffolding of some sub-hypodermal or parenchymal cells. In contrast, the images of the rice husks in the enzymatic pretreatments showed a degree of alteration of the components of the inner layer that gradually and orderly uncovers the cells of the sub-hypodermis or parenchyma. Thus, although the treatments with copper extracts in the pictures appear to have a higher degree of degradation in the microstructure of the rice husks, future experiments in which this degree of decomposition is quantified need to be performed.

3.5 Conclusions

In this study, enzymatic extracts from *P. ostreatus* cultures in SmF were produced. Evaluating the laccase activity of different concentrations of compounds derived from lignocellulose (LDCs) such as CMC, xylose, and lignin, with and without copper sulfate. In this evaluation, the inducing effect of the laccase activity of LDCs depended on their concentration and interactions between them and copper. Two effects of LDCs were distinguished in laccase activity: on the one hand, high concentrations of CMC and xylose increase the laccase activity by copper, and on the other hand, high concentrations of lignin decreased the laccase activity by copper. Consequently, the laccase activity was increased 5-fold in media with high concentrations of CMX, xylose, and copper; however, most of this enzymatic activity enhancement was due to the addition of copper to the culture medium only (a 4-fold increase); while high concentrations of lignin in the medium reduced this copper laccase activity up to 4-fold. Moreover, the presence of lignin in the cultures induced a new isoform of laccase in the culture media.

The percentages of lignin loss in the rice husks treated with GY, CX, and L-extracts, with copper and high laccase activity were 24, 29, and 37%, respectively. Despite the inhibited laccase activity and the induction of additional laccase isomorphs, in the extracts produced with a high concentration of lignin with and without copper, these treatments show the largest compositional functional group and microstructure differences which suggest that previous lignin metabolisms in the culture of *P. ostreatus* could produce molecules that improve delignification. An attenuation of bands associated with carbonyl functional groups and aromatic skeletal was evident in the spectra of the treatment of rice husks with lignin and copper, and a greater degree of degradation of the microstructure of the husks with this treatment was also observed.

Chapter 4

Laccases and other Lignocellulolytic Enzymes in the Transcriptome of *Pleurotus ostreatus* used for Biological Pretreatment Systems of Rice Husks in SmF

Abstract: Rice husks (RH) is a lignocellulosic biomass difficult to break down and an attractive reservoir of sugars in the cellulose and hemicellulose fractions protected by lignin. This study used white-rot basidiomycete *Pleurotus ostreatus* to treat rice husks in submerged cultures modified to induce laccase activity. Changes in the composition of the rice husk and the transcripts of lignocellulolytic enzymes were evaluated in selected biological pretreatment systems with different laccase activity. To establish the composition of the modified culture medium that increased laccase activity, the effect of the proportions of three composition factors in the presence and absence of copper sulfate, was evaluated using a special class of response surface experiments: a mixture design. Mixtures with differences in laccase activity of an order of magnitude were selected. Changes in chemical functional groups in the infrared spectra and lignocellulose composition were compared. It was found that nearly 50 % of lignin but only 30% of cellulose and hemicellulose were removed by the end of the culture in the medium with the highest laccase activity. Cellulose elimination was similar regardless the presence or absence of copper. Finally, the *P. ostreatus* transcriptome using RNA-seq was analized to determine changes in the expression of lignocellulolytic enzyme gene transcripts, highlighting the active carbohydrate enzymes (CAZy). The results showed that the rice husks have a potent CAZy inducing power unaffected by the presence of the laccase-inducer copper; however, copper positively affected the transcription of laccases, *lacc10*, *lacc2*, and *lacc6* genes, while rice husks positively affected the transcription *lacc5* gene. The findings provide a strategy to design biological rice husk pretreatment systems based on natural enzyme cocktails. [4]

4.1 Introduction

Lignocellulose biomass is highly resistant to degradation, and only fungi and some bacteria have developed the enzymatic arsenals required to degrade it [159], [160]. This enzymatic armory must respond to several lignocellulose peculiarities in plant

[4] Full author list

Dinary Durán-Sequeda[1,2], Sofia Ortiz[1], Liyeira Anaya[1], Daniel Durán[1], Luis Cruz[1], Manuel Alfaro[2], Lucía Ramírez[2], Rocío Sierra[1], and Antonio G. Pisabrarro[2]

[1] Product and Process Design Group (GDPP), Department of Chemical and Food Engineering, Universidad de los Andes, Colombia; e-mail@e-mail.com

[2] Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre (UPNA), 31006 Pamplona, Navarre, Spain

biomass, including the proportions and composition of the polymers and their particular structure in cellulose, hemicellulose, and lignin. Several studies with omics approaches are being carried out focused on genomes, transcriptomes, proteomes, and exo-proteomes of these organisms. All of them help us reveal the critical enzymes involved in lignocellulose degradation[161]. This knowledge potentially confers added value to lignocellulosic biomass residues to produce biofuels, other chemical platform products, or enzymes for biotechnological processes [162].

Lignocellulolytic enzymes, a wood degradation-related group of enzymes, have been extensively studied in white and brown rot basidiomycete fungi [163]. Currently, they are classified as Carbohydrate-Active EnZymes (CAZy), a group that includes all enzymes involved in the modification, degradation, or biosynthesis of carbohydrates and their derived, as well as lignin modifying enzymes (LMEs) [164]. The CAZy database (www.cazy.org) provides information on two broad protein categories that participate in lignocellulose degradation: the Catalytic and Associated Modules. There is a single group of Associated Modules, the Carbohydrate-Binding Modules (CBMs), while there are five groups of Catalytic Modules: Glycoside Hydrolases (GH), glucosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), and auxiliary activities (AA) [165]. As their names indicate, most enzymes (CBM, GH, GT, PL, and CT) are involved in carbohydrate degradation, while the AA group is associated with LMEs and lytic polysaccharide-monooxygenases (LPMOs). The AA enzymes can directly or indirectly act on the degradation and modification of the aromatic or aliphatic components in lignocellulose in the lignin polymer. The types, amount, and how CAZys are secreted vary among fungi, substrates, and environmental conditions. Therefore, enzyme cocktails in the fungal secretome could have different results in lignocellulose pretreatment.

Recent publications about transcriptomes and exoproteomes in white-rot fungi reveal the existence of dynamic mechanisms in which several factors determine the control in the secretion of enzymes related to the degradation of lignocellulose biomass. These factors include i) genetic factors (genes and gene families) encoding groups of lignocellulolytic enzymes that vary in different white-rot fungi [19], [29], [166]–[169]; ii) nutritional factors, provided by the composition of the culture media, that show that the expression profiles of these genes change according to their composition and especially with their lignin content [168]–[172]; and iii) stress response factors by which white-rot fungi respond to other organisms, growth in co-culture or to the presence of xenobiotics compounds or the presence of metals such as copper [173], [174]. Despite this diversity of factors, the results of omics studies show that at least two profiles of enzymatic activities are necessary for the complete recycling of carbon from the lignocellulose biomass by rot fungi: i) hydrolase activity mediated by GHs enzymes involved in the degradation of cellulose and hemicelluloses by hydrolyzing the glycosidic bond, and ii) AA-mediated oxidase activity involving degradation of lignin or by lytic polysaccharide monooxygenase (LPMO), which can degrade cellulose by oxidation [175]. Interestingly, in white-rot fungi, the most abundant enzymes seem to be the oxidoreductases associated with the degradation of lignin, and the most widely reported as up-regulated by the presence of lignin are the laccases [168], [170], [171], [175], [176]. Moreover, in some cases, the culture times when laccase activity peaks coincide with lower cellulases or hemicellulases activities and *vice versa* [169], [170], [172], [175]. In another case, the induction of laccases by copper sulfate also induces other oxidoreductases, glycoside hydrolases, and chitinases [174], [177]. All these

results suggest that laccase regulation mechanisms could also affect the regulation of other lignocellulolytic enzymes.

Rice husks is a recalcitrant lignocellulosic by-product of paddy rice processing [1], [2]. The rice husks biomass comprises cellulose, hemicellulose, lignin, ash, and a small fraction of extractive compounds [7]. Cellulose content varies between 38-50%, hemicellulose between 23-32%, and lignin between 15-25% [8]. It has also been found that the ash content can reach around 20% of the weight of dry paddy rice [7]. Therefore, this lignocellulosic biomass is an important deposit of sugar.

Pleurotus ostreatus is a laccase-producer white-rot fungus and a biological model for studying these enzymes since its genome encodes 12 laccase genes whose expression responds to different culture conditions [28].

In this study, a rice husks-containing medium was used as a substrate for *P. ostreatus* grown in submerged cultures (SmF) to evaluate the proportions of rice husks and laccase inducers (such as copper sulfate) on the laccase activity released into the culture media. Two culture media with different compositions were selected in which laccase activity was significantly affected, and changes in composition and the chemical functional group in the infrared spectrum by FTIR were evaluated. Component and chemical functional group changes in the rice husks were associated with laccase activity, affecting cellulose and hemicellulose fractions. For this reason, we performed a transcriptomic analysis focused on CAZymes and compared their expression in the presence and absence of copper as a laccase inducer. The conditions of maximum laccase activity inducible by copper were used in this study. Our results showed that the use of culture media supplemented with lignocellulosic biomass could be a strategy to reduce the costs of laccase production and to add value to the lignocellulosic residues. This strategy allows for the production of enzymatic cocktails rich in laccase and other lignocellulolytic enzymes. Furthermore, this modified culture media strategy could help designing biological pretreatment processes for highly recalcitrant lignocellulosic biomass such as rice husks.

4.2 Methods

4.2.1 Fungal strain and culture conditions

The *Pleurotus ostreatus* strain ANDES-F515, provided by the Laboratory of Mycology and Phytopathology of the Universidad de Los Andes-LAMFU and deposited in the ANDES Natural History Museum (MHN ANDES), was isolated in the Bosque de la Merced, Santa Bárbara village, Bojacá, Cundinamarca, Colombia. The dikaryotic mycelium was maintained and conserved on Malt Extract Agar at 4 °C, with periodic replication of the growth zone for eight days of incubation at 25 ° C in the dark.

The submerged (SmF) cultures were performed in 250 ml flasks containing 100 mL of culture medium. All SmF cultures were inoculated with five 4 mm diameter plugs taken from the growth zone of eight days Malt Agar plate cultures and were incubated at 25°C, 150 rpm, in the dark for 21 days. All experiments were performed in duplicate.

4.2.2 Experimental Design of the biological pretreatment systems or modified culture media

The design of the biological pretreatment systems was carried out using a grade 2 extreme vertex mixture experimental design. The components of the mixtures were glucose, yeast extract, and milled rice husks. The restrictions established for the upper and lower limits of the amount and proportions, in the mixture are shown in Table 4-1

Table 4-1. Limits of components in the mixture design of extreme vertices

Components gL^{-1}	Amount		Proportion	
	Lower	Upper	Lower	Upper
Yeast extract	5.00	15.00	0.05	0.15
Glucose	5.00	45.00	0.05	0.45
Rice husks	40.00	80.00	0.40	0.80

In the simplex design plot (Figure 4-1), each vertex of the triangle represents one of the three components of the mixture. This simplex design plot shows that there are 13 points in the design space. The proportions of the components are selected for each point in such a manner that they sum to one, according to the bounds shown in Table 4-2, which were adjusted to accommodate specified constraints. The specific composition for each experimental mixture is shown in Section 3, Table 4-2. This design was tested in both conditions in the absence and presence of copper sulfate 1 mM.

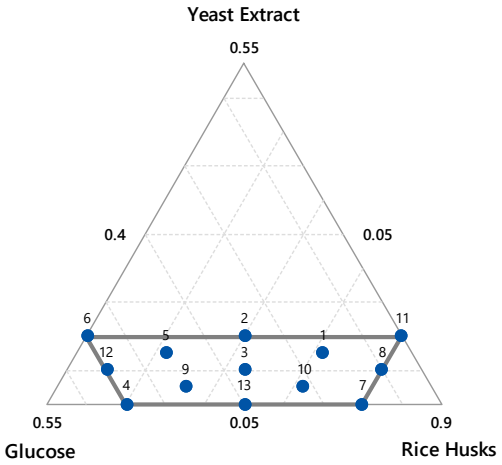


Figure 4-1. Simplex Design Plot in proportions.

4.2.3 Biomass production

Fungal biomass production was gravimetrically determined at 21 days of culture. The culture was filtered through a (previously dried and weighed) filter paper using a vacuum filter system, the collected, filtered mycelium was dried at 45°C for 72 h and weighed [73].

4.2.4 Spectrometric analyses

Laccase activity was determined with ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) as the substrate[95]. The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 5.0), and 10 µL aliquots of an appropriately diluted enzyme sample. Oxidation of ABTS was monitored by following the increase in *A*436 (ε 29.3 $mM^{-1}cm^{-1}$). A laccase activity unit was defined as the enzyme required to oxidize 1 µmol ABTS per minute at 25 °C. The DNS method measured reducing sugars using the 3,5-dinitrosalicylic acid (DNS) reagent at 540 nm [96].

4.2.5 Zymograms

Non-denaturing electrophoresis conditions (native-PAGE) were used to visualize the isoenzymes present in the experimental samples [98]. The stacking and running gels contained 4% and 9% acrylamide and were adjusted to pH 6.8 and 8.8 with Tris-HCl buffer, respectively. The running buffer was Tris-Glycine pH 8.3. The isozymes were revealed by staining the gel after running with 2mM of ABTS solution.

4.2.6 Rice husks compositional analysis

After enzymatic treatment, the concentrations of lignin and constitutive sugars (glucose and xylose) in rice husks were determined following the NREL/TP510-42618 protocol [138]. This protocol consisted of biomass separation into the constituting polymers using an aqueous acid phase (H_2SO_4, 72 % w/w) where each of the rice husks samples was hydrolyzed. For this, 300.0±10.0 mg of the rice husks sample were mixed with the acid solution for 1h at 30°C. Next, each sample was diluted by adding 84.00±0.04 mL of deionized water, placed in a 250 mL Schott flask, and put in an autoclave for 1h at 121° C. After the concentrated and diluted hydrolysis, the holocellulose found in the liquid phase was quantified using HPLC fitted with a column for sugars AMINEX HPX-87H (Bio-RAD), with a refractive index detector. The lignin in the solid phase was quantified gravimetrically

4.2.7 Fourier Transform Infrared Spectrometry (FTIR)

Infrared spectra were recorded with a Nicole 380 FT-IR Spectrometer (Thermo Scientific). The samples were mixed with KBr and pressed as tablets. The absorption spectra were recorded in the region from 4000 to 400 cm^{-1} wavelengths. OriginPro8 software was used to analyze the results.

4.2.8 RNA isolation and transcriptional analyses

The fungal biomass produced in the cultures containing 45 gL^{-1} glucose and 15 gL^{-1} yeast extract with or without 1 mM copper sulfate and with or without 40 gL^{-1} rice husks were harvested on day 14^{th} of culture for RNA isolation. The mycelium was collected by centrifugation and filtration, immediately frozen in liquid nitrogen, and grounded in a mortar to a fine powder. Then, 100 ng of the powder was transferred to a 1.5 mL microcentrifuge tube for total RNA extraction using a Fungal RNA EZN.A Kit

(Omega Bio-Tek, Norcross, GA, USA) according to the manufacturing guidelines. Finally, the integrity and quantity of RNA were validated using Bioanalyzer (version 2100) and Qubit 2.0 fluorometer.

4.2.9 mRNA-seq analysis

Illumina compatible libraries were prepared to be sequenced using the Illumina Nova Seq 6000 system from an mRNA isolate originating from RNA total. Following sequencing, RNA-seq data were filtered for quality assurance using FastQC and trimmed with BBDuk to remove adapters and low-quality reads (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). www.genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html was used as a *P. ostreatus* genome reference for aligning the resulting reads using STAR v2.3.16 [101]. The parameters used to achieve a single hit mapping were: --outReadsUnmapped Fastx --outFilterMismatchNoverLmax 0.04 --outFilterMultimapNmax 1. The mkPC15 v2.0 reference genome was assembled entirely in twelve scaffolds (34.3 Mb genome size)[102]. In total, 12,330 genes were annotated in this genome [103]. The expression levels were quantified using Python script rpkmforgenes.py (www.sandberg.cmb.ki.se/media/data/rnaseq/rpkmforgenes.py) to calculate values of reads per kilobase of transcript per million mapped reads (RPKM).

4.2.10 Differential gene expression analysis and CAZy gene annotation

Differentially expressed gene (DEG) analyses were performed using the EdgeR Bioconductor package and a dispersion parameter of 0.1. These analyses determined the transcriptional changes in the two culture conditions by comparing the gene expression values based on read counts. The gene expression values with Log_2 fold changes of read counts with a p-value < 0.01 and an FDR (False Discovery Rate) < 0.05 as the cut off for statistical significance were used. A DEG with log_2 Fold Change ≥ -2 was established as an upregulated gene, and log_2 Fold Change ≤-2 was established as a downregulated gene.

The CAZy gene annotations were based on the JGI automated annotation in the PC15 v2.0 reference genome, which uses the CAZy database (http://www.cazy.org/). 509 transcripts from the PC15 v2.0 genome were matched with CAZy genes from JGI automated annotations portal. These CAZy genes identified were used to found CAZy Differentially expressed genes (DEG) in each condition evaluated.

4.2.11 Statistics analysis

Minitab® version 18 software was used to construct the statistical design, evaluate statistical significances, obtain the regression models, and find the simultaneous local optimum of one or more response variables.

4.3. Results

4.3.1 Characterization of laccase activity in different biological pretreatment systems in submerged fermentation.

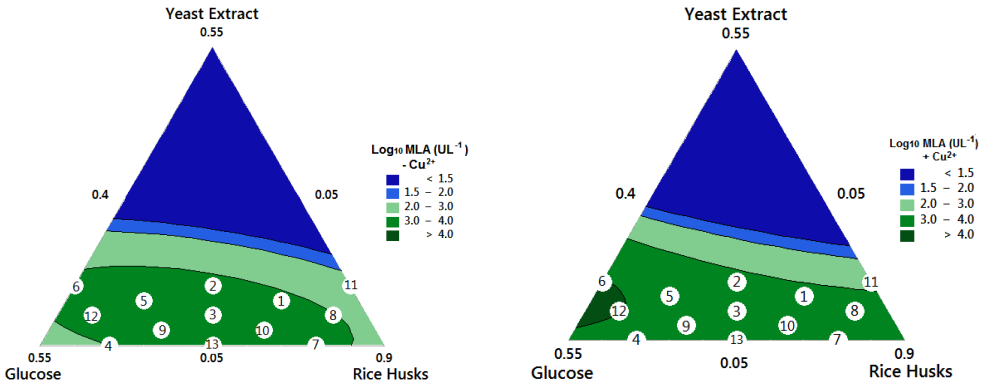
Thirteen modified culture media were designed for the biological pretreatment of rice husks in submerged fermentation, with glucose and yeast extract, to evaluate the effect of culture medium composition on the laccase activity of *P. ostreatus* in the absence and presence of copper sulfate at 1 mM. Table 4-2 shows the composition of these culture media according to the mixture statistical design used and the results of the maximum laccase activity obtained in these media at day 21 of culture. Figure 4-2 shows the contours plots. These results showed that the maximum laccases activities in the mixtures varied from 150 to 14000 UL^{-1}. In the absence of copper sulfate, the culture media with the lowest and highest laccase activity were GYR281063 and GYR051580, respectively (see nomenclature designations Table 4-2). The difference in activities between these media was higher than an order of magnitude. While in culture media with copper sulfate, the mixture with the highest laccase activity was GYR451540 and the mixture with the lowest activity was also the mixture GYR051580. The difference in activities between these media was also higher than an order of magnitude.

In the statistical analysis of the mixture design, the proportions of the components of the mixture were fitted to a suitable quadratic regression model (p-value <0.05 and R^2 0.8723 and 0.8920) to explain the effect of the modified culture medium composition on the laccase activity with and without copper, respectively. In both cases, the effect of the interaction of glucose and yeast extract was statistically significant (value of p <0.05), and only without copper the interaction of the proportions of glucose, and rice husks were also statistically significant (value of p <0.05). These results suggest that rice husks also could have an inducer role of laccase activity depending on nutritional conditions; however, this possible inducer effect seems to be lower than the laccase inducer effect by copper sulfate.

Table 4-2. Maximum laccase activity (MLA) of *P. ostreatus* in modified culture media with different proportions of glucose, yeast extract, and rice husk from a mixture design in SmF.

Mixture number	Culture medium name (GYR)*	Components gL^{-1}			Variable response - Cu^{2+}		+ Cu^{2+}	
		Glucose	Yeast extract	Rice husks	MLA (UL^{-1})	Log_{10} MLA	MLA (UL^{-1})	Log_{10} MLA
1	GYR161271	16.25	12.50	71.25	1954	3.29	2661	3.43
2	GYR251560	25.00	15.00	60.00	2641	3.42	1269	3.10
3	GYR281063	27.50	10.00	62.50	2913	3.46	5277	3.72
4	GYR450550	45.00	5.000	50.00	1227	3.09	5847	3.77
5	GYR361351	36.25	12.50	51.25	1968	3.29	7633	3.88
6	GYR451540	45.00	15.00	40.00	1438	3.16	13943	4.14
7	GYR150580	15.00	5.000	80.00	1442	3.16	3671	3.56
8	GYR101080	10.00	10.00	80.00	1501	3.18	2615	3.42
9	GYR360856	36.25	7.500	56.25	1927	3.28	3813	3.58
10	GYR210871	21.25	7.500	71.25	2475	3.39	4403	3.64
11	GYR051580	5.000	15.00	80.00	157	2.20	564	2.75
12	GYR451045	45.00	10.00	45.00	2372	3.38	6461	3.81
13	GYR300565	30.00	5.000	65.00	2317	3.36	3406	3.53

*The culture medium name indicates its composition; for example, GYR161271 contains 16, 12, and 71 gL^{-1} of glucose, yeast extract, and rice husks, respectively.



(**a**) (**b**)

Figure 4-2. Mixture Contour Plot of maximum laccase activity: (a) Laccase activity in culture media without copper; (b) Laccase activity in culture media with copper sulfate 1mM. The number inside the white circle is the mixture number shown in Table 4-2. Blue and green are the logarithm on base 10 for the predicted maximum laccase activity as the statistical model.

4.3.2 Characterization of two biological pretreatment systems: GYR451540 and GYR051580 in submerged fermentation.

Figure 4-3 shows the laccase activity profiles in cultures made using the GYR 451540 and GYR051580 mixtures in the absence and presence of copper sulfate and the laccase isoenzyme and total protein profiles in these cultures at several days. Over time, the laccase activity profiles show that laccase activity increases with culture time; therefore, the maximum laccase activity for all systems occurred at the end of the culture time. The isoforms present in the liquid phase of these pretreatment systems were compared with those present in the liquid phase of culture media of the same composition in glucose and yeast extract without rice husks at the same time of culture. These profiles showed at least three bands or laccase isoforms in the liquid phases of some cultures (two isoforms with electrophoretic mobility around 40 kDa and another with an estimated size of 70 kDa) whose intensity and number varied in different culture media. In the GYR451540 culture, the three isoforms were present both in the absence and presence of copper sulfate, whereas in GYR051580, the isoform with the lowest electrophoretic run was weakly present, as it also happened in the media without rice husks. The total native protein profile shows that the bands with the highest intensity correspond to the migration profiles of the laccase isoenzymes.

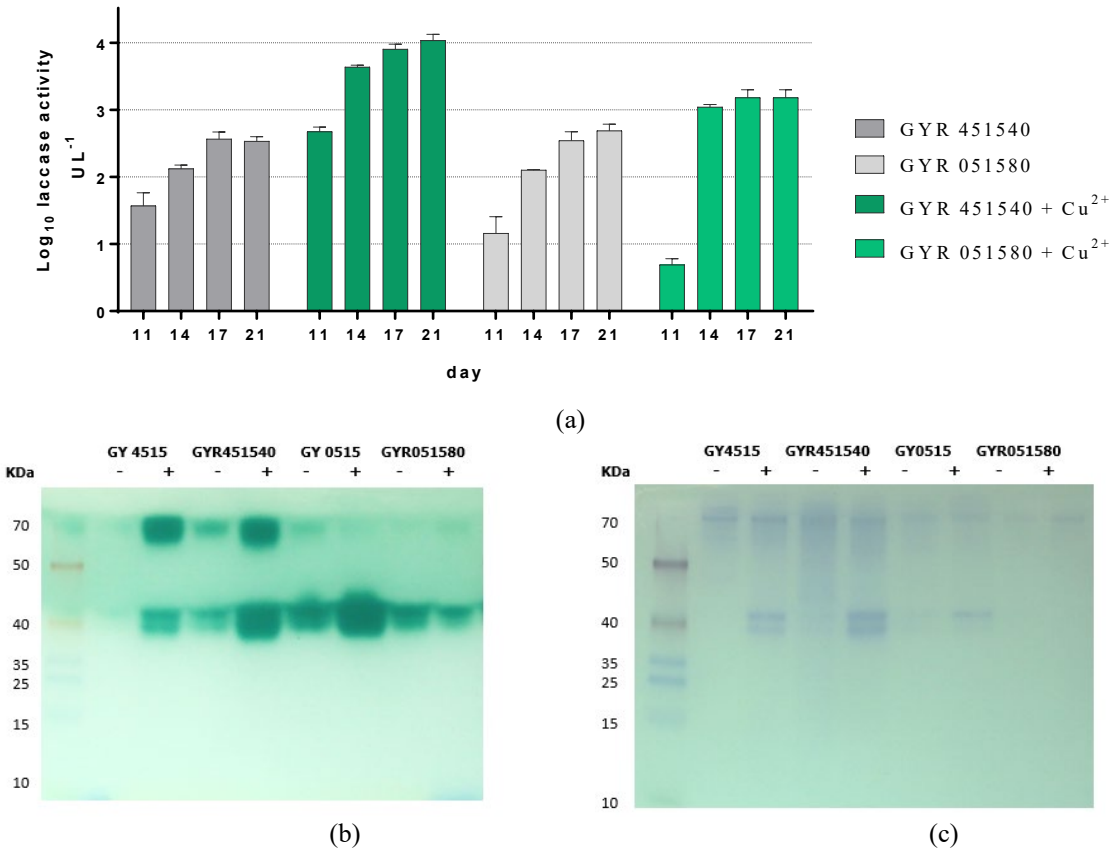


Figure 4-3. Laccases in two biological pretreatments systems, GYR451540 and GYR051580: (**a**) Laccase activity profile at different culture time; (**b**) Zymograms of laccase Isoenzymes; (c) Native-PAGE of proteins in the supernatant of the corresponding cultures with (+) or without (-) copper as laccase inducer.

.

The change in the concentration of reducing sugars and the final biomass production was evaluated in the GYR451540 and GYR051580 cultures during 21 days of culture in the absence or presence of copper sulfate (Figures 4-4). In all culture media, the concentration of reducing sugars decreased with the cultivation time. Although this decrease was gradual in the GYR451540 from days 11 to 21, it was more abrupt in the GYR051580 system. In this last culture, the concentration of reducing sugars stayed similar to the initial value after 11 days of cultivation with copper, whereas it was lower by 86% on day 11 in the cultures lacking copper. At the end of the culture time in the systems GYR451540, the reducing sugar concentration was around zero in the absence or presence of copper; however, in the GYR051580 systems, it remained around 1 gL^{-1}.

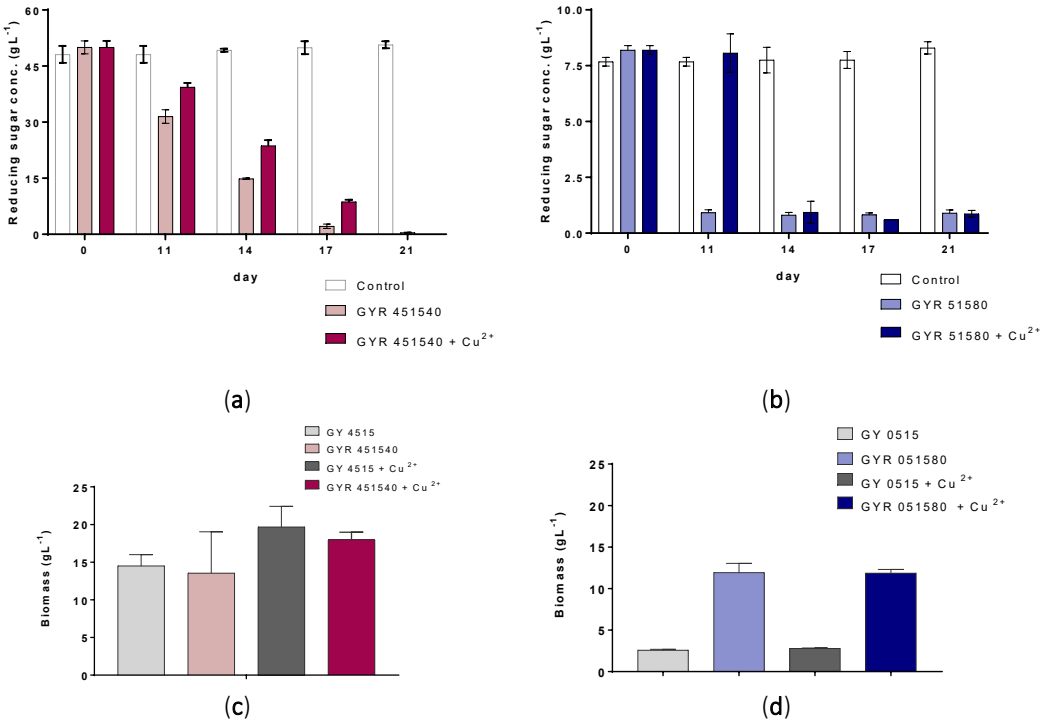


Figure 4-4. Reducing sugars and fungal biomass in GYR451540 and GYR051580 biological pretreatment systems. (**a**) Reducing sugar concentration in GYR451540 in the absence (red light) or presence of 1mM copper sulfate. (**b**) Reducing sugar concentration in GYR051580 in the absence (blue light) or presence of 1mM copper sulfate (blue dark); (**c**) Final fungal biomass production in GYR451540. (**d**) Final fungal biomass production in GYR051580. Control in (a) and (b) panels are culture media GYR451540 or GYR051580 without *P. ostreatus*

Finally, the fungal biomass production at the end of the culture made with or without rice husks showed differences depending on the initial glucose concentration in the system. In the GYR451540 system, which initially had 45 gL^{-1} glucose (with and without copper), the biomass production was similar to that obtained in the media without rice husks. In the GYR051580 system, which initially had 5 gL^{-1} glucose (with and without copper), the biomass production in the presence of rice husks was almost five times higher than in the media without rice husks. This result suggests that in the GYR051580 medium, the decrease in the concentration of reducing sugars by day 14 of culture could be inducing the bioconversion of the structural sugars in the holocellulose fraction of rice husks to fungal biomass.

4.3.3 FTIR spectra of the rice husks after the pretreatment.

The FTIR spectra of the rice husks recovered from the GYR451540 and GYR051580 pretreatment systems at different culture times are shown in Figure 4-5. The overlap between the spectra of the untreated and recovered rice husks at different cultivation times showed that the coupling between the bands around 3400 cm^{-1}, 2900 cm^{-1}, and 1100 cm^{-1} changed with time, and these changes seemed more evident in copper treatments. A focus of different fragments of the 1740 region at 1500 cm^{-1} is shown in Figure 4-6, where the spectra of the rice husks treated in both systems GYR451540 and GYR051580 with copper sulfate overlap. These regions showed a similar number of appearance bands conserved close to the same position in the

untreated rice husks spectrum from GYR051580 treatment, whereas most of them appeared attenuated in the GYR451540 treatment. The main difference between these treatments was observed in the region around 1560, where a deep band appeared in the GYR051580 copper treatment, but this band was more tenuous in the GYR451540 copper treatment.

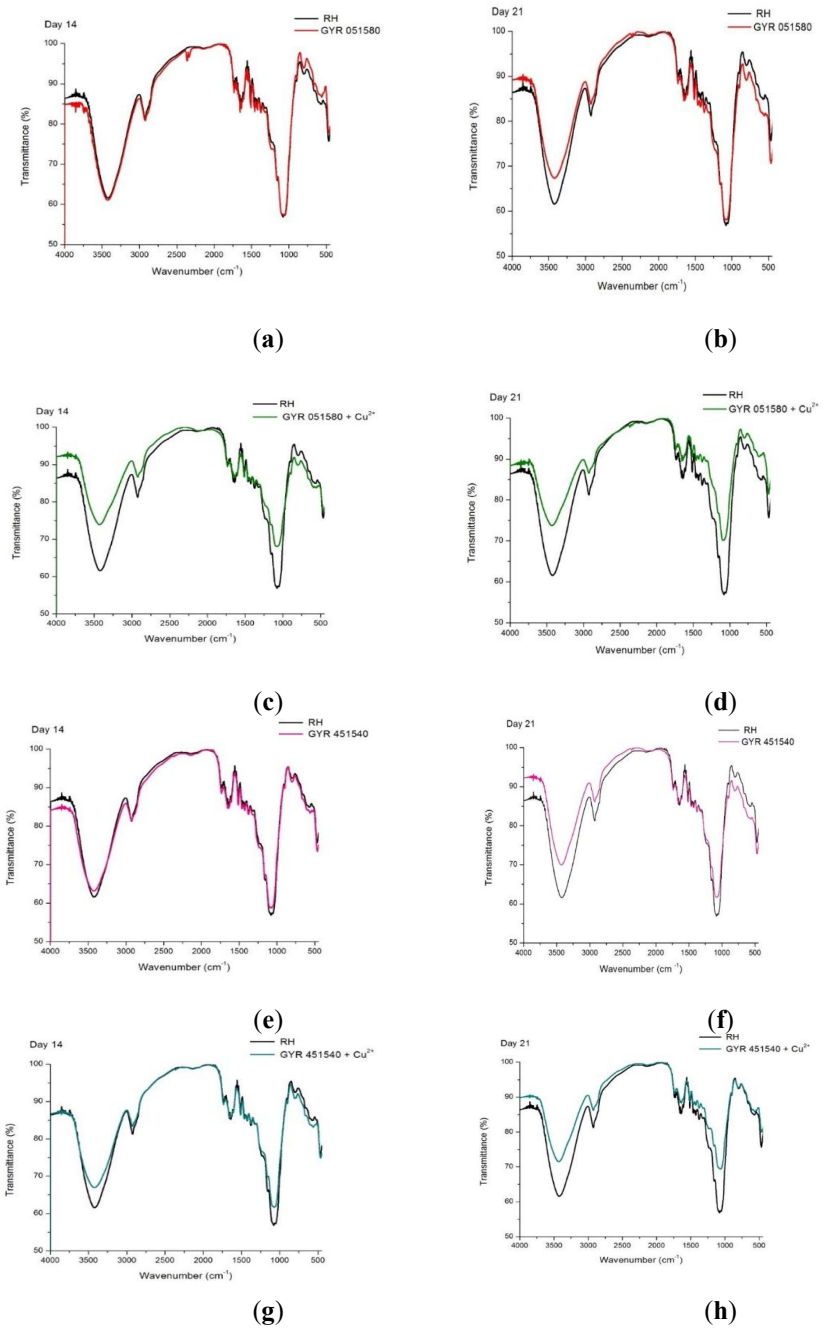


Figure 4-5. Rice husks (RH) FTIR spectra overlapping: (a) RH untreated vs. RH from GYR051580 day 14; (b) RH untreated *vs*. RH from GYR051580 day 21; (c) RH untreated *vs*. RH from GYR051580 + Cu^{2+} day 14; (d) RH untreated *vs*. RH from GYR051580 + Cu^{2+} day 21; (e) RH untreated *vs*. RH from GYR 451540 day 14; (b) RH untreated *vs*. RH from GYR451540 day 21; (c) RH untreated *vs*. RH from GYR451540 + Cu^{2+} day 14; (d) RH untreated *vs*. RH from GYR451540 + Cu^{2+} day 21.

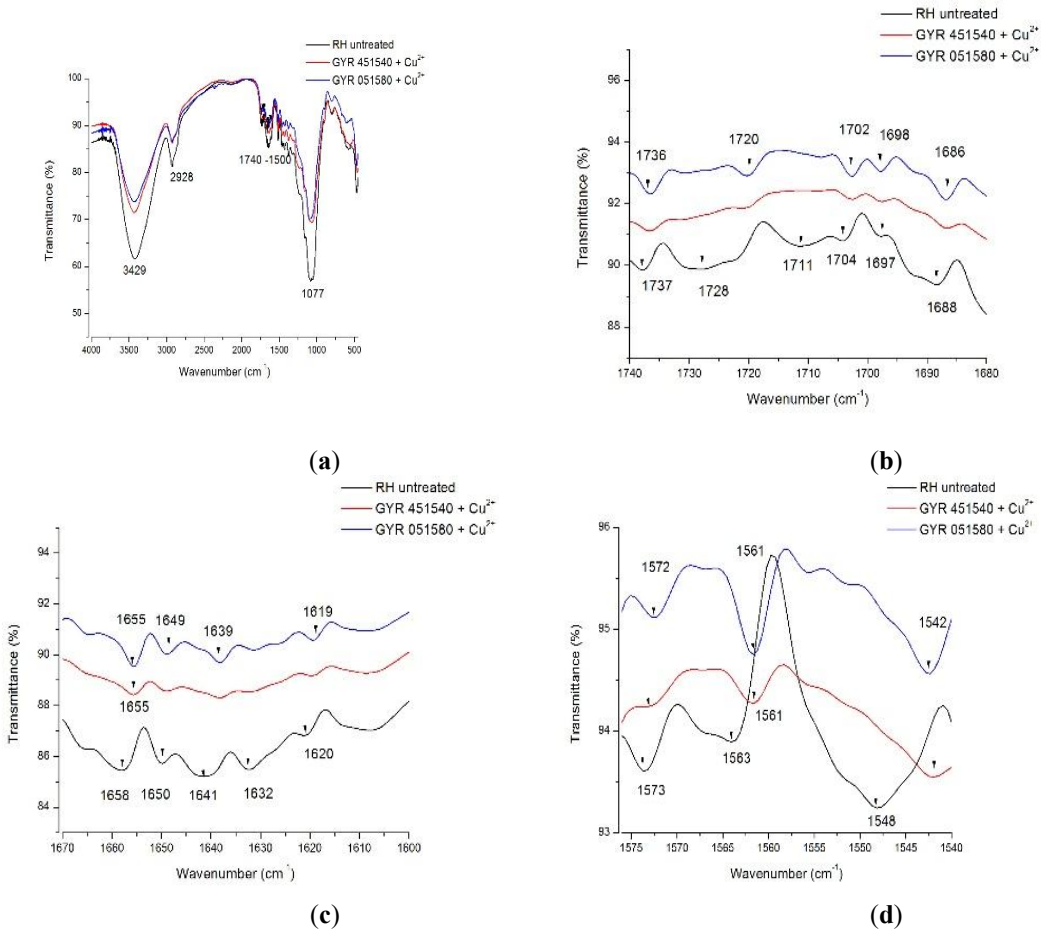


Figure 4-6. Rice husks (RH) FTIR spectra overlapping at functional group regions: (a) untreated *vs*. treated with copper day 21 on whole spectra. (b) 1740-1680 cm^{-1} region. (c) 1670-1600 cm^{-1} region. (d) 1575- 540 cm^{-1} region.

The analysis of the changes in the lignocellulosic composition of rice husks recovered from 21 days of treatment in GYR051580 and 14 and 21 days of treatment in GYR451540 with and without copper are shown in Figure 4-7. This analysis showed that the most significant changes in the composition of the cellulose, hemicellulose, and lignin fractions occurred in the husks recovered at the end of the culture. However, these changes depended on the treatment system and the presence of copper sulfate. At the end of the cultivation time, cellulose loss was mainly associated with the concentration of glucose and rice husks in the pretreatment system rather than the presence or absence of copper sulfate: 30% cellulose loss in GYR451540 and 10% in GYR051580. The percentage of hemicellulose loss in the treated husks was higher than 25% when copper was present. On the contrary, in the treatments without copper, the loss depended on the composition of the culture medium. In the GYR051580 treatment, this loss was close to 20% at the end of the culture. The lignin loss in the husks of the different treatments ranged from 25% to 50%. These results did not seem to depend on the presence of copper sulfate in the GYR051580 system. In contrast, the highest losses occurred in the copper systems in the GYR451540 treatments at different cultivation times. These results indicate that lignin loss could be associated with laccase activity in the GYR451540, but that was independent on the laccase activity in GYR051580.

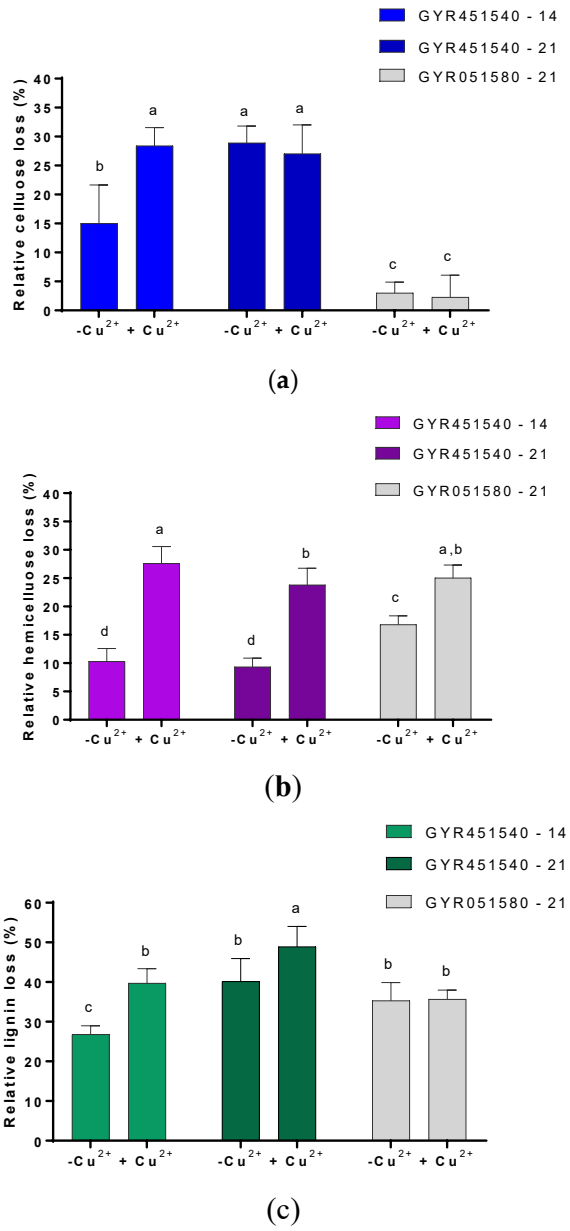


Figure 4-7. Lignocellulose relative loss in rice husks after biological treatment systems GYR451540 and GYR051580: (a) Cellulose; (b) Hemicellulose; (c) Lignin. The relative lignocellulose composition loss was calculated concerning untreated rice husks. Means that do not share a letter are significantly different (P-value <0.05).

4.3.4 Transcripts of CAZymes gen analysis in biological pretreatment systems GYR451540 with or without copper

The lignocellulose polymers compositional analysis (Figure 4-7) revealed that the rice husks recovered from the GYR451540 pretreatment systems were more modified with and without copper than those from GYR051580 systems. The rice husks from GYR451540 systems also showed more changes in the FTIR spectra. One of the main differences between these treatments was the lignin elimination when 1mM copper

sulfate was present in the system. The effect of the presence of copper on cellulose was negligible while the extent of hemicellulose removal was copper-dependent. Therefore, other lignocellulolytic enzymes, especially CAZYs in the GYR451540 systems, were explored with and without copper sulfate. For this, we compared the transcriptomes of *P. ostreatus* in culture media with and without rice husks, with and without copper. The results are shown in Figures 4-8. These comparisons show that whereas the addition of husks to synthetic media induces the differential expression of hundreds of genes, copper sulfate addition to culture media containing rice husks induced the differential expression of only about twenty genes. In the list of down-regulated and up-regulated genes, 107 transcripts annotated in the mkPC15 genome as CAZymes were identified from a total of 509 CAZymes genes. Of these 107 transcripts, 77 transcripts corresponded to different genes, suggesting that several of these genes were regulated in several analyzed conditions.

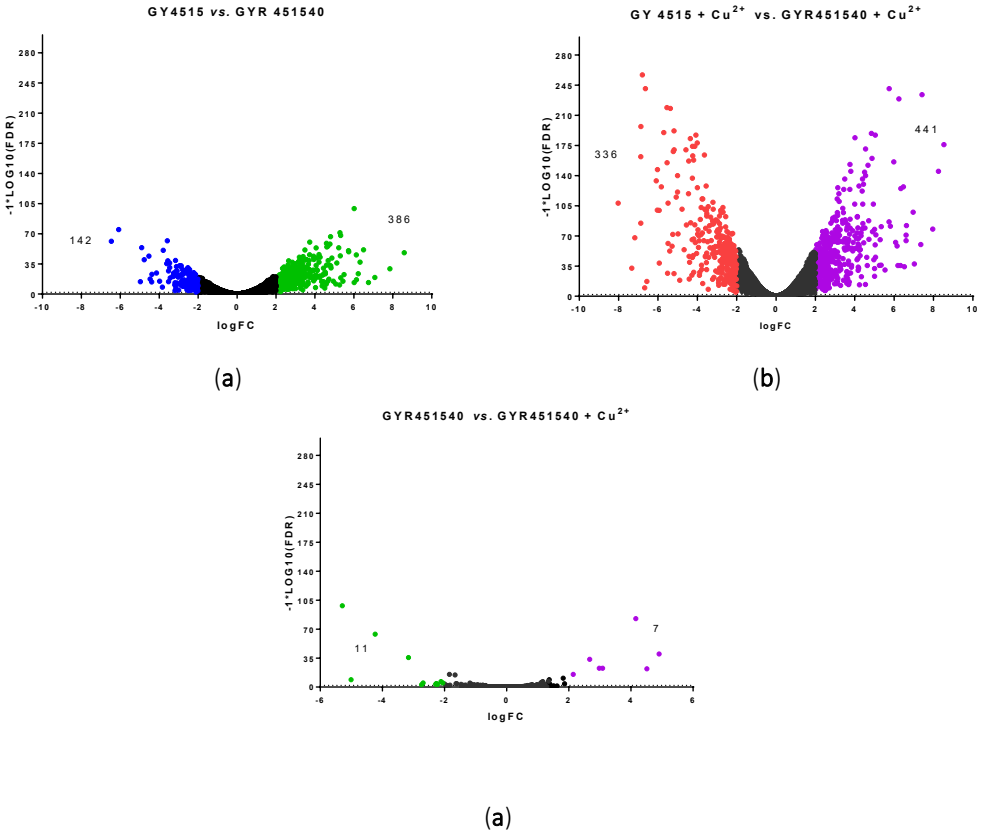
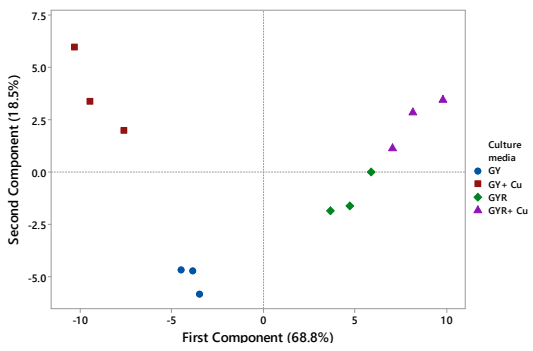


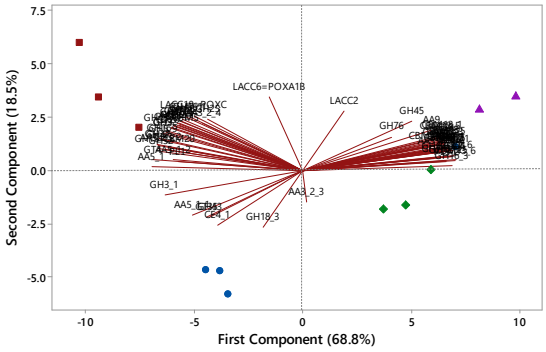
Figure 4-8. Volcano plot highlighting the amount of differentially expressed genes (DEG), downregulated (left), and upregulated (right) in *P. ostreatus* from three different analyzes: (**a**) DEG in culture media GY4515 vs. GYR451540; (**b**) DEG in culture media GY4515 + Cu^{2+} *vs*. GYR451540 + Cu^{2+}; (**c**) DEG in culture media GYR451540 *vs*. GYR451540 + Cu^{2+}.

A principal component analysis was performed to establish the correlated CAZymes transcripts and their distribution in the evaluated culture media (Figure 4-9). The score plot shows that the groupings of the data in the graph follow three distributions. In the first component, a cluster containing the media with scale, with and without copper. In the second component, two clusters separate the synthetic media with copper towards the positive part of the component and the synthetic media without copper on the negative part of the component. However, two large groups of CAZymes gene transcripts have the most significant effect on each component corresponding with

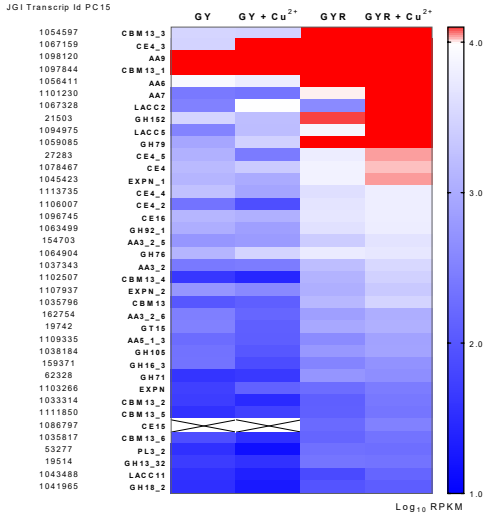
the distribution of the culture media without rice husk and copper in the second component and with the distribution of the media with the rice husks and copper in the first component. Few transcripts influence the portion of the second component where the copper-free and copper-free synthetic media are distributed. All the transcripts were separated into two heat maps (Figure 4-9, c and d). In these heat maps, the expression levels of each gene are located in descending order concerning the highest expression in the synthetic culture media with copper sulfate (panel c) and the highest expression in the culture media with rice husks and copper sulfate (panel d). So at the top of the gene list are the transcripts with the highest expression values. The results show that of the nine genes most expressed in the synthetic medium, GY4515 + Cu^{2+}, only three are at similar expression levels in the GYR451540 + Cu^{2+} medium. While in this list, the sixteen genes most expressed in the GYR451540 + Cu^{2+} medium shared seven with a similar expression level in the GYR451540 media without copper.



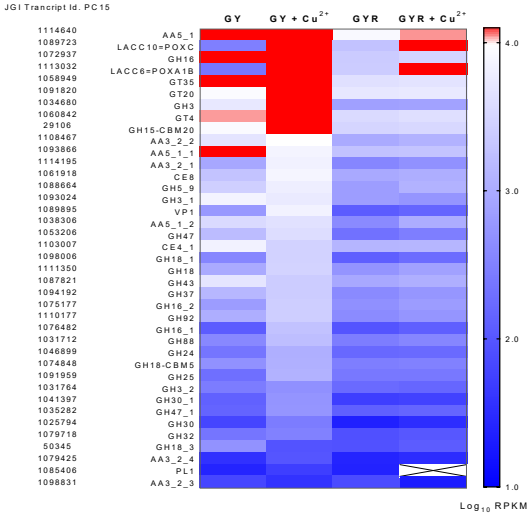
(a)



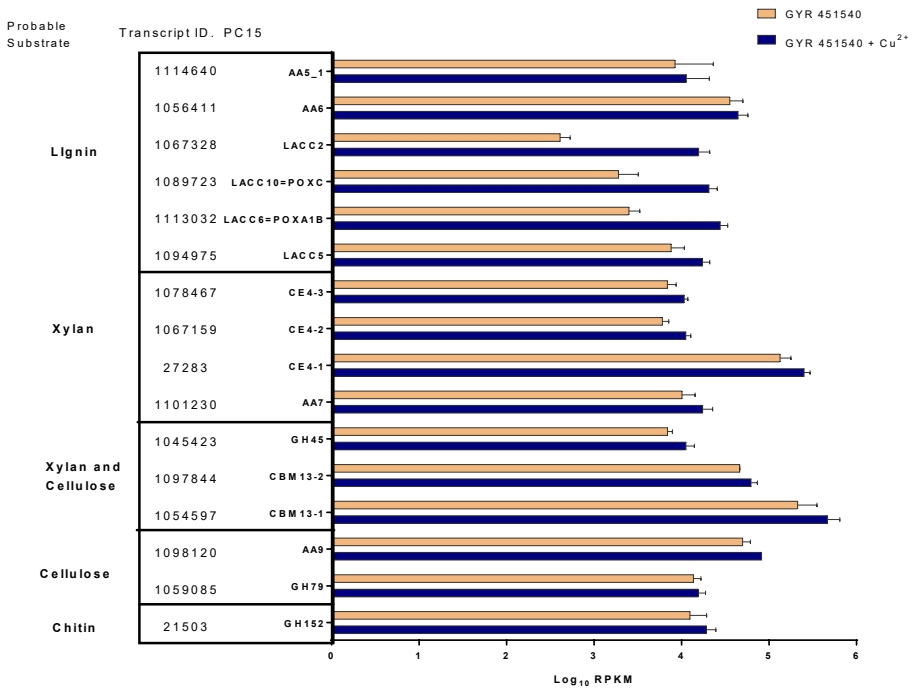
(b)



(c)



(d)



(e)

Figure 4-9. CAZymes transcriptional profile analysis of *P. ostreatus* in different culture media: (**a**) Principal Component Analysis (PCA), clusters CAZymes transcripts by culture media in scatterplot; (**b**) PCA, clusters CAZymes transcripts in biplot; (**c**) Heatmap analysis of CAZymes transcripts from first PCA component; (**d**) Heatmap analysis of CAZymes transcripts from second PCA component; (**e**) Top sixteen CAZymes overexpressed in GRY 451540 and GYR451540 + Cu^{2+} systems

The list of CAZyme gene transcripts identified with expression levels greater than 4, their function, and possible substrates are shown in Table 4-3. Finally, in Figure 4-9, the expression levels of the CAZY genes from Table 4-3 in the GYR451540 and GYR451540 + Cu^{2+} systems are also compared. From this comparison, we found that of the six CAZymes genes with possible action on lignin, three transcripts of laccase genes, *lacc*2, *lacc10*, and *lacc6,* had expression levels lower than 4 in GYR451540 media without copper. While the transcripts of the other three lignin-modifying CAZymes genes, *AA5_1*, *AA6*, and *lacc5,* had an expression level close to four or at the same level as the same gene transcripts in the copper GYR media. Other CAZYs transcripts whose possible substrates are xylan or cellulose were identified with expression levels as Log RPKM greater than four in both culture systems.

Table 4-3. Carbohydrate Active Enzymes description from CAZy database (http://www.cazy.org/)

Transcript ID (PC15)	CAZy name	Short CAZY name	Catalytic or binding module	Functions	Probable substrate
21503	Glycoside Hydrolase Family 152	GH152	β-1,3-glucanase	Hydrolysis of (1→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans	Fungal cell wall
1114640	Auxiliary Activity Family 5 / Subf 1	AA5_1	Oxidase with oxygen as acceptor Galactose oxidase Glyoxal oxidase Alcohol oxidase Raffinose oxidase	AA5_1 enzymes can contribute to lignin degradation through extracellular peroxide generation, which is needed for	Lignin

				manganese peroxidase (MnP)) [178].	
1056411	Auxiliary Activity Family 6	AA6	1,4-benzoquinone reductase	Oxidoreduction of benzoquinone to hydroquinone	Lignin
1067328 1089723 1113032	Auxiliary Activity Family 1 / Subf 1	Lacc2 Lacc10 Lacc6	AA1_1 are multicopper oxidases that use diphenols and related substances as donors with oxygen as the acceptor. Laccase / p-diphenol:oxygen oxidoreductase	Oxidation of hydroxyl groups in several compounds and reduction of oxygen	Lignin
1094975	Auxiliary Activity Family 1 / Subf 2	Lacc5	AA1_2 Ferroxidases		Lignin
1067159 1078467 27283	Carbohydrat e Esterase Family 4	CE4	Acetyl xylan esterase Chitin deacetylase Chito-oligosaccharide deacetylase	Deacetylation of xylans and xylo-oligosaccharides Hydrolyses the N-acetamido groups of N-acetyl-D-glucosamine residues in chitin.	Xylan Chitin
1101230	Auxiliary Activity Family 7	AA7	Glucooligosaccharid e oxidase (GOOX)	GOOX found in this family oxidizes the reducing end glycosyl residues of oligosaccharides linked by alpha- or beta-1,4 bonds and glucose.	Xylan Cellulose
1045423	Glycoside Hydrolase Family 45	GH45	Endoglucanase Xyloglucan-specific endo-β-1,4- glucanase / endo-xyloglucanase; Endo-β-1,4-mannanase	Also, hydrolyses 1,4-linkages in β-D-glucans containing 1,3-linkages.	Cellulose Xylan Other Hemicellulos es
1054597 1097844	Carbohydrat e-Binding Module Family 13	CBM1 3	Modules of approx. 150 residues found in Glycoside hydrolases, glycosyltransferase s, xylanase, arabinofuranosidas, GalNAc transferase 4.	These modules can bind mannose, xylan, GalNAc.	Xylan Cellulose
1098120	Auxiliary Activity Family 9	AA9	AA9 are copper-dependent lytic polysaccharide monooxygenases, Lytic cellulose monooxygenases (LPMOs)	AA9 cleavage of cellulose chains with oxidation of carbons C1 and/or C4 and C-6	Cellulose
1059085	Glycoside Hydrolase Family 79	GH79	β –glucuronidase, hyaluronoglucuronidas e heparanase baicalin, β–glucuronidase, β-4-O-methyl-glucuronidase	Glycosidase family of enzymes that catalyze breakd own of complex carbohydrates	Cellulose

4.4 Discussion

This study compared some biological pretreatment systems with different laccase activities to determine compositional changes in rice husks and the CAZymes likely involved in this process. To select the systems to compare, we initially evaluated the effect of the culture medium composition on the laccase activity in the absence or presence of the laccase inducer copper sulfate. These culture media contained glucose, yeast extract, and rice husks. The different compositions evaluated were established through a mixture design of extreme vertices restricted to minimum glucose and yeast extract concentrations. Since we previously observed that *P. ostreatus* could grow and colonize the rice husks in solid culture but grows poorly in submerged culture without an initial source of glucose and nitrogen.

Several multifactorial aspects involved in the strategy of producing laccases in submerged culture (SmF) using lignocellulosic-modified culture media have been the subject of other studies using *P. ostreatus* and other withe-rot fungi [179]–[181]. Using a basal culture medium with glucose, peptone, yeast extract, 1mM copper sulfate, and 2% of various lignocellulosic materials, the laccase activity also varied from 6,543 to 9,300 UL^{-1} in SmF of *Trametes hirsuta* [182]. With a high laccase activity 41640 UL^{-1} on day 12th culture, laccase production has been scaled up to 102 L bioreactors in SmF, using 4% wheat bran and *Cerrena unicolor* C-139, in basal media of 5.5 gL^{-1} glucose, 2 gL^{-1} peptone, 2 gL^{-1} of yeast extract, and the laccase inducer copper sulfate [183]. Similary, Our results showed that changes in the glucose and yeast extract initial concentrations in the basal medium can negatively or positively affect laccase activity despite the presence or not of lignocellulosic material in the modified medium. For example, if the initial glucose conditions are insufficient, the laccase activity could be reduced by up to one order of magnitude; when compared to the activity observed in glucose-sufficiency conditions with yeast extract where the laccase activity by copper sulfate is increased up to order of magnitude (Table 4-2 and Figure 4-2).

From evaluating the effect of the culture medium on laccase activity, we chose two systems with the highest and lowest laccase activity to compare laccase production, fungal biomass production, and glucose consumption. These systems were GYR451540 and GYR051580 (Figure 4-2). These systems differ in the initial glucose and rice husks concentrations, accounting for 4% of lignocellulosic biomass in the GYR451540 system and 8% in GYR051580 (Table 4 2). Based on the rice husks elemental composition analysis of carbon and nitrogen previously performed (data not shown), the estimated C: N ratio in both cultures was approximately 18: 1. When the fungal growth of *P. ostreatus* in these two systems GYR451540 with or without copper was compared, it was found that in the GYR451540 systems, the final biomass production was similar to the final biomass in culture media without rice husks, GY4515. However, the final biomass production in the GYR051580 systems was fourfold higher than in the culture media with rice husks. These results suggest the bioconversion of rice husk sugars to fungal biomass in GYR051580 systems. A signal of this possible bioconversion in a submerged system with *P. ostreatus* was indirectly showed when, in a medium with 20 gL^{-1} of glucose and 0.5 gL^{-1} of ammonium sulfate, the addition of 20 gL^{-1} of orange peel increased the growth rate by 52% [184].

Lignocellulose biomass pretreatment using *P. ostreatus* has been more often studied in rice straw pretreatment than rice husks in solid fermentation [23], [185]–[187]. In many of those studies, combining different physical or chemical pretreatments or changing the culture variables, the percentage of lignin removal from rice straw have been 33% [185], 41% [23], and up to 81% [186] for various lignocellulose feedstock and 40% in rice husks [187]. These studies also emphasize selectivity as lignin removal and the cellulose ratio, which varied in 4:1, 2:1, 1:1, and 3:1[23], [185]–[187]. In submerged culture, our comparisons of the compositional analysis of rice husks treated in the GYR451540 and GYR051580 systems with and without copper showed that this lignin: cellulose removal selectivity changed from 2:1 to 8:1, with a removal of 50% and 35% lignin, respectively (Figure 4-7).

In other biological pretreatments, high selectivity in lignin removal produced cellulose 3% with 18% removal of lignin and 21% removal of hemicellulose, occurred with an increase in laccase and xylanase that was higher than that of endo- and exo-glucanases during the culture time [188]. A reduction in the band depth of 3400 cm^{-1} when comparing the FTIR spectra of the rice husks of both GYR451540 and GYR051580 copper pretreatments shows an important modification in the hydroxyl groups (Figure 4-5). Additionally, due to the differences in the lignin and cellulose removal selectivity in these two systems, it is possible that the reduction of the bands in the regions 1740-1680 cm^{-1} and 1680-1630 cm^{-1} (Figure 4-6), aldehyde, ketone, ester, or carboxylic acid probably resulting from both laccase action on aromatic ring structure of lignin and xylanases or cellulases actions in the carbonyl group [189], [190] in GYR451540 treatment. While in GYR051580, the appearance of these bands could be more related to the exposure of these functional groups in hemicelluloses by xylanases actions. In the 1575-1530 cm^{-1} region where ring stretching associated with ring aromatic lignin or furans in cellulose is adsorbed, the main difference between the FTIR spectra of the rice husks of the GYR451540 and GYR051580 systems with copper was the appearance of a band around 1562 cm^{-1} which is much deeper in the spectrum of GYR051580. This band, 1562 cm^{-1}, still needs to be assigned to a lignocellulose compound [191].

In the transcriptomic analysis, we only selected the GYR451540 systems in the presence and absence of copper sulfate. The focus of this analysis was on the CAZymes annotated in the mkPC15 genome. In general, this analysis showed that a small number of genes are differentially expressed when the transcriptome of *P. ostreatus* in GYR451540 was compared with copper and without copper. These suggest that rice husks had a more significant influence on the *P. ostreatus* transcriptome regulation than copper sulfate (Figure 4-8). It has already been observed that the presence of wood increased the secretome complexity than glucose in dikaryon and monokaryons strains of *P. ostreatus* [192]. However, the transcripts of *lacc2*, *lacc10*, and *lacc6* were the only differentially expressed genes by adding copper on the top sixteen CAZymes over-expressed in the rice husks systems. The *lacc5* transcript, recognized as a ferroxidase, was overexpressed under both conditions, GYR451540 with or without copper. Transcripts encoding auxiliary CAZymes, *AA5_1*, and *AA6*, were also found in the group of enzymes that could contribute to lignin modification. *AA5_1* (jig #ID, 134564, in mkPC9) was among the three most abundant proteins in the mkPC9 secretome when grown on synthetic medium or media with poplar wood lignocellulose non-timber wheat straw. While AA6, a flavodoxin (jig #ID, 115319, in mkPC9), was also in the mkPC9 secretome but only in lignocellulose media [30].

The high glucose concentrations in the GYR451540 systems with and without copper sulfate could induce Carbon Catabolite Repression (CCR) and inhibit the overexpression of CAZymes genes [82]. However, the comparative analysis of the *P. ostreatus* transcriptome in culture media with and without rice husk and high glucose concentrations showed that several CAZymes genes were overexpressed in both the GYR451540 systems. Furthermore, the loss of an important fraction of cellulose and hemicellulose in the compositional analysis of the husks from these systems suggests that the secretion of these enzymes occurred. Studying the mkPC9 *P. ostreatus* strain showed that the CRE mutant knockout resulted in a less efficient biological pretreatment of wheat straw. In contrast, the *cre1* mutant, with overexpression, failed to improve efficiency, indicating that the secretion level of CAZymes was not exclusively dependent on CRE activity [193]. In the same sense, our results suggest that although high concentrations of glucose do not inhibit the transcription of other CAZymes, they influence the activity, isoenzymes, and inducing effect of laccase by copper, which influences the cellulose: lignin removal selectivity. The other CAZymes, CE4, CBM13, GH79, were detected in the mkPC9 secretome. However, the transcripts of three important enzymes that participate in the bioconversion of rice husks, AA9 (LPMOs), AA7 (GOOX), and GH45 (endoglucanases), overexpressed in our study, were not detected in the exoproteome of mkPC9 culture conditions [30].

Finally, our results show that the use of culture media modified with lignocellulosic biomass could represent a strategy to reduce laccase production costs by adding value to lignocellulosic residues and represents a strategy to produce enzymatic cocktails rich in laccase and other lignocellulolytic enzymes. Furthermore, this culture media-modified strategy could help design biological pretreatment systems for highly recalcitrant lignocellulosic biomass such as rice husks.

4.5 Conclusions

In this study, the effect of the composition of different culture media modified with rice husks in the presence and absence of copper sulfate on the laccase activity of *P. ostreatus* in submerged culture was evaluated. The inducing effect of the laccase activity of copper sulfate was affected by the rice husks concentrations in the culture media. Therefore, two culture media with different copper-induced laccase isoenzymes and activity were selected to compare the compositional changes of the rice husks recovered from these two systems, showing that the culture medium with the highest laccase activity presented the greatest lignin loss that was almost 50%; however, high lignin losses of around 35% were also observed in the modified media with the highest concentration of rice husks and the lowest magnitudes of laccase activity even in the presence of copper sulfate.

Other lignocellulose components were also affected during the treatment; percentages of cellulose loss around 30% and hemicellulose loss of 28% were achieved regardless of laccase activity. The analysis of the *P. ostreatus* transcriptome in synthetic media modified with rice husks in the presence and absence of copper sulfate showed that rice husks also induce transcripts of laccase and other CAZymes, which could explain the changes in lignocellulose of the rice husks observed in the absence of copper sulfate and with laccase activity two orders of magnitude lower than that induced by copper sulfate in modified media of the same composition.

Chapter 5

Comparative analysis of the *Pleurotus ostreatus* transcriptome in synthetic culture media, with rice husks and copper sulfate in SmF

Abstract: This study compared the *Pleurotus ostreatus* transcriptome in different culture media with laccase inducers in submerged fermentation. Copper sulfate and rice husks were used as laccase inducers to identify possible genes that regulate these enzymes laccase. For this purpose, a synthetic culture medium based on glucose and yeast extract was used to produce fungal biomass in the presence and absence of copper sulfate and the presence and absence of rice husks or both substances. The results show that copper induces laccase activity two orders of magnitude more than rice husks; however, rice husks could induce one extra isoenzyme laccase, and at the transcriptional level, rice husks induced l*acc2*, *lacc5*, *lacc6*, and *lacc10* as copper sulfate but at different relative order transcriptional using real-time qPCR. The comparative analysis of the transcriptome focused on identifying the most up and down-regulated genes for each pair of conditions tested and on the identification of genes centrally upregulated and down-regulated in all conditions tested. The *lacc2, lacc6*, and *lacc10* genes were in the top ten of the upregulated in copper sulfate cultures, while *lacc5*, *lacc6*, and *lacc10* were among the centrally upregulated genes in the presence or absence of copper sulfate or rice husk or both. It was found that a large number of transcripts correspond to unknown conserved proteins. The genes identified were grouped into four groups: structural components and binding protein, nitrogen metabolism and uptake nutrients, copper uptake, ion transports, and copper metabolism. The analysis of the expression level of these genes in the evaluated conditions allowed the identification of genes possibly associated with the morphological changes observed in the media with rice husks, CBM13, and Vmh2, transcripts associated with nitrogen metabolisms such as the NMR regulation factor, and copper transporter as CTRs involved in the copper uptake. Laccases and other copper-dependent proteins were overexpressed in the conditions evaluated in the presence of copper sulfate, which suggests that possibly several of the transcripts mentioned above are associated with laccase regulation. Other studies are needed to evaluate these possible associations.[5]

5.1 Introduction

Bioprocesses are based on detecting the environment and the consequent cellular response that all microorganisms and living organisms have developed in their evolutionary processes [78], [194]. This bidirectional detection and response process to the environment occurs sequentially through complex cellular pathways of reception,

[5] Full author list

Dinary Durán-Sequeda[1,2], Gúmer Pérez[2], Manuel Alfaro[2], Lucía Ramírez[2], Rocío Sierra[1], and Antonio G. Pisabrarro[2]

[1] Product and Process Design Group (GDPP), Department of Chemical and Food Engineering, Universidad de los Andes, Colombia; e-mail@e-mail.com

[2] Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre (UPNA), 31006 Pamplona, Navarre, Spain

transduction, and response to environmental signals [195]. In a bioreactor, the microorganisms receive the signals from the culture medium and the culture conditions and, in response, produce biomass, proteins, or substances of industrial interest.

In fungi, membrane proteins mediate the signal reception stage in this interaction between the organism and the environment [195]. On the one hand, some integral membrane proteins are part of membrane transport systems that transport ionized and hydrophilic molecules, such as salts, carbohydrates, or amino acids, that cannot pass freely through the membrane. These transport systems have been classified into two general groups: channels and carriers, including transporters, porters, or permeases. In this case, some molecule's intracellular and extracellular concentrations can be the signal that triggers a response [196]. Transport systems also act bi-directionally. In the case of metals, since many of them are cofactors of some enzymes that require metals such as Fe, Zn, Mn, or Cu, the metal transport systems are finely regulated to allow the taking of metals from the environment, maintain homeostasis, and avoid toxicity [197].

On the other hand, other membrane proteins belong to a protein system that participates in a signaling complex called the G-protein signaling system or Guanidine nucleotide-binding proteins [194]. This system has been described mainly in detecting glucose concentration and participates in detecting other external signals, including pheromones or stress conditions [198]. The G protein signaling complex comprises three components: a G protein-coupled receptor (GPCR), a transmembrane protein that forms seven loops, and whose two ends are located outside the cell; in the cytoplasm part of the complex, a heterotrimeric G protein is made up of three subunits, alpha (α), beta (β), and gamma (ϒ), and the last components is a GTP molecule bound to the alpha subunit [199]. The mechanism begins when the ligand in the medium (glucose or nitrogen) binds to the GPCRs; these induce a conformational change that activates the GTPase domain of α subunit, which separates from the trimer. Inside the cell, adenylate cyclase (AC) has a GTP-binding site attached to α subunit that activates its catalytic capacity and allows AC to convert ATP to cAMP. cAMP is a second messenger that activates gene transcription and regulates the R subunit of cAMP-dependent Protein Kinase downstream. Four cAMP molecules bound to the R subunit of PKA release the catalytic subunit of this protein. PKA participates in the control of glucose metabolism and the regulation of the cell cycle [26].

In submerged fermentation (SmF), filamentous fungi form compact and spherical structures of mycelial mass, called pellets [200]. The pellets have a spherical shape surrounded by a peripheral area from which filaments of different thicknesses and lengths protrude [201]. In addition to the pellets, mycelial or disperse mycelial hyphae structures can be found in fungal SmF cultures as prevalent morphology [9, 8]. Whether a filamentous fungus acquires one or another morphology depending on various factors such as initial inoculum concentration, pH, stirring speed, and the composition of the culture medium, among others [202]–[204]. The fungus morphology has several implications in the bioprocess: on the one hand, it is related to changes in viscosity and limitation in mass transfer in the culture; on the other hand, it is related to the production of specific metabolites [201], [204], [205].

Pleurotus ostreatus is a filamentous fungus belonging to the white-rot basidiomycetes group as it can produce several ligninolytic and lignin-modifying

enzymes (LMEs) [30]. The biotechnological potential of *P. ostreatus* today is centered on its ability to produce laccases [19], [59]. These multicopper oxidase enzymes are the main LMEs produced by *P. ostreatus* in the presence of copper sulfate [116], [121], and lignocellulosic substrates [30].

This study presents the macroscopic and molecular identification of a *P. ostreatus* isolate and compares its transcriptome when grown in synthetic media (Glucose, yeast extract, GY) and synthetic media modified with rice husks (GYR) in the presence and absence of 1mM copper sulfate (Cu^{2+}), and in SmF. This comparison focused on the transcripts differentially expressed between the synthetic medium and the modified media in the presence and absence of copper sulfate to identify transcripts associated with the effect on laccases of copper sulfate and rice husks.

5.2 Methods

5.2.1 Fungal strain and culture conditions

The *P. ostreatus* strain ANDES-F515, provided by the Laboratory of Mycology and Phytopathology of the Universidad de Los Andes-LAMFU and deposited in the ANDES Natural History Museum (MHN ANDES), was isolated in the Bosque de la Merced, Santa Bárbara village, Bojacá, Cundinamarca, Colombia. The dikaryotic mycelium was maintained and conserved on Malt Extract Agar at 4 °C, with periodic replication of the growth zone for eight days of incubation at 25 ° C in the dark.

Molecular identification was performed by analyzing two internal transcribed spacer sequences (ITS)[206]. In this analysis, the fungal DNA was first obtained using the EZNA SP Fungal DNA Mini Kit D5524-01. Then total fungal DNA was used as a template for ITS amplification by PCR, which was carried out using the universal primers ITS1_FW 5'-TCCGTAGGTGAACCTGCGG-3', ITS2_RV 5'-CGTAGCTACTTCTTGCGTCG-3', and ITS4b_RV 5'-GACCTGGCACATGTTCAGAGGAC-3' [206][207]. The amplification protocol was as follows: denaturation 5 min at 95 °C, 29 cycles of 1 min at 95 °C, 30 s at 53 °C, 1 min at 72 °C. The process ended with a final extension step of 10 min at 75 ºC. The PCR amplified products were run on a 1% agarose gel from which they were cut and purified using an E.N.Z.A® gel extraction kit and sequenced by the Sanger method. The ITS1-ITS2 DNA sequences and the ITS1-ITS4b sequences were compared with the compilation available in the GenBank/EMBL/DDBJ database using BLASTN (http://www.ncbi.nlm.nih.gov/blast).

The SmF cultures were performed in 500 mL flasks containing 200 mL of synthetic culture medium composed of 45 gL^{-1} glucose and 15 gL^{-1} yeast extract (GY culture). The rice husks was added at 40 gL^{-1} and the copper sulfate at 0.25 gL^{-1} (1mM). All SmF cultures were inoculated with five 4 mm diameter plugs taken from the growth zone of eight-day Malt Agar plate cultures and incubated at 25°C, 150 rpm, in the dark for 14 days. All experiments were performed in triplicate.

5.2.3 Laccase activity determination

Laccase activity was determined with ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) as the substrate [95]. The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 5.0), and 10 µL aliquots of an appropriately diluted enzyme sample. Oxidation of ABTS was monitored by following the increase in *A*436 (ε 29.3 $mM^{-1}cm^{-1}$). A laccase activity unit was defined as the enzyme required to oxidize 1 µmol ABTS per minute at 25 °C.

5.2.4 Zymograms

Non-denaturing gel electrophoretic conditions (native-PAGE) were used to visualize the isoenzymes present in the culture supernatants [98]. The stacking and running gels contained 4% and 9% acrylamide and were adjusted to pH 6.8 and 8.8, respectively, with Tris-HCl buffer. The running buffer was Tris-Glycine pH 8.3. The isozymes were revealed by staining the gel with 2mM of ABTS solution.

5.2.4 RNA isolation and transcriptional analyses

The fungal biomass produced all synthetic and modified culture media with or without 1 mM Cu2SO4 were harvested on day 14th of culture for RNA isolation. The mycelium was collected by centrifugation and filtration, immediately frozen in liquid nitrogen, and grounded in a mortar to a fine powder. Then, 100 ng of the powder was transferred to a 1.5 mL microcentrifuge tube for total RNA extraction using a Fungal RNA EZN.A Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturing guidelines. Finally, the integrity and quantity of RNA were validated using Bioanalyzer (version 2100) and Qubit 2.0 fluorometer.

5.2.5 Real-time qPCR

Reverse transcription (RT) was performed using 800 ng per sample of the total RNA to obtain cDNA in a 20 µL volume using an iScript cDNA synthesis kit (Bio-Rad, Alcobendas, Spain). According to manufacturing instructions in a thermal cycler, incubation of a complete reaction mix was carried out (MJ Research, Inc). RT products were diluted 1:20 and kept at -20 °C until used. Real-time qPCRs were performed in a CFX96 real-time system (Bio-Rad Laboratories, SA) using SYBR green dye to detect product amplification [59]. Each reaction mixture was set to a final volume of 20 µL containing 10 µL iQ SYBR green supermix (Bio-Rad Laboratories, SA), 2 µL of 5 µM stock forward and reverse primers (Table 2-2, in section 2.2.4, Chapter 2), 1 µL of diluted RT product, and 5 µL of sterile water. Cycling conditions were as follows: denaturation 5 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 63 °C, 15 s at 72 °C, and a final step with a step increase of 0.5 °C every 5 s from 65 to 95 °C in a linear gradient. Each reaction was performed in triplicate, and nontemplate controls (NTCs) were included for each primer set. An experimentally validated inter-plate calibrator (IPC) was used to compensate for inter-plate variation. Crossing-point (Cp) values and relative fluorescence units were recorded, and the latter was used to calculate amplification efficiencies by linear regression using the LinReg program [99]. Forward (Fw) and reverse (Rv) primers sequence for the laccase and reference genes were those described by Castanera *et al*.[28], [59], [99].

5.2.6 mRNA-seq analysis

Illumina compatible libraries were prepared to be sequenced using the Illumina Nova Seq 6000 system from an mRNA isolate originating from RNA total. Following sequencing, RNA-seq data were filtered for assurance quality using FastQC and trimmed with BBDuk to remove adapters and low-quality reads (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). The www.genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html was used as a *P. ostreatus* genome reference for aligning the resulting reads using STAR v2.3.16 [101]. The parameters used to achieve a single hit mapping were: --outReadsUnmapped Fastx --outFilterMismatchNoverLmax 0.04 --outFilterMultimapNmax 1. The mkPC15 v2.0 reference genome was assembled entirely in twelve scaffolds (34.3 Mb genome size)[102]. In total, 12,330 genes were annotated in this genome [103]. The expression levels were quantified using Python script rpkmforgenes.py (www.sandberg.cmb.ki.se/media/data/rnaseq/rpkmforgenes.py) to calculate values of reads per kilobase of transcript per million mapped reads (RPKM).

5.2.7 Differential gene expression analysis and gene annotation

Differentially expressed gene (DEG) analyses were performed using the EdgeR Bioconductor package and a dispersion parameter of 0.1. These analyses determined the transcriptional changes in the two culture conditions by comparing the gene expression values based on read counts. The gene expression values with Log_2 fold changes of read counts with a p-value < 0.01 and an FDR (False Discovery Rate) < 0.05 as the cut off for statistical significance were used. A DEG with $\log_2$ Fold Change ≥ -2 was established as an upregulated gene, and $\log_2$ Fold Change ≤-2 was established as a downregulated gene.

The genes annotations were based on the JGI automated annotation to transcript identifications of the mkPC15 v2.0 reference genome. JGI automated annotation uses the following databases: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), InterPro (IPR), Eukaryotic Orthologous Groups (KOG) and Enzyme Commission numbers (EC number), Transporter Classification Database (TCDB), Carbohydrate-Active EnZymes (CAZymes) and MEROPS database (proteolytic enzymes) [104]. For the unannotated genes, the Basic Local Alignment Search Tool (BLAST) was used to find local similarity between JGI sequences unannotated and The National Center for Biotechnology Information (NCBI) standards database sequence [105], [106].

5.3 Results and discussion

5.3.1 Morphological and molecular identification of P. ostreatus ANDES-F515

Figure 5-1 shows the fruiting bodies of the *P. ostreatus* ANDES-F515 isolate. In this figure, at least four basidiocarps can be seen. The largest basidiocarp has a short stipe with a wide rolled cap with wavy edges and a whitish color. The lamellae are white and descend to the stipe. Three lower and anterior basidiocarps show that the stipe is

off-center. All these characteristics correspond to the genus *Pleurotus*. Additionally, the molecular characterization of the species by the analysis of ITSs produced a significant alignment of the ITS1-ITS4b sequence obtained from the DNA of the fungus with the accession MT778826 *P.ostreatus* available in the GenBank / EMBL / DDBJ database using BLASTN (http://www.ncbi.nlm.nih.gov/blast). This alignment had 95.86% identity, 97% query coverage, and an E value of 0.0. Other *P. ostreatus* sequences also had good homology, and although the ITS1-ITS4b sequence also had a significant alignment with other fungal strains such as *Laccocephalum mylittae*, the basidiocarp of this species has very different morphological characteristics.

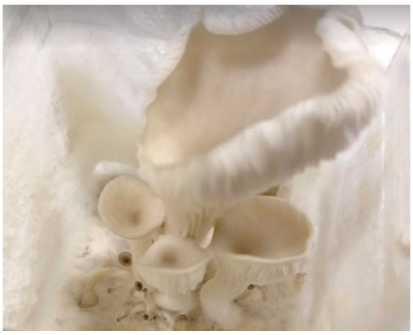
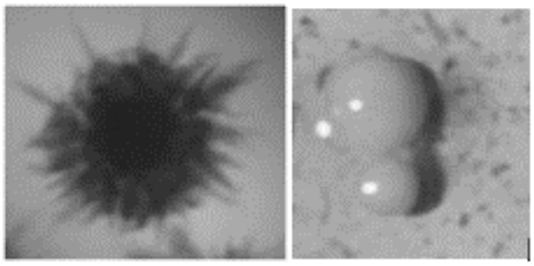


Figure 5.1. The fruiting body of *P. ostreatus* ANDES-F515

5.3.2 Morphological characteristics of P. ostreatus ANDES-F515 in submerged cultures supplemented with rice husks

The pellets obtained from the GY culture medium (glucose-yeast extract) without rice husks and those obtained from the GYR culture medium (GY with rice husks) are shown in Figure 5-2. One of the main differences observed between GY synthetic media and GYR rice husks modified media was the morphology of the pellets. These visible differences in the pellets were independent of the presence of copper sulfate. On the one hand, the pellets produced in the GY medium had the typical spherical shape surrounded by a peripheral zone from which filaments of different thicknesses and lengths protruded. On the other hand, the pellets produced in the GYR medium had a spherical, compact, and smooth shape. The size of the pellets was variable during the first 8 days of culture.



(a) (b)

Figure 5.2. *P. ostreatus* growth in SmF: (a) Pellets in GY synthetic culture media; (b) Pellets in synthetic media modified with rice husks (GYR).

The morphological changes observed in the pellets in SmF cultures of *P. ostreatus* can result from many factors, including inoculum size and culture conditions

such as mechanical agitation [208]. In SmF of several filamentous ascomycetes, the relationship between the change in morphology, mainly from dispersed mycelium to pellets, and the secretion of enzymes or metabolites of interest has been reported [49], [209]. In some cases, these changes occurred due to changes in the type of carbon source [210], affecting enzyme secretion [211]. In another white-rot basidiomycete fungus, *Phenerochaete chrysosporium* in SmF, the pellet morphology was associated with lignin peroxidase secretion [212], [213]

Some studies have demonstrated the effect of mechanical agitation in submerged cultures of *P. ostreatus*, and the effect of hydrodynamic stress on laccase activity has been evaluated. Initially, agitation was found to affect laccase activity, perhaps due to stimulation of proteases [73]. Subsequently, it was found that increasing agitation reduces the size of the pellets, which favors fewer diffusion limitations and an increase in metabolically active biomass, which positively affects *lacc10* transcription levels [214].

5.3.3 Laccase activity and laccase gene expression in P. ostreatus SmF supplemented with rice husks and copper

The synthetic culture medium glucose-yeast extract (GY) composition resulted from the study of various GY media in which different concentrations of both compounds were tested. From this evaluation, the GY medium of composition (45 gL^{-1} glucose, 15 gL^{-1} of yeast extract: GY 4515) yielded the highest biomass production. Similarly, the amount of rice husks added to the GYR medium resulted from evaluating various rice husks concentrations to increase biomass and laccase production in the presence of 1 Mm copper sulfate. We selected these culture media to study the laccase activity and laccase gene transcription in the presence of these inducers.

The laccase activity released to the supernatant in SmF cultures of *P. ostreatus* supplemented with rice husks and copper is shown in Figure 5-3 (a). Copper addition increased the laccase activity recovered in the supernatant both in the absence (GY cultures) and the presence of rice husks (GYR). In the absence of copper sulfate, the basal laccase activity recovered was similar in the GY and the GYR media, although, in the presence of copper, the activity recovered from the GY media was higher than that from the GYR, suggesting that the macromolecular lignocellulose present in the rice husks is not a potent laccase inducer in these culture conditions.

The zymographic study of the laccase activities recovered is shown in Figure 5-3 (b). Three sets of activity bands were detected with apparent mobilities of 35, 40, and 75 kDa. The 35 and 40 kDa bands were detected in all the samples, albeit the inducer increased their intensity. Moreover, an additional band of 75 kDa (presumably corresponding to Lacc6) appeared in the copper-supplemented cultures, although its induction was more robust when the rice husks were absent in the media. Interestingly, a high molecular size band was consistently observed in the GRY cultures supplemented with copper absent in all the other conditions. The nature of this band deserves further investigation as it has not been identified yet.

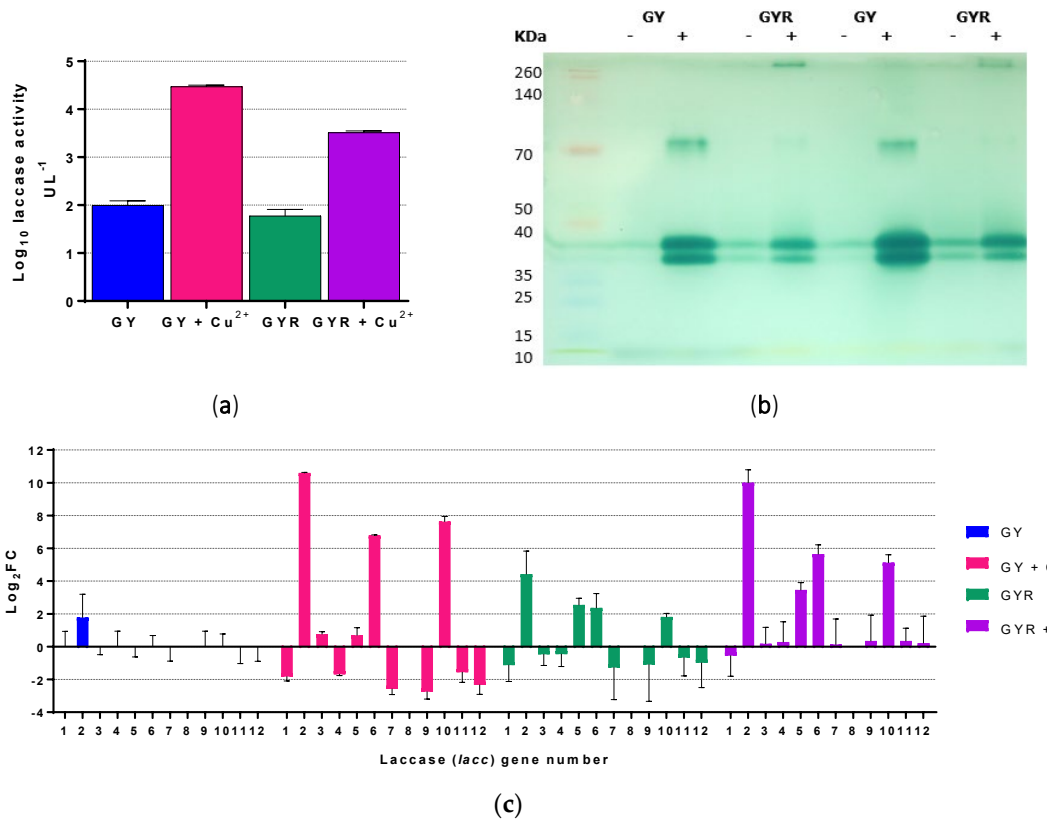


Figure 5.3. Laccases in GY and GYR culture media of *P. ostreatus*: (**a**) Laccase activity; (**b**) Isoenzymes of laccases in the liquid culture extract, media without copper (-), with copper (+); (**c**) relative transcription of twelve laccase genes in *P. ostreatus*. The transcript levels were expressed as fold changes (FC) compared to the housekeeping genes.

We carried out a qPCR analysis of the 12 laccase gene transcription in these conditions to validate the results obtained. The results are shown in Figure 5-3 (c). We could detect laccase genes transcription in eleven of the twelve laccases genes (all but *lacc8*), although at very different levels. An intense copper inducer effect was observed for *lacc2*, *lacc6,* and *lacc10* in the presence and the absence of rice husks. In these three cases, the inducer effect was slightly higher in the absence than in the presence of rice husks, which fits with the activity and zymographic results discussed above. Interestingly, an apparent laccase expression inducer effect was seen for the rice husks in *lacc2, lacc*5, *lacc6*, and *lacc10,* even in the absence of copper. In these cases, the rice husks inducer effect is weaker than the copper effect (compare lanes GY, GYR, and GY+Cu^{2+}), and these two inducer effects seem not to be additive suggesting that they are somehow competitive pathways. The other six laccase genes, *lacc4*, *lacc7*, *lacc9*, *lacc11,* and *lacc12,* were downregulated expression at different levels in GY+Cu^{2+} and GYR experimental conditions.

5.3.4 Transcriptome analysis of P. ostreatus SmF supplemented with rice husks and copper

To study the effect of the addition of rice husks or copper to SmF cultures of *P. ostreatus* made in GY 4515, the complete transcriptomic profiles of the samples corresponding to cultures without inducer (GY), supplemented with either one of them

(GY+ Cu^{2+}, and GYR), and supplemented with both of them (GYR + Cu^{2+}) were obtained.

Figure 5-4 shows the volcano plot distribution of the pairwise comparisons of the transcriptomes recovered from cultures made in the above conditions using an FDR <0.05, log_2 FC≥2 as a threshold to differentially expressed genes (DEGs) downregulated (log_2FC≤-2) and upregulated (log_2FC≥2). The addition of rice husks (GYR) had a more substantial effect than the addition of copper (GY+Cu^{2+}), as shown by the larger number of DEG detected when compared to the basal condition (GY): whereas the expression of 142 genes was downregulated upon husks addition, only 90 were so upon copper supplementation. A similar result can be seen in the upregulated genes where 386 were detected upon rice husks, and copper comparatively upregulated just 189. The effect of the combined addition of rice husks and copper (GYR+Cu^{2+}) revealed 172 downregulated and 412 upregulated genes in comparison with the basal state (GY). These results highlight that the addition of rice husks produces a more generalized metabolic effect than the addition of copper and that this broader effect of the lignocellulose is somehow dominant over the effect of the mineral inducer, as the combined effect of both supplements is more similar to the sole effect of rice husks. These results also indicate the specificity of copper as a laccase inducer since its effect on the expression of these genes is larger than the corresponding effect at the whole genome level.

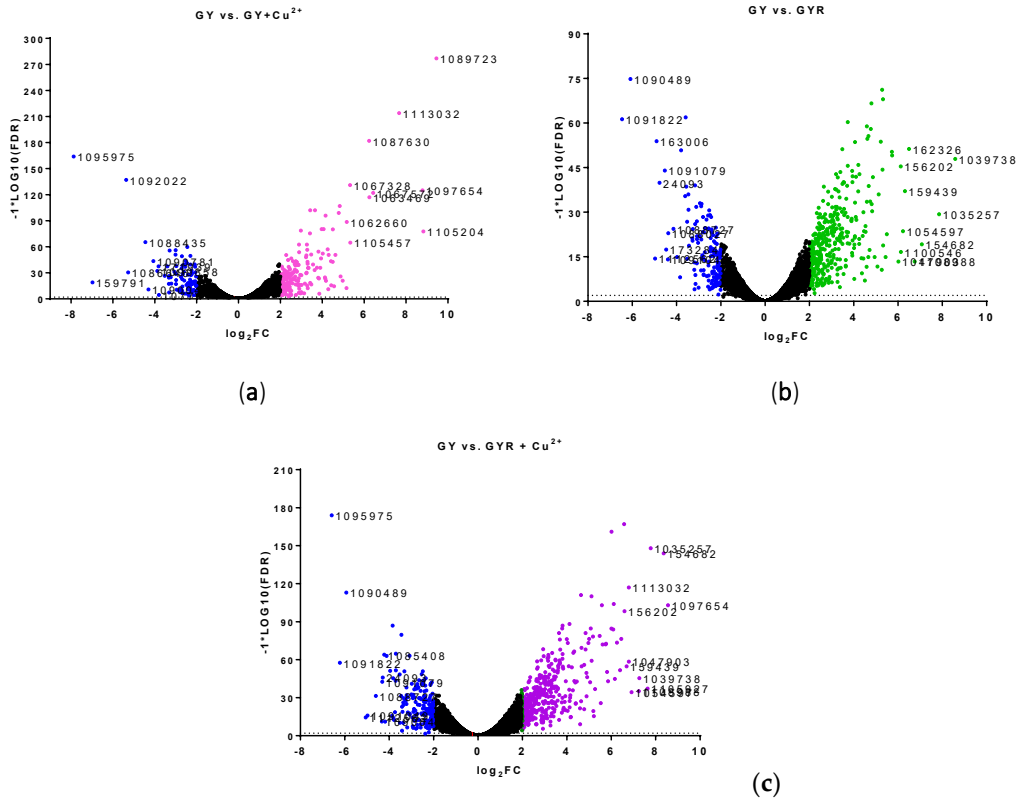


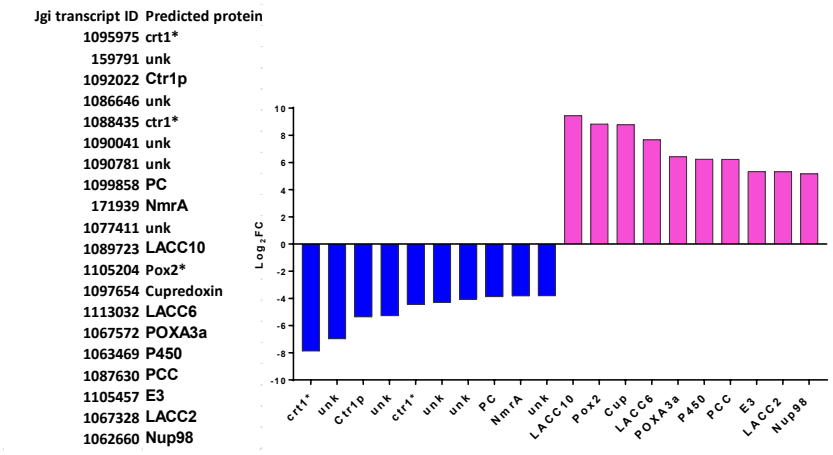
Figure 5.4. Volcano plots highlighting the differentially expressed genes (DEG), downregulated (log_2FC≤-2), and upregulated (Log_2FC≥2) in *P. ostreatus* from three different transcriptome comparisons: (**a**) GY vs. GY+Cu^{2+}; (**b**) GY *vs*. GYR; (**c**) GY *vs*. GYR+Cu^{2+}

From this analysis, the ten most downregulated and upregulated transcripts identified in each comparison are shown in Figure 5-5. The genes were called after the automatic annotation of *P. ostreatus* PC15 available at www.genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html. Of the 60 transcripts corresponding to the top ten upregulated and downregulated genes in the three pairwise comparisons, 28 coded for genes with unknown function. The unknown genomic sequences were aligned against GenBank/EMBL/DDBJ database using BLASTN (http://www.ncbi.nlm.nih.gov/blast). This analysis allowed aligning only three of the 28 unknown sequences to genes annotated in the GenBank. The identified genes corresponded to transcripts 1095975, 1088435, and 1105204.

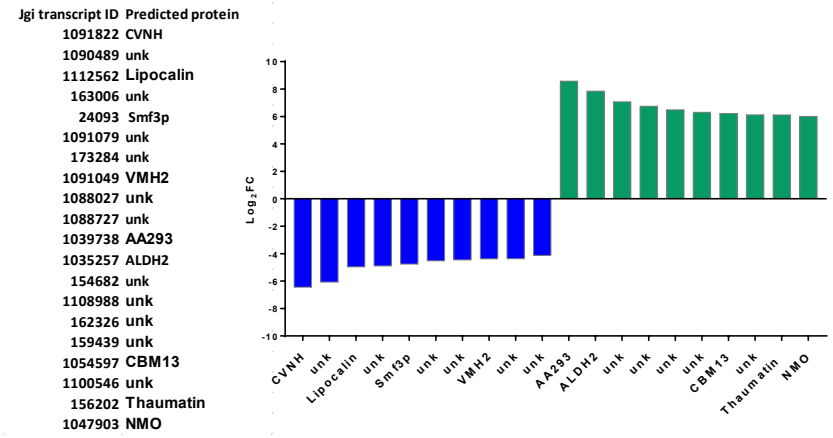
In the presence of copper sulfate (Figure 5-5, a), three genes involved in copper transport were among the top ten downregulated compared to the basal state (GY), being the copper transporter *ctr1* the most negatively regulated transcript in these conditions. This result suggests that *P. ostreatus* controls the entry of copper into the cell by effectively reducing the expression of the transporters. Copper is an essential cofactor for laccase activity, but it is also a potentially toxic element with a high unspecific ability to bind to different proteins. The high concentration of copper used in this analysis (1 mM) could make it essential for the fungal homeostasis to control the entry of this inducer. On the other hand, five laccase-related (*lacc10*, *pox2*, *lacc6*, *poxA3a*, and *lacc2*) appeared most upregulated by copper's presence. These results fit with those of the qPCR experiment discussed above. Besides these genes, a cupredoxin coding gene is also among the top ten upregulated genes in the presence of copper. All these results reinforce the idea discussed above of the specificity of the copper effect on the whole gene transcription in *P. ostreatus* in these culture conditions.

The effect of rice husks addition (Figure 5-5, b) reveals a less specific effect. More than half of the top ten upregulated and downregulated genes were annotated as unknown, and none of them was related to the laccase enzyme family. This result strengthens the hypothesis put forward above of a general metabolic effect of adding lignocellulose to de culture medium compared to the more specific effect of copper addition.

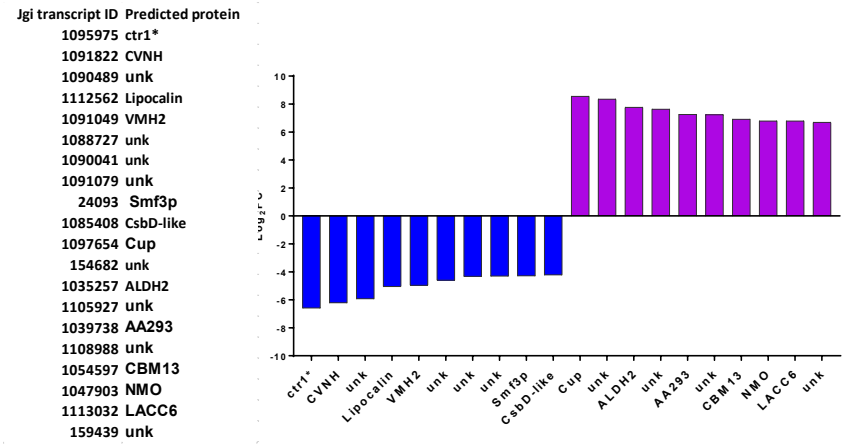
Finally, it was of particular interest to compare the up and downregulated genes upon the combined addition of the two inducers because, as we discussed above, the effect of rice husks addition was somehow dominant over the effect of copper addition. This comparison between the basal GY and GYR+Cu^{2+} is shown in Figure 5-5, panel c. As expected, the pattern of up and downregulated gene expression upon the combined addition of rice husks and copper was more similar to that produced by rice husks than to the one resulting after the addition of copper. Only two of the downregulated genes in GYR+Cu^{2+} were also downregulated in GY+Cu^{2+}, whereas six were upregulated in the GYR culture. Similarly, only two of the genes upregulated in GY+Cu^{2+} were also upregulated in GYR+$Cu^{2+,}$ and seven were upregulated in GYR. It is noteworthy that the most downregulated gene in the GYR+Cu^{2+} culture was *ctr1* coding for a copper transporter, and the more upregulated the gene coding for a cupredoxin. These two genes were also the more relevant in the case of the GY+Cu^{2+} cultures. We suggest that this result reflects the reaction of the fungus against the high copper concentration in these conditions.



(a)



(b)



(c)

Figure 5.5. The top ten of differentially expressed genes downregulated (Log_2FC <-2) and upregulated (Log_2FC ≥2) genes in the transcriptome of *P. ostreatus* in culture media:(**a**) GY *vs*. GY+ Cu^{2+}; (**b**) GY *vs*. GYR ; (**c**) GY *vs*. GYR+Cu^{2+}.

Our next aim was to identify the transcripts that were up and downregulated in all culture conditions. For that purpose, we grouped all the down-regulated and upregulated transcripts with $log_2FC \geq 2$ in Venn diagrams to identify their relationships. The results are shown in Figures 5-6. If we focus on the 251 downregulated genes (left panel), we can see that 90 of them are related to copper, 142 to the presence of rice husks, and 172 to the combined presence of both inducers. Of the 90 genes downregulated by copper, the expression of 64 of them (71.1 %) is specifically repressed by the addition of copper sulfate. In comparison, only 14 of the 142 genes (9.9 %) repressed by the addition of rice husks are specifically downregulated. If we focus on the upregulated genes, the picture is similar: 136 of the 189 (72 %) of the genes upregulated by copper are specific, whereas only 37 of the 386 whose expression is upregulated by rice husks (9.6 %) were specific for this inducer. These results again support the idea of a more general change in the transcription profile when rice husks are added than when copper is supplemented. In the first case, a general adaptation of the metabolism occurs, whereas in the second, it can respond to the stress caused by the presence of the metal ion.

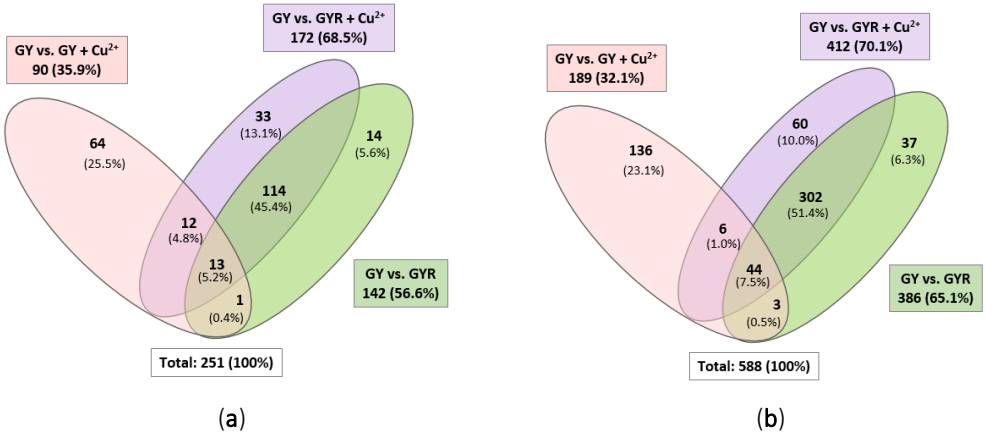


Figure 5.6. Venn diagram of DGE numbers in the *P. ostreatus* transcriptome in glucose- yeast extract (GY) culture media with copper ($GY+Cu^{2+}$), rice husks (GYR), and both rice husk and copper ($GYR+Cu^{2+}$): (**a**) Downregulated; (**b**) Upregulated

Focusing on the genes up and downregulated in the three conditions, six out of the 13 genes consistently downregulated (Figure 5-7, a) coded for unannotated proteins. All of them but gene 159791 showed similar levels of downregulation. There were 44 genes upregulated in the three conditions, 25 unannotated (Figure 5-7, b) that displayed different upregulation patterns. For seven of them, upregulation seemed to be more robust when rice husks were present in the culture medium (genes 1089499, 1101109, 1090872, 1087565, 1092192, 1087569, and 6510). The 19 consistently upregulated genes for which an annotation was available (Figure 5-7, c) showed variable upregulation patterns, although seven showed the same pattern associated with rice husks addition described above. This group of consistently upregulated genes included three laccases (*lacc5*, *lacc6,* and *lacc10*) and three copper-related genes (genes 1097654, 1089715, and 1034299) whose upregulation, genes except for *lacc5*, seemed to be more robust upon the addition of copper (compare the bars for $GY+Cu^{2+}$ and $GYR+Cu^{2+}$ with that of GYR). These results suggest that the rice husks can partially induce copper transporters and laccases by a pathway presumably different from copper induction. These commonly regulated transcripts and the more over-expressed

transcripts were compiled with their predicted functions in Table 5-1. This table excludes transcripts of unknown or uncharacterized functions.

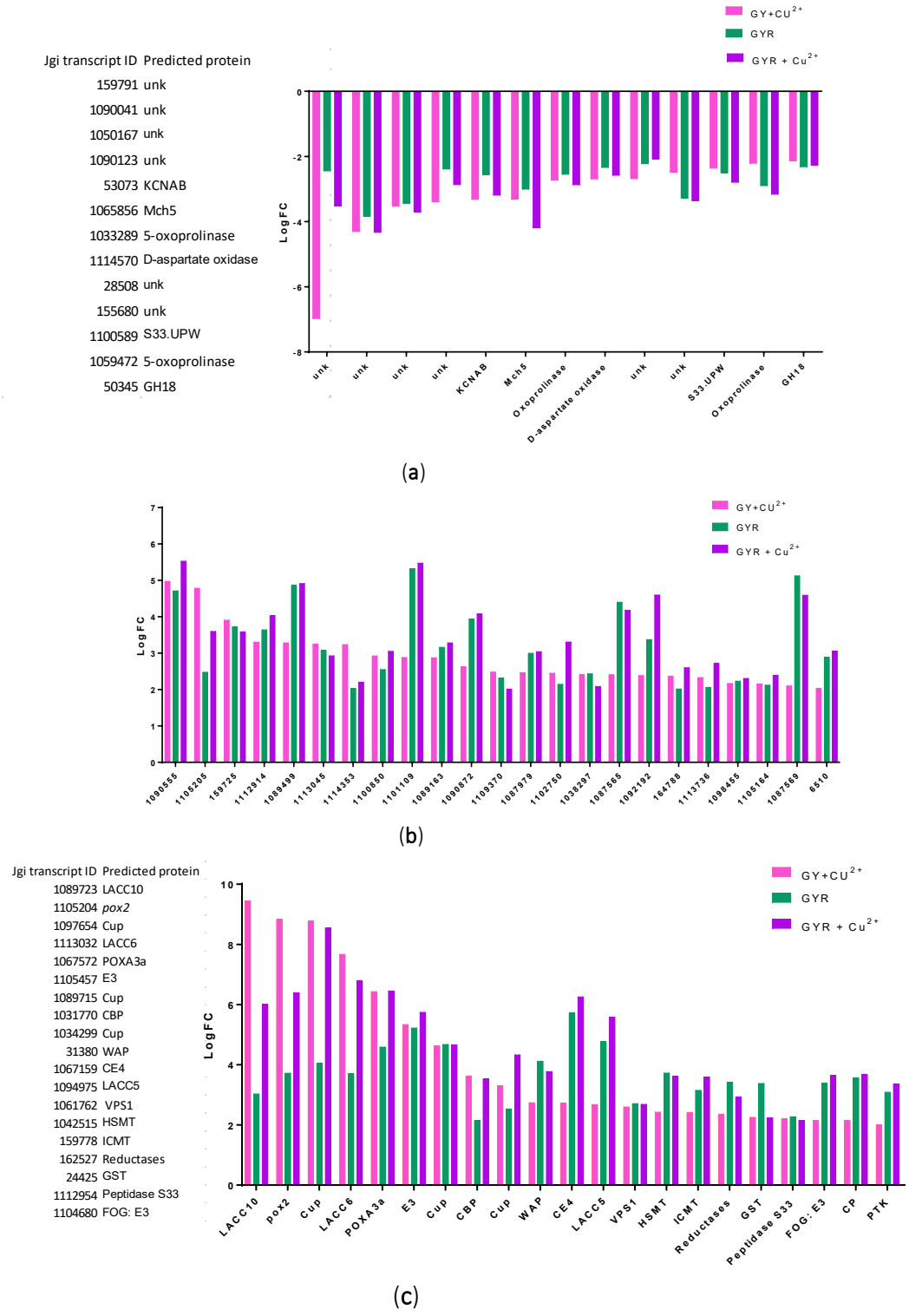


Figure 5.7. Common differentially expressed genes (DEG) in GY, GY with copper, GY with rice husks, and GY with rice husks and copper culture media of *P. ostreatus*: (**al**) unknown and annotated downregulated DEG ; (**b**) unknown upregulated DEG (**c**) annotated upregulated DEG.

Table 5-1. The top-ten and common down-regulated and up-regulated genes in the pairwise *P. ostreatus* transcriptomes. Exclude unknown genes.

Gene ID	Protein	Predicted protein or function	Log$_2$FC		
			GY vs. GY+Cu^{2+}	GY vs. GYR	GY vs. GYR+Cu^{2+}
1089723	LACC10	Multicopper oxidases, laccase LACC10=POXC	9.4	3.0	6.0
1105204	*pox2*	Phenol oxidase 2 gene (pox2) (unannotated gen)	8.8	3.7	6.4
1097654	Cup	Cupredoxin homologous superfamily type	8.8	4.0	8.6
1113032	LACC6	Multicopper oxidases, laccase LACC6=POXA1B	7.7	3.7	6.8
1067572	POXA3a	Small subunit of laccase POXA3a	6.4	4.6	6.5
1063469	P450	Cytochrome P$_{450}$ CYP2 subfamily	6.2		
1087630	PCC Ca^{2+}	Ca^{2+}-modulated nonselective cation channel polycystin	6.2		2.6
1105457	E3	Predicted E3 ubiquitin ligase	5.3	5.2	5.7
1067328	LACC2	Multicopper oxidases, laccase LACC2	5.3		5.6
1062660	Nup98/Cu^{2+}	Nuclear pore complex, Nup98 component, and Cupredoxin domain	5.2		
1089715	Cup	Cupredoxin homologous superfamily type	4.6	4.7	4.7
1031770	PC-Cu	Blue (type 1) copper domain	3.6	2.1	3.5
1034299	Cup	Cupredoxin homologous superfamily type	3.3	2.5	4.3
31380	WAP	WAP-type 'four-disulfide core' domain, antiproteinase	2.7	4.1	3.8
1067159	CDAs, CE4	Chitin deacetylase, Carbohydrate Esterase Family 4 (CE4)	2.7	5.7	6.3
1094975	LACC5	Multicopper oxidases, laccase LACC5	2.7	4.8	5.6
1061762	VPS1	Vacuolar sorting protein 1, VPS1, dynamin, and related proteins	2.6	2.7	2.7
1042515	HSMT	Homocysteine S-methyltransferase	2.4	3.7	3.6
159778	ICMT	Isoprenylcysteine carboxyl methyltransferase (ICMT) family	2.4	3.2	3.6
162527	ACP	3-oxoacyl-[acyl-carrier-protein] reductase	2.4	3.4	2.9
24425	GST	Glutathione S-transferase	2.3	3.4	2.2
1112954	S33	Peptidase S33, prolyl aminopeptidase	2.2	2.3	2.2
1104680	FOG:E3	FOG: Predicted E3 ubiquitin ligase	2.1	3.4	3.6
1069722	CP	Cerato-platanin like	2.1	3.6	3.7
1105411	PTK	Protein tyrosine kinase	2.0	3.1	3.4
50345	Chts, GH18	Chitinase, Glycoside Hydrolase Family 18	-2.1	-2.3	-2.3
1059472	Oxoprolinase	Oxoprolinase	-2.2	-2.9	-3.2
1100589	S33.UPW	Peptidase S33, prolyl aminopeptidase	-2.4	-2.5	-2.8
1114570	DAO	D-aspartate oxidase	-2.7	-2.3	-2.6
1033289	Oxoprolinase	Oxoprolinase	-2.7	-2.5	-2.9
1065856	Mch5	High affinity facilitated diffusion, riboflavin-regulated riboflavin uptake system	-3.3	-3.0	-4.2
53073	KCNAB	Voltage-gated shaker-like K+ channel, subunit beta/KCNAB	-3.3	-2.6	-3.2
171939	NmrA-like	Nitrogen metabolite repression in fungi	-3.8		
1099858	PC	Polyketide cyclase / dehydrase and lipid transport	-3.9		
1088435	*ctr1*	predicted Copper transporter gene (unannotated gen)	-4.4		-2.4
1092022	Ctr1p	High-affinity copper transporter, Ctr1p	-5.4		-4.1
1095975	*ctr1*	predicted Copper transporter gene (unannotated gen)	-7.9		-6.6
1091822	CVNH	CyanoVirin-N Homology domain		-6.5	-6.2
1112562	Lipocalin	Lipocalin family type		-5.0	-5.1
24093	Smf3p	Intracellular heavy metal transporter, Smf3p		-4.8	-4.3
1091049	VMH2	Specific vegetative mycelium hydrophobin 2		-4.4	-5.0
1085408	CsbD-like	general stress response protein		-3.8	-4.2
1054597	CBM13	Carbohydrate-Binding Module Family 13		6.2	6.9
1039738	AA293	predicted metallo-protease		8.6	7.3
1035257	ALDH2	Aldehyde Dehydrogenase (NAD+)		7.9	7.8
156202	Thau	Thaumatin family, stress tolerance		6.1	6.6
1047903	NMO	Nitronate monooxygenase		6.0	6.8

5.3.5 Transcriptome values for the laccase genes in P. ostreatus SmF supplemented with rice husks and copper

This section focuses on and summarizes the transcriptome values for the 12 laccase genes annotated in the genome of *P. ostreatus*, as found in the experiments described in this chapter. The expression values and profiles are shown in Figure 5-8. We found the expression of all the annotated laccase genes except *lacc8*. This gene is annotated in the genome sequenced of monokaryons mkPC15, but it is absent in the genome of monokaryon mkPC9. We have not carried out a whole-genome sequence of the strain used in this work, but as there was no transcription signal for this gene, we suggest that *lacc8* is not present in the ANDES-F515 dikaryotic strain used in this work and that it is an mkPC15-specific laccase gene. All the other laccase genes showed expression in the GY medium without specific inducers (log_{10} RPKM <3). In the cultures carried out without inducers, there was not a clearly predominantly expressed laccase gene. Upon copper addition as an inducer, the transcription of genes *lacc2*, *lacc6,* and *lacc10* was higher, becoming the predominant laccases under these conditions. The addition of rice husks slightly increased the laccase gene transcription as a whole. Interestingly, under these conditions, the more expressed laccase gene was *lacc5*, slightly predominant over *lacc6* and *lacc10*. The *lacc5* gene codes for an enzyme with signatures of ferroxidase and, consequently, cannot be considered a canonical laccase. The combined addition of copper and husks promotes laccase genes *lacc2*, *lacc6, lacc10,* and the non-canonical *lacc5* as the more expressed. These results also suggest that of all the laccases evaluated, *lacc2* is the one that responds most specifically to induction by copper.

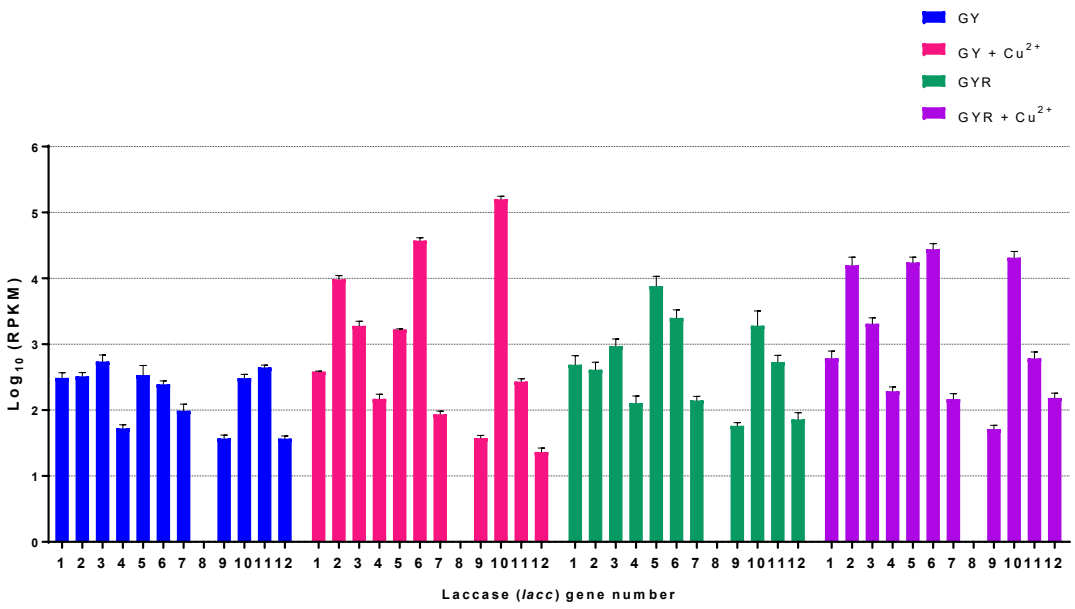


Figure 5.8. Profile of the transcript levels of twelve laccases genes in transcriptome of P. ostreatus in different culture media. The transcript levels were expressed as log_{10} of reads per kilobase of transcript per million mapped reads (RPKM).

5.3.6 Functional grouping of the top and common differentially regulated genes

To further compare the similarities and differences of the expression levels of most regulated genes in the culture media with or without copper sulfate and rice husks, we grouped most of those shown in Table 5-1 into four classes according to their predicted function. These groups are shown in Figure 5-9. The first group clusters the transcripts coding for structural components or binding protein (Figure 5-9, a). The transcripts associated with proteins that participate in nitrogen metabolism or nutrient uptake were grouped on the second. (Figure 5-9, b), on the third one, the transcripts are associated with ion transport (Figure 5-9, c), and the last group includes various transcripts associated with copper metabolism (Figure 5-9, d).

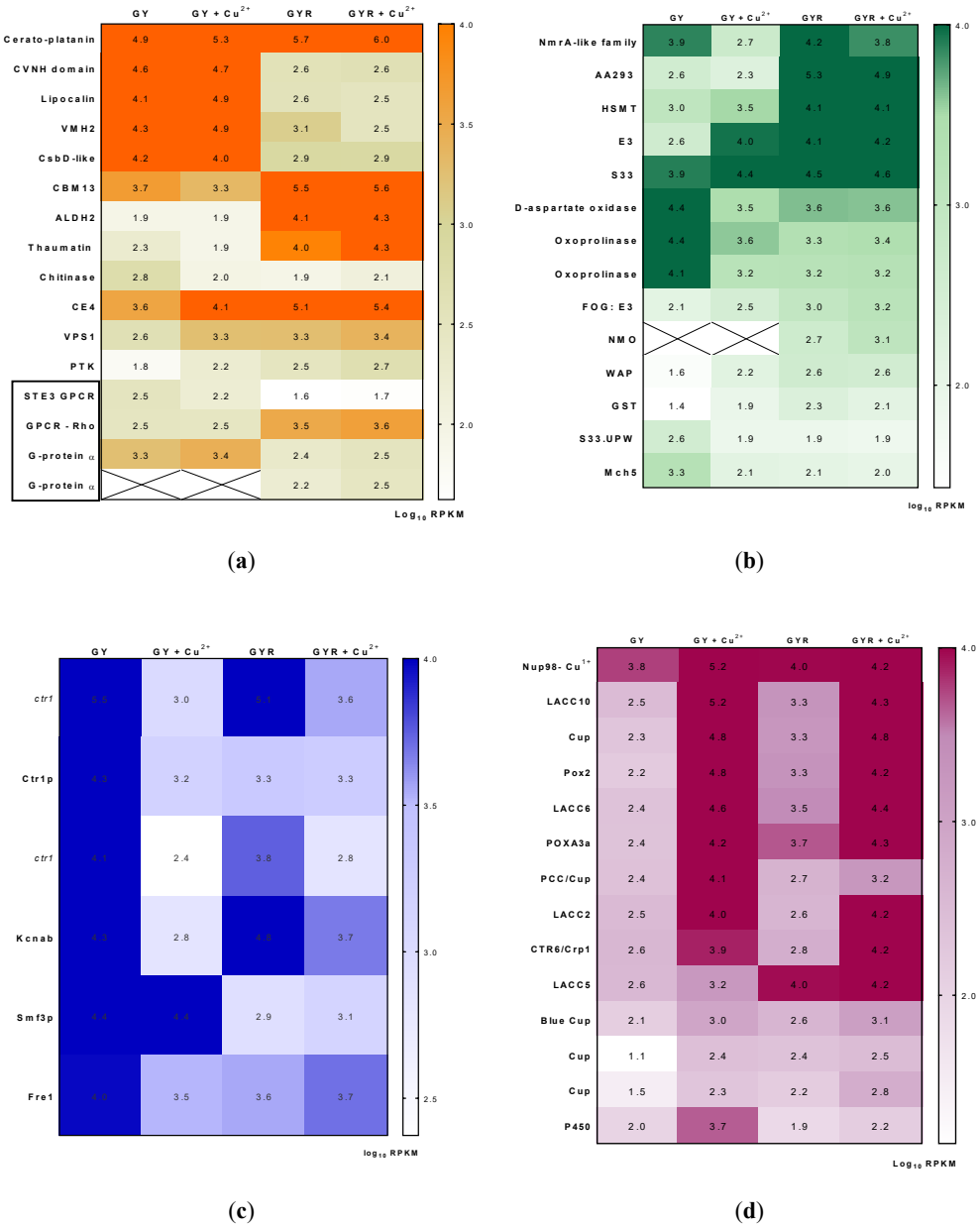


Figure 5.9. Level of expression of differentially expressed genes associated with several predicted functions in the transcriptome of *P. ostreatus* in different culture media: (**a**) Structural components and binding protein, in the box four proteins of G-proteins signaling system were included; (**b**) nitrogen metabolism and uptake nutrients;

(**c**) copper uptake and ion transports; (**d**) copper metabolism. The level of expression is presented as log10 RPKM.

In the group of structural proteins and binding proteins (Figure 5-9, a), the most overexpressed gene in the presence of inducers was coding for a cerato-platanin like protein. Cerato–platanins are cell wall fungal hydrophobin proteins [215]. On the other hand, a group of four transcripts, including vegetative hydrophobin Vmh2, and other binding proteins, was overexpressed in non-rice husks culture media. In contrast, in the presence of alone rice husks, CBM13, ALDH2, and thaumatin predicted genes appeared overexpressed. *P. ostreatus* Vmh2 is a hydrophobin that assembles differently under different environmental conditions [216],[217], and glycans increase its solubility [218]. In the culture media with rice husks, the presence of these glycans in the nutritional environment surrounding the fungus is suggested by the increase in CBM13, which could explain why, in rice husks, this hydrophobin is down-regulated. Although in the predicted transcriptome of monokaryons mkPC15 and mkPC9, the most overexpressed hydrophobin would be Vmh3, although its overexpression level changes with the type of culture [219]. This study observed that the type carbon source could also regulate this type of vegetative hydrophobins.

A gene coding for chitin deacetylase is overexpressed while that of chitinase is down-regulated. In addition to structural modifications, depolymerization and de-N-acetylation of chitin by chitinases and chitin deacetylases could also be associated with cell signaling and morphogenesis [220]. An important detail about chitin deacetylases was that their enzymatic activity was increased in the presence of metal ions, mainly Cu^{2+} and Co^{2+} [221]. In our case, the presence of Cu^{2+} or other metals in the rice husks could be related to the increase in the positive regulation of this transcript in all culture media conditions with laccase inducer.

One of the main differences between the cultures with and without rice husks is the morphology of the pellets. Therefore, because two transcripts encoded for GTP-related proteins, a protein tyrosine kinase (PKT) and a vacuolar sorting protein -GTPase dynamin (VPS1) were overexpressed, in Figure 5-9, four proteins from the G protein signaling system STE3-GPCR (gene ID 1025941), Rho-GPCR (gene ID 173720) G protein α-subunit_1 (gene ID 1064731) G protein α-subunit_2 (gene ID 1051028) were additionally check and included in this analysis. However, the possible interaction between these four proteins is unknown. The results show that copper and rice husks positively regulated PKT and VPS1; however, in the rice husks media, the G protein α-subunit_2 and STE3-GPCR were down-regulated while another G protein α-subunit_1 and Rho-GPCR appeared upregulated in these cultures.

The relationship of the G-protein signaling system in *P. ostreatus* in some cellular processes has briefly been studied in a few studies. One of them has shown that in the fungal proteome of the mkPC9 strain, some protein kinases were found to change their expression in the presence of different lignocellulose derived [222]. In another study, this same monokaryon mkPC9 also showed that the overexpression of a gene encoding cAMP-dependent protein kinase A (PKA) catalytic subunit resulted in a higher transcription level of laccase [223]. On the other hand, non-selective inhibition of protein kinase by the antibiotic staurosporine affected the growth and hyphal morphology of *P. ostreatus* [224]. Finally, mkPC9 transformant mutated in a gene coding G protein α-subunit increased the Intracellular cAMP and the laccase activity by the inducer $CuSO_4$ and ferulic acid [225]. These results (Figure 5-9, a) suggest that several GPCRs mechanisms could be regulated in the evaluated conditions, one related to laccase

regulation and others related to the morphological changes of the pellets in submerged culture in the presence of rice husks lignocellulose; however other studies are needed.

Copper sulfate-induced laccase activity in *P. ostreatus* and other fungi has been positively associated with yeast extract's organic nitrogen source [110]. Figure 5-8 (b) shows that the transcripts coding for proteins associated with nitrogen uptake and metabolism were differently downregulated and upregulated. In culture media without laccase inducers, the negative regulation of several genes related to nitrogen metabolism was increased. These genes correspond to two oxoprolinases and one D-aspartate oxidase. The activity of these two enzymes increases the levels of L-glutamate and ammonium, both responsible for the activation of the nitrogen metabolic repression (NMR) mechanism. The NMR regulator is increased in the inducer-free conditions and rice husks, apparently because of an increase in peptidase, metalloprotease activity, and even mechanisms of intracellular degradation via the proteasome. Although in the media that only contained copper sulfate as a laccase inducer and some transcripts with peptidase and ubiquitin ligase activity were also increased, NMR was not overexpressed.

Figure 5-8 (c) shows that the transcripts of proteins associated with cations transport and copper uptake and metabolisms were differently down and upregulated. Five transcripts with a function associated with cation transport were overexpressed in GY media. Three transcripts were identified as Ctr1 transporters, a potassium channel (Kcnab), and a manganese and iron transporter (Smf3p). Only one of the three copper transporters (ctr1) was overexpressed in copper-free GYR media as Kcnab did. Smf3p has been implicated with a potential role in Cu^{+} uptake [226]. This transporter overexpression occurred in both copper and non-copper GY media but was reduced in GYR media. These transporters tended to be negatively regulated by copper under conditions of copper excess, as the results of Figure 5-8 show. This negative regulation of ctr1 by copper in *P. ostreatus* has been previously shown [123]. Because the pentahydrated copper sulfate generates Cu^{2+} ions that the Ctr1p cannot transport, the reduction of Cu^{2+} to Cu^{+} is mediated by Fre1, and we found a Fre1 (gene ID 1034376) downregulated DGE transcript in the *P. ostreatus* F515 transcriptome (Figure 5-8 panel c). The transcription levels of this reductase were also increased in the GY medium and tended to decrease in the copper or rice husks containing media. We also included the data from the upregulated Cu^{+} transporting (copper detoxification) ATPase, CRT6/ Cr1p (gene ID 1037970), reported as an extracellular copper transporter (Figure 5-8 panel d). In the *Candida albicans* transcriptome, it has also been found that Ctr1p levels decrease under conditions of excess copper at the same time that CTR6/Cr1p levels increase [226]. Recently, it has been found that the availability of nitrogen affects the transcriptome of the fungus *Cryptococcus neoformans*; in case of nitrogen limitation, a type of copper transporter is the most upregulated gene CRTs, which suggests that the regulation of this type of transporters occurs not only due to the availability of copper but also due to nutritional factors [227].

Finally, an increase in the expression levels of several transcripts coding for proteins containing copper-binding domains was found in cultures with copper sulfate with or without rice husks (Figure 5-8, d). These results suggest the occurrence of various cellular processes associated with the metabolism of this metal, such as Cu^{2+} reduction, transport from outside the cell, intracellular and nuclear transport of this metal, and the transcription of enzymes with metal response elements. The increase in these transcripts occurred almost exclusively in the cultures in which copper sulfate was added except for *lacc5*, whose maximum transcription levels were found in the rice husk media.

5.4 Conclusions

In this paper, we compared the transcriptome of *P. ostreatus* F515 in four different culture media. A reference medium based on glucose and yeast extracts supplemented with copper, rice husks, and copper and rice husks. The mycelium grown in these culture media showed differences in the morphology of the pellets influenced by the rice husks and the laccase activity influenced mainly by copper sulfate. The results of the transcriptome comparison showed that three laccases genes, *lacc5*, *lacc6*, and *lacc10*, are upregulated by the presence of copper and husks. The expression levels of these genes could explain the differences in the magnitude of laccase activity, the number, and the intensity of laccase isoforms found in the supernatants of these cultures.

We also found that the expression of genes coding for carbohydrate-binding proteins is affected by the rice husks. This association can be the basis of the morphological changes found in the culture pellets because of the downward regulation of cell wall components, such as that of the vegetative hydrophobin Vmh2.

We also found that the addition of rice husks to the culture media influences the levels of gene expression related to nitrogen metabolism, a high regulation of NMR in rice husks media, high levels of expression of peptidase and metalloproteinase genes could be associated with lower values of laccase activity in media with rice husks.

The increase of the laccase transcripts coincided with the downregulation of several copper transporters and the copper reductase Fre1. In the non-rice husks media, these transcripts had higher expression levels than in the rice husks media. In conclusion, the laccases genes *lacc2*, *lacc5*, *lacc6*, and *lacc10* respond to the presence of copper sulfate and rice husks. Some of these mechanisms could be associated with copper transporters that could be regulated by nutritional factors such as carbohydrates and nitrogen metabolism. However, further studies need to be performed to establish these relationships.

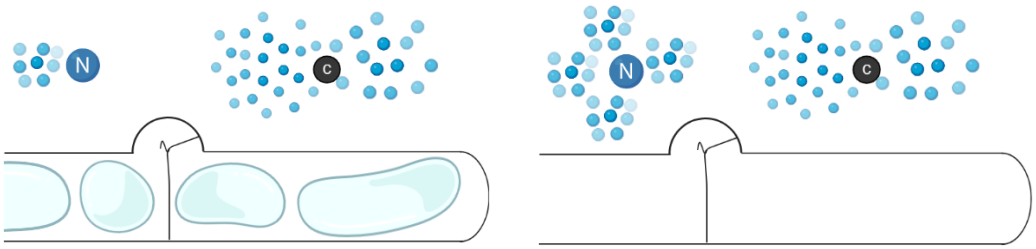
Chapter 6

General Discussion & Complementary Experiments

The objective of this work was to determine the best composition of the culture medium to increase the laccase activity by *P. ostreatus* in submerged fermentation aimed to the pretreatment of rice husks and to compare the modifications in the husks due to the increase of these enzymes in various biological pretreatment strategies.

6.1 Effect of nitrogen sufficiency and limited nutritional conditions on the laccase activity of P. ostreatus

Our results suggest that the increase in laccase activity depends on nutrient-sufficient conditions, especially on the availability of an organic nitrogen source. Nitrogen sufficiency is likely necessary to increase the expression of copper transporters (CTRs) involved in the uptake of this metal and responsible for the induction of laccase by copper sulfate. We show that the laccase activity increased up to two orders of magnitude in the nutrient-sufficient culture media (45gL^{-1} glucose, 15 gL^{-1} yeast extract) upon adding 1mM copper sulfate to the culture. In comparison, with the addition of this laccase inducer in the nitrogen-limited media (45 gL^{-1} glucose, 4 gL^{-1} yeast extract), the laccase activity was lower than the activity found in cultures without copper sulfate. We also observed that these two nutritional statuses, nutrient-sufficient and nitrogen-limited, are associated with changes in the vacuole patterns in hyphae of the fungus, such as those illustrated in Figure 6-1.



(**a**) (**b**)

Figure 6.1. Vacuole patterns in hyphae of the fungus *P. ostreatus* in two nutritional conditions: (a) nitrogen-limited as a culture medium glucose 45 gL^{-1} and yeast extract 4 gL^{-1} (GY4504); (b) nutrient-sufficient as a culture medium glucose 45 gL^{-1} and yeast extract 15 gL^{-1} (GY4515). Carbon source (C) is glucose, and nitrogen source (N) is yeast extract.

Additionally, we compared the effect of the nitrogen-limited (GY4504) and nitrogen-sufficient (GY4515) conditions in the production of biomass and laccase activity in the dikaryotic strains F515 and dkN001, and the monokaryons mkPC15 and mkPC9 (which, combined, form dkN001), to determine if different *P. ostr*eatus strains share the negative effect of organic nitrogen limitation in laccase activity. We observed

that all these strains produced some grade of vacuolated terminal hyphae in nitrogen-limited culture media (data not shown). The comparisons between the dikaryotic and monokaryotic *P. ostreatus* strains and their response as biomass production and laccase activity in the nitrogen-limited (GY4504) and sufficient (GY4515) conditions are shown in Figure 6-2. The results show that the biomass production in the two culture media (GY4504 and GY4515) with and without copper was primarily similar for the four strains evaluated, suggesting that all these strains similarly assimilate nitrogen. *P. ostreatus* F515, N001, and mkPC9 are fast-growing while mkPC15 is slow-growing. However, using the same glucose concentration, biomass production was drastically reduced in nitrogen-limited culture media. In dkN001 and mkPC15 strains, this reduction was independent on the copper addition in the cultures; still, in F515 and mkPC9 strains, a possible adverse effect on biomass production by copper sulfate (1 mM) was observed when the culture medium was nitrogen-limited because the biomass production was additionally reduced by about 50% compared with N001 and mkPC15 in those cultures of the same composition.

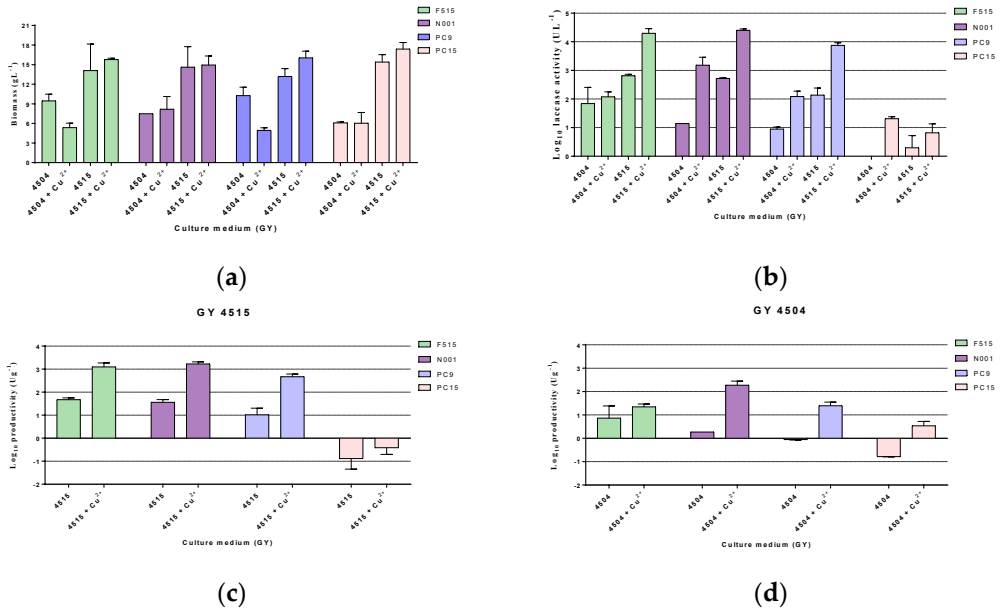


Figure 6.2. Effect of nitrogen-limited and nitrogen-sufficient nutritional conditions on dikaryotic and monokaryotic strains of *P. ostreatus*: (a) biomass production; (b) maximum laccase activity; (c) productivity in nitrogen-sufficient culture medium GY4515; (b) productivity in nitrogen-limited culture medium GY4504.

On the other hand, the results of laccase activity show a similarity in response to the composition of the culture medium and copper between the fast-growing strains and the slow-growing mkPC15 strain. Due to the effect of the composition of the culture medium on biomass production, we compared the laccase activity in terms of productivity (Figure 6-2 panel c and d). We observed that under nutrient sufficiency conditions (GY4515, panel c), the productivity of laccase activity per gram of biomass produced tends to increase due to copper. This increase was around 1.5 orders of magnitude greater than the medium without copper for fast strains. Although the productivity of laccase activity per gram of biomass produced tends to increase due to copper under nitrogen-limited conditions, the orders of magnitude of this increase are variable for each strain, suggesting that the nitrogen-limiting threshold could be different for each one of them. However, the productivity of laccase activity per gram of biomass produced in a medium with sufficient nutrients GY4515 is at least an order of magnitude greater than the productivity of laccase activity per gram of biomass produced in a

medium limited in nitrogen GY4504, demonstrating that nitrogen limitation negatively affects copper-induced laccase activity in several *P. ostreatus* strains.

The nitrogen assimilation mechanisms appear to be conserved in mkPC15 because nitrogen-sufficient concentrations increase biomass production more than nitrogen-limited concentrations; however, the laccase induction mechanisms by copper appear altered in this *P. ostreatus* monokaryotic strain. In previous studies for mkPC15, it was found that no laccase activity was induced by lignocellulose extracts, although lignocellulose extracts induced increased fungal biomass production [59]. That same study also showed that lignocellulose extracts induced *lacc2*, *lacc5*, and *lacc6*, but not *lacc10* transcription [59]. Our study found that all these laccase genes *lacc2*, *lacc5*, lacc6, and *lacc10*, can be regulated by copper, maybe by metal responsive elements in their promoter sequences [56], [57], [149], [228]–[230]. This copper laccase regulation is possible by the copper uptake mediated by CTRs, which transport copper from the environment to the inside cell. The expression of the *ctr1* gene was detected in the vegetative mycelium during all stages of culture in the mkPC15 strain [123]. Therefore we believe that other mechanisms of regulation of CTRs independent of the nitrogen concentration could occur in this strain, making the mkPC15 strain an attractive model to study in the future the regulation mechanisms of CTRs and their potential role in regulating laccase by copper and other inducers.

6.2 Rice husks modifications by two biological pretreatment strategies

To determine the effect of the increase in the laccase activity present in the culture media on the rice husk modifications, two biological pretreatment strategies of this lignocellulosic biomass were evaluated: 1) enzymatic treatment of the rice husks with crude extracts from *P. ostreatus* cultures and 2) treatment of rice husks in modified culture media. The results suggest that the high magnitude of laccase activity in the treatment is not the only factor associated with the compositional, chemical, and structural modifications observed in the rice husks in both strategies of biological pretreatments.

For the strategy of enzymatic treatment of rice husks with crude extracts of *P. ostreatus* cultures, the results showed that the modifications in the lignocellulose fractions and the selectivity depend on the composition of the culture medium where the extract was produced. The highest delignification percentages and high selectivity ratio in lignin removal per holocellulose removal fractions were achieved in rice husks treated with extracts produced in cultures supplemented with lignin and copper at the same time (Table 6-1). Both results suggest the presence of metabolites derived from lignin in the extract (L+ Cu^{2+} extract); on the one hand, these metabolites could improve delignification via laccase activity; on the other hand, they could increase selectivity by containing possible cellulases and xylanases inhibitory substances. Despite these results, an important percentage in removing hemicellulose, cellulose, and lignin was obtained with the rest of the extracts evaluated, which suggests a basal secretion of lignocellulolytic enzymes or other factors in the treatments contributed to the compositional, and structural and morphological modifications in the rice husks which were observed independent of the magnitude of the laccase activity in the extracts.

For the second strategy of treatment of rice husks in *P. ostreatus* cultures made using modified culture media, we compared the transcriptomes recovered from different conditions. Rice husks induced the expression of genes coding for proteins involved in

lignin degradation such as laccases (*lacc5*, *lacc6*, and *lacc10*) and other oxidases, carbohydrate-active proteins such as CBM13, and xylan and cellulose-degrading or modifying enzymes such as carbohydrate esterases (CE), glucooligosaccharide oxidases (GOOX), endoglucanases, and lytic polysaccharide monooxygenases (LPMOs). The overexpression of these genes could explain the compositional changes found in rice husks from modified culture media. The removal percentages of cellulose, hemicellulose and lignin in this strategy of biological pretreatment depended more on the time of the culture than on the laccase activity in the culture. However, the purification and characterization of the xylanase, cellulose, and laccase activities should be done in future studies

Table 6-1 shows the treatment process characteristics and the results of the main compositional modifications of the rice husks recovered from four selected treatments using strategies of enzymatic treatment and modified culture media. For the enzymatic treatment strategy, the treatments performed with the L-extracts with and without copper are compared. These extracts were produced in a basal medium with glucose and yeast extract, using LCDs and copper sulfate as inducers. The high concentration of lignin used in these culture media negatively affected fungal biomass production and laccase activity but induced a laccase isoform that we have not identified yet. The difference in laccase activity between these extracts was greater (one order of magnitude) in those with copper sulfate than in those without cooper. In this case, the difference in laccase activity is associated with an increase of almost 57% in the lignin loss in the rice husks treated with these extracts and with changes in the FTIR spectra. In these spectra, the treatment with the extract without copper showed a change in the depth of the bands at 1077, 897, and 800 cm^{-1} related to changes in the silicon fraction, while the treatment with copper was associated with the disappearance of a peak at 1559 cm^{-1} and the appearance of a faint band related to C = C vibrations in skeletal Aromatic at 1560 cm^{-1}.

The treatments of the rice husks in the modified culture media were also carried out in a basal medium of glucose and yeast extract. In this case, the composition of the treatments differed in the concentration of glucose, rice husks, and the use of the copper sulfate laccase inducer. The main bioprocess difference between these treatments was biomass production. The culture medium modified with rice husks with a lower initial glucose concentration (GYR 051580) produced six-fold more biomass concentration than predicted theoretically. The biomass yield coefficient experimental obtained was four-fold higher than the theoretically expected on the initial glucose concentration. This result shows the bioconversion potential of rice husks lignocellulose biomass in fungal biomass.

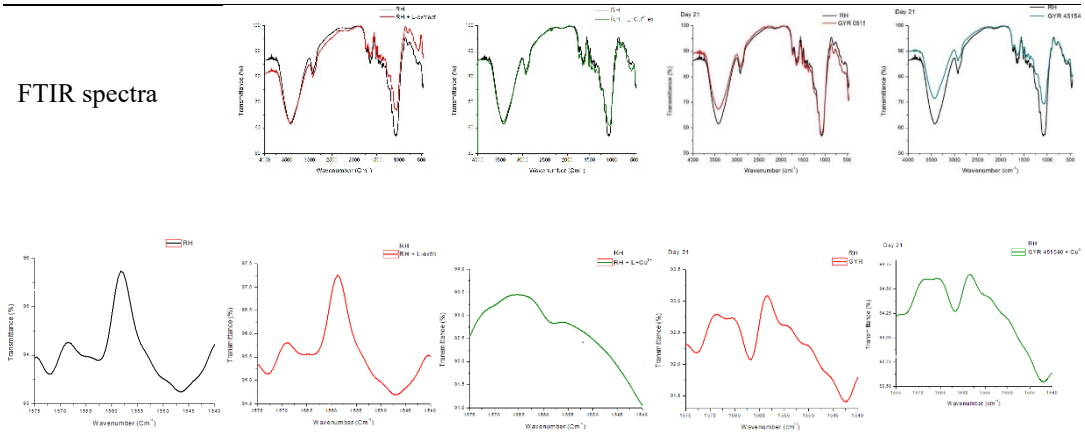
On the other hand, the medium with the highest initial glucose concentration (GYR451540+Cu^{2+}) produced the highest biomass concentration with an experimental performance coefficient equal to the theoretical expected. The bioconversion in the GYR451540 treatment with copper could be delayed by the high initial glucose concentration and the culture time since only at day 21 of culture, final glucose concentration was observed to be almost zero in the culture broth. In future work, it would be interesting to measure the growth kinetics and evaluate this culture at a longer treatment time.

Although the laccase activity in the GYR051580 treatment was lower by more than one order of magnitude than in the GYR451540 system with copper and fewer laccase isoforms were identified, the percentage of lignin removal (more than 30%) was

high and similar to the best results obtained using enzymatic treatments that had a high laccase activity (L+Cu^{2+} -extract). In this GYR051580 system, hemicellulose removal was larger than cellulose in the GYR451540+Cu^{2+} culture medium. In this last treatment, there was a higher percentage of delignification and a lower selectivity than in the GYR 051580 medium. The modifications in the rice husks FTIR spectra in modified media showed a change in depth of the band at 3419 cm^{-1} associated with hydroxyl groups, which suggests a structural change in this material. In addition, changes in the depth of the bands at 2922 and 1077 cm^{-1}suggesting changes in type C – H stretching and Si-O-Si stretching were also observed in the treatment with the higher activity observed GYR451540 with copper. In both the GYR051580 and GYR451540 treatment with copper, a band at 1560 cm^{-1} of greater depth was observed in the L-extract enzymatic treatment with copper, despite the differences in the order of magnitude of laccase activity between these treatments. In future work, it would be interesting to evaluate whether the appearance of this band is related to the action of laccases, the delignification of rice husks lignocellulose by the synergistic action of lignocellulolytic enzymes, or other factors of the biological treatment process that may contribute to this observed change.

Table 6-1. Comparative summary of processes and results of the rice husk treatment using two biological pretreatment strategies with *P. ostreatus*.

	RH in Enzymatic treatment		**RH in Modified culture media (GYR)**	
	L-extract	**L+Cu^{2+} -extract**	**051580**	**451540+ Cu^{2+}**
Basal culture media composition (gL^{-1})	Glucose (40) Yeast extract (20)	Glucose (40) Yeast extract (20)	Glucose (5) Yeast extract (15)	Glucose (45) Yeast extract (15)
Inductor concentration (gL^{-1})	Lignin (5) CMC (0.5) Xylose (0.5)	Lignin (5) CMC (0.5) Xylose (0.5) $CuSO_4$•$5H_2O$ (0.25)	Rice husks (80)	Rice husk (40) $CuSO_4$•$5H_2O$ (0.25)
Rice husks loaded (%, w/v)	5	5	8	4
Treatment time (days)	3	3	21	21
Predicted biomass, X (gL^{-1}) *	16	16	2	18
Experimental biomass, X (gL^{-1})	10.5 ± 0.7	16 ± 0.4	12 ± 1	18 ±1
Final glucose concentration, (gL^{-1})	29 ± 1.8	28 ± 2.8	0.90 ± 0.13	0.370 ± 0.065
$Y_{X/S}$ **	1.0	1.5	1.6	0.4
Total protein concentration	554 ± 28	776 ± 5	494 ± 23	324 ± 10
Log_{10} laccase activity (UL^{-1})	2.4 ± 0.4	3.8 ± 0.2	2.7 ± 0.1	4.0 ± 0.1
Hypothetical isoenzyme	Unknown	LACC6	LACC2	LACC5

	LACC2 LACC10	Unknown LACC2 LACC10	LACC10	LACC6 LACC2 LACC10
Relative lignin loss (%)	24 ± 4	37 ± 2	35 ± 5	49 ± 5
Selectivity (lignin removed /holocellulose removed)	0.4	2.5	1.8	0.9
FTIR spectra				

*The predicted biomass was estimated from a biomass yield of 0.4 multiplied by the initial glucose concentration
** $*Y_{X/S}$: the biomass yield coefficient or apparent growth yield was estimated as the relation between units (gL^{-1}) of biomass (X) per unit (gL^{-1}) of the glucose (S) [231].

6.3 Comparative transcriptome analysis

In the transcriptome analysis of *P. ostreatus* F515, our objective was to determine the first genes regulated by copper and lignocellulose and the common genes regulated by these laccase inducers to establish possible features and relationships between these genes and laccase regulation in culture media in nutrient-sufficient conditions, with copper sulfate, rice husks, and both components.

Some laccase genes, small subunits of laccase, and cupredoxin are the primary and commonly regulated genes. In the group of differentially expressed and identified transcripts, both upregulated and downregulated, they suggest that one of the main differences between the evaluated culture conditions is regulating transmembrane proteins, other proteins in the cell wall, and proteases. A summary illustrating some of the differences found from this comparison is shown in Figure 6-2. This figure shows that one of the main proteins regulated in copper sulfate and rice husks are copper transporters and hydrophobins, respectively. We believe that these differences could be related to the main differences observed between cultures: on the one hand, the regulation of CTRs with the induction of laccase by copper sulfate and the regulation of hydrophobins, including Vmh2 with morphological changes associated with the rice husks in the culture; however, in both cases the proposed cell models deserve to be tested in future studies, identifying these proteins either using immune-technics or by microscopy studies. On the other hand, the comparative analysis of the *P. ostreatus* transcriptome in nutrient-sufficient conditions, with copper sulfate, rice husk, and both components, showed that many unidentified transcripts are differentially expressed

under the evaluated conditions, for which the role that these transcripts play in the laccase regulation by copper sulfate and rice husks is yet to be elucidated.

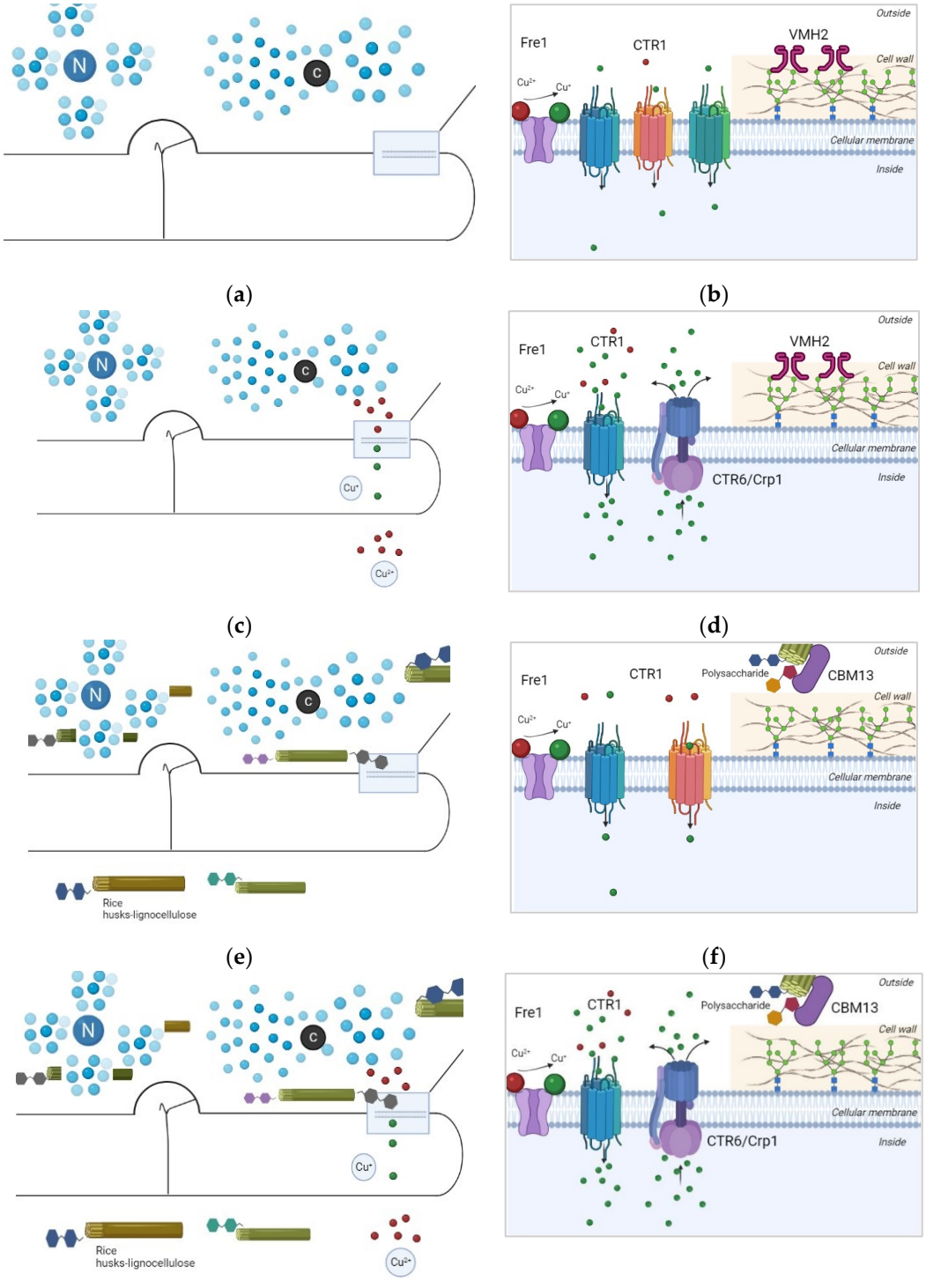


Figure 6-3. Four cell models of *P. ostreatus* in nutrient-sufficient conditions culture: (**a**) and (**b**) GY4515 culture media; (**c**) and (**d**) GY4515 + Cu^{2+} (**e**) and (**f**) GY4515 + Rice husks ; (**g**) and (**h**) GY4515 + Rice husks + Cu^{2+}

6.4 Effect of the composition of the culture medium on the regulation of laccase isoenzymes.

Laccases Lacc10, Lacc6, and Lacc2 constitute the typical isoenzymatic pattern of *P. ostreatus* in many analyzed conditions [98]. In cultures with copper sulfate, the results suggest that under carbon-sufficient conditions, organic nitrogen is required for the induction of the laccase genes, *lacc2*, *lacc6*, and *lacc10* by copper, possibly by a nitrogen pathway that mediates the regulation of CTRs; once the copper is inside the cell, other mechanisms of regulation of these isoenzymes could occur through mechanisms independent of copper and associated with glucose concentration. In copper and nitrogen sufficiency, low glucose concentrations negatively regulate Lacc6, while Lacc2 and Lacc10 are positively regulated. The results showed that under conditions of nitrogen sufficiency but carbon limitation (GY0515 culture medium), the induction by copper of the isoform with the lowest electrophoretic flow (presumably Lacc6) is not detected in the zymogram in the extracts of 12 days of culture. In contrast, the other two isomorphs (presumably Lacc10 and Lacc2) were stained with greater intensity in the cultivation conditions evaluated. Similar behavior for Lacc6 was observed by Palmieri *et al*. (2001), who found that although intracellularly the mRNA and the protein concentration of Lacc6 increased over time, in the culture broth, the concentration of this protein and its enzymatic activity decreased after three days of fungal culture, this decrease was explained by an increase in extracellular protease activity [232]. These results imply that while copper transcriptionally regulates *lacc2, lacc6,* and *lacc10*, the post-translational modifications, secretion, and proteolytic degradation of these isoenzymes occur independently of this metal and are selectively influenced by other factors, in this case by glucose concentration.

Similar to that described above, the results suggest that several mechanisms of regulation of laccases are involved in the rice husks media and are responsible for an increase in the *lacc5*, *lacc6*, and *lacc10* transcripts but low enzymatic activity. While the mechanisms of transcriptional induction of laccases by rice husks or lignocellulose are not entirely elucidated, they could be associated with conditions of carbon sufficiency, diversity of carbon sources, or lignin derived. Meanwhile, the low laccase activity in the rice husks media could be explained by an increase in protease transcripts that, from the results, suggest a higher extracellular protease activity in these culture media. Additionally, in the media with rice husks and copper, the results of the transcriptomic analysis also showed that the decrease in the induction of laccase by copper in the presence of rice husks could also be explained by a decrease in the CTRs transcripts expression in these media. Furthermore, the isoenzymatic pattern of *P. ostreatus* in media with rice husks and copper showed a big protein with laccase activity that did not migrate to the running gel in the native PAGE. It is possible that this protein could be lacc5. Although lacc5 is not a laccase in *sensu stricto*, it has been classified as a multi-copper oxidase, ferroxidase [54], [233], the predicted protein for Lacc5 is 630 amino acids much larger than Lacc2, Lacc6, or Lacc10 that contain between 522 and 533 amino acids [58]. The purification and identification of this enzyme should be considered in future studies since, when induced by rice husks, it could be involved in its degradation.

Another isoform of laccase was found in culture media where lignin was used, but its identity is unknown. This isoform was stained late in the zymogram, after 30 minutes in 2mM ABTS solution. In future work, the indirect identification of this isoform may be carried out through qPCR using the same primers used in this investigation or the identification of the protein after purification and sequencing.

pox2 case: Lacc10 (POXC) was previously called POX2 [228]. *lacc10*, transcript ID 1089723, is the most overexpressed transcript in the presence of copper sulfate, followed by transcript ID 1105204, which has no annotation in the JGI. When we performed a local alignment of each of these sequences against the GenBank database, both sequences had a significant alignment with the sequence ID Z49075.2 deposited as *pox2*. Although the identity between the alignments was higher than 95%, in both cases, the score for *lacc10*, transcript ID 1089723, was higher than 2000, while the unknown ID 1105204 was lower than 500. The number of amino acids for Lacc10 was 533, while the amino acid number for ID 1105204 was 71. Both sequences are on the same chromosome, but on opposite strands, *lacc10* is on the negative strand, and the unknown sequence is on the negative strand. In order not to lose track of the sequence ID 1105204, it was named the *pox2* gene. We believe that this could be a case of gene duplication; however, further studies will be necessary to identify this non-annotated and copper-regulated gene along with other laccase genes.

Lacc2 case: Lacc2 (POXA3) consists of the two proteins POXA3a and POXA3b formed by heterodimerization of the large subunit of POXA3 laccase *sensu stricto* with a smaller subunit differentially glycosylated [233]. The transcription of the large subunit *lacc2* was positively regulated by copper, as were *lacc5*, *lacc6*, and *lacc10*, although at a lower level. The results also showed that the small subunit of laccase POXA3a was overexpressed with rice husks and copper within the transcripts more regulated by copper. As suggested previously by Giardina *et al*. (2003) [23], these results show no transcriptional co-regulation in our experimental conditions. Although the role of the small subunit laccase, POXA3a, is unclear, it is suggested that this subunit is involved in stabilizing the large subunit Lacc2. The quick loss of the large subunit native structure in the absence of the small subunit could cause the lower activity measured in the heterologous expression of heterodimeric laccase from *P. ostreatus* in *Kluyveromyces lactis* [218].

In summary, the culture medium composition influences the regulation of copper transporters (CTRs) in the cell membrane of *P. ostreatus* and the entry into the cell of this metal. In this fungus, the regulation of these CTRs seems to depend on organic nitrogen concentration in the medium. When there is copper inside the cell, this metal mainly induces the transcription of the *lacc2*, *lacc5*, *lacc6*, and *lacc10* genes. Other factors could also regulate the production of these transcripts in the culture medium composition, such as the concentration of glucose, nitrogen, or lignocellulose, which ultimately controls the laccases secretion and laccase activity of various isoforms. The laccase activity and laccases isoforms in the extracts or culture broth can cause modifications in the lignocellulose of the rice husks that seem to depend more on the synergistic action between the laccases and other ligninolytic enzymes than on the magnitude of the laccase activity and the number of isoforms.

Chapter 7

Conclusions

The increase of biomass production of *P. ostreatus* F515 in SmF, culture media with nutrient-sufficient conditions in both carbon and organic nitrogen are required for an effective rice husks pretreatment. This nutrition-sufficient culture media composition is around 45 gL^{-1} of glucose and 15 gL^{-1} of yeast extract. Without copper sulfate or rice husks, the changes in the glucose and the yeast extract concentrations are insufficient to explain the laccase activity in the culture medium.

In a carbon-nitrogen-sufficient, copper sulfate 1mM culture media laccase activity is up to two orders of magnitude higher than the same culture media without copper sulfate, depending on the culture time. This increase appears to depend on the overexpression of copper transporters under nutrient-sufficient conditions and is associated with the overexpression of the *lacc2*, *lacc6*, and *lacc10* genes.

The laccase activity by copper sulfate is affected by CMC, xylose, and lignin concentration in a culture medium with sufficient carbon and nitrogen. CMC and xylose increase this laccase activity while lignin reduces the laccase activity induced by copper but induces a different isoform detected in the culture medium.

The increase in laccase activity by the rice husks depends on the glucose and yeast extract concentration in the culture medium. Rice husks reduce the copper-induced laccase activity by up to one order of magnitude depending on the culture time, possibly due to negative CTR regulation. Rice husks induce changes in the pellet morphology, possibly associated with changes in the wall cell and membrane proteins. Rice husk induces overexpression of *lacc5* and other genes to lignocellulolytic enzymes and other related proteins.

The highest percentages of lignin removal by enzymatic treatments or rice husks pretreatment in modified culture media occurred in culture media with higher laccase activity; however, these losses seemed more related to the rice husk lignocellulose or LDCs than copper sulfate. Probably, the rice husks lignocellulose or LDCs induce other laccase isoenzymes, other lignocellulolytic enzymes, or metabolites that could work together with *lacc2*, *lacc6*, and *lacc10* laccase to improve the delignification.

Conclusiones

Para aumentar la producción de biomasa de P. ostreatus F515 en SmF, se requieren medios de cultivo con condiciones suficientes de nutrientes tanto en carbono como en nitrógeno orgánico. La composición de este medio de cultivo fue de 45 gL^{-1} de glucosa y 15 gL^{-1} de extracto de levadura. Sin sulfato de cobre o cáscara de arroz, los cambios en las concentraciones de glucosa y extracto de levadura son insuficientes para explicar cambios en la actividad lacasa en el medio de cultivo.

En medios de cultivo con suficiente carbono-nitrógeno, el sulfato de cobre 1 mM aumenta la actividad lacasa hasta dos órdenes de magnitud que en el mismo medio de cultivo sin sulfato de cobre. La magnitud de la actividad lacasa depende del tiempo de cultivo. En medios de cultivos suficientes en carbono-nitrógeno el aumento de la actividad lacasa inducida por cobre parece depender de la sobreexpresión de transportadores cobre (CRTs) que ocurren en condiciones de nutrientes suficientes y está asociada con la sobreexpresión de los genes *lacc2*, *lacc6* y *lacc10*.

La actividad lacasa del sulfato de cobre se ve afectada por la concentración de CMC, xilosa y lignina en un medio de cultivo con suficiente carbono y nitrógeno. La CMC y la xilosa aumentan esta actividad lacasa mientras que la lignina reduce la actividad lacasa inducida por el cobre pero induce una isoforma diferente detectada en el medio de cultivo.

El aumento de la actividad lacasa por las cascarillas de arroz depende de la concentración de glucosa y extracto de levadura en el medio de cultivo. Las cáscaras de arroz reducen la actividad lacasa inducida por el cobre hasta en un orden de magnitud dependiendo del tiempo de cultivo, posiblemente debido a la regulación negativa de algunos genes *Ctr*. Además, las cascarillas inducen cambios en la morfología del hongo, posiblemente asociados con cambios en las proteínas de la membrana y de la pared celular. También se encontró que las cascarillas de arroz induce la sobreexpresión de lacc5 y otros genes en enzimas lignocelulolíticas y otras proteínas relacionadas.

Los porcentajes más altos de eliminación de lignina por tratamientos enzimáticos o pretratamiento de cáscara de arroz en medios de cultivo modificados ocurrieron en medios de cultivo con mayor actividad lacasa; sin embargo, estas pérdidas parecían más relacionadas con la lignocelulosa de la cáscara de arroz o a la suplementación de los medios de cultivos con compuestos derivados de la lignocelulosa que al propio efecto inductor de lacasas del sulfato de cobre. Probablemente, las cáscaras de arroz lignocelulosa o LDC inducen otras isoenzimas de lacasa, otras enzimas lignocelulolíticas o metabolitos que podrían trabajar junto con lacc2, lacc6 y lacc10 lacasa para mejorar la deslignificación.

References

[1] C. A. M. Moraes *et al.*, "Review of the rice production cycle: By-products and the main applications focusing on rice husk combustion and ash recycling," *Waste Manag. Res.*, vol. 32, no. 11, pp. 1034–1048, 2014.

[2] R. Pode, "Potential applications of rice husk ash waste from rice husk biomass power plant," *Renew. Sustain. Energy Rev.*, vol. 53, pp. 1468–1485, 2016.

[3] J. S. Lim, Z. Abdul Manan, S. R. Wan Alwi, and H. Hashim, "A review on utilisation of biomass from rice industry as a source of renewable energy," *Renew. Sustain. Energy Rev.*, vol. 16, no. 5, pp. 3084–3094, 2012.

[4] FAO, "FAOSTAT." [Online]. Available: http://www.fao.org/faostat/en/#data/QC. [Accessed: 21-May-2021].

[5] Fedearroz, "Producción de Arroz Paddy Seco en Colombia por Semestres Desde 2000 hast a 2018 Toneladas," 2018. [Online]. Available: http://www.fedearroz.com.co/new/apr_public.php. [Accessed: 11-Apr-2018].

[6] B. D. Park, S. Gon Wi, K. Ho Lee, A. P. Singh, T. H. Yoon, and Y. Soo Kim, "Characterization of anatomical features and silica distribution in rice husk using microscopic and micro-analytical techniques," *Biomass and Bioenergy*, vol. 25, no. 3, pp. 319–327, 2003.

[7] C. D. C. Lamb, B. M. Z. D. Silva, D. de Souza, F. Fornasier, L. B. Riça, and R. D. C. D. S. Schneider, "Bioethanol production from rice hull and evaluation of the final solid residue," *Chem. Eng. Commun.*, vol. 6445, pp. 1–13, 2018.

[8] M. N. Salimi, S. E. Lim, A. H. M. Yusoff, and M. F. Jamlos, "Conversion of rice husk into fermentable sugar by two stage hydrolysis," *J. Phys. Conf. Ser.*, vol. 908, no. 1, 2017.

[9] C. Kongmanklang and K. Rangsriwatananon, "Hydrothermal Synthesis of High Crystalline Silicalite from Rice Husk Ash," *J. Spectrosc.*, vol. 2015, pp. 2–7, 2015.

[10] S. Chakma, A. Ranjan, H. A. Choudhury, P. K. Dikshit, and V. S. Moholkar, "Bioenergy from rice crop residues: Role in developing economies," *Clean Technol. Environ. Policy*, vol. 18, no. 2, pp. 373–394, 2016.

[11] P. Unrean, B. C. Lai Fui, E. Rianawati, and M. Acda, "Comparative techno-economic assessment and environmental impacts of rice husk-to-fuel conversion technologies," *Energy*, vol. 151, pp. 581–593, 2018.

[12] H. Tan and S. R. Wang, "Experimental study of the effect of acid-wash pretreatment on biomass pyrolysis," *Ranliao Huaxue Xuebao/Journal Fuel Chem. Technol.*, vol. 37, no. 6, pp. 668–672, 2009.

[13] E. Menya, P. W. Olupot, H. Storz, M. Lubwama, and Y. Kiros, "Characterization and alkaline pretreatment of rice husk varieties in Uganda for potential utilization as precursors in the production of activated carbon and other value-added products," *Waste Manag.*, vol. 81, pp. 104–116, 2018.

[14] Y. Wang *et al.*, "Conversion of rice husk into fermentable sugar and silicausing acid-catalyzed ionic liquid pretreatment," *Environ. Sci. Pollut.*

Res., vol. 28, pp. 40715–40723, 2021.
[15] J. O. Madu and B. O. Agboola, "Bioethanol production from rice husk using different pretreatments and fermentation conditions," *3 Biotech*, vol. 8, no. 1, pp. 1–6, 2018.
[16] F. R. Amin *et al.*, "Pretreatment methods of lignocellulosic biomass for anaerobic digestion," *AMB Express*, vol. 7, no. 72, pp. 1–12, 2017.
[17] S. Aslanzadeh, M. M. Ishola, T. Richards, and M. J. Taherzadeh, *An Overview of Existing Individual Unit Operations*. Elsevier B.V., 2014.
[18] N. Akhtar, K. Gupta, D. Goyal, and A. Goyal, "Recent Advances in Pretreatment Technologies for Efficient Hydrolysis of Lignocellulosic Biomass," *Environ. Prog. Sustain. Energy*, vol. 35, no. 2, pp. 489–511, 2016.
[19] M. Alfaro, J. A. Oguiza, L. Ramírez, and A. G. Pisabarro, "Comparative analysis of secretomes in basidiomycete fungi," *Journal of Proteomics*, vol. 102. Elsevier B.V., pp. 28–43, 2014.
[20] J. C. Sigoillot *et al.*, "Fungal Strategies for Lignin Degradation," in *Advances in Botanical Research*, 1st ed., vol. 61, Elsevier Ltd., 2012, pp. 263–308.
[21] C. Wan and Y. Li, "Fungal pretreatment of lignocellulosic biomass," *Biotechnol. Adv.*, vol. 30, no. 6, pp. 1447–1457, 2012.
[22] S. Beg, S. I. Zafar, and F. . Shah, "Rice Husk Biodegradation by Pleurotus ostreatus to Produce a Ruminant Feed," *Agric. Wastes*, vol. 17, pp. 15–21, 1986.
[23] M. Taniguchi, H. Suzuki, D. Watanabe, K. Sakai, K. Hoshino, and T. Tanaka, "Evaluation of Pretreatment with Pleurotus ostreatus for Enzymatic Hydrolysis of Rice Straw," *J. Biosci. Bioeng.*, vol. 100, no. 6, pp. 637–643, 2005.
[24] NCBI, "Taxonomy browser (Thiotrichales)," *Taxon. Database*, p. http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwta, 2011.
[25] K. Deepalakshmi and S. Mirunalini, "Pleurotus ostreatus : an oyster mushroom with nutritional and medicinal properties," *J Biochem Tech*, vol. 5, no. 2, pp. 718–726, 2014.
[26] U. Kües and R. Fischer, "The Mycota - Growth, Differentiation and Sexuality," in *The Mycota I : Growth, Differetiation and Sexuality.*, Third Edit., K. Esser, Ed. Springer, 2006, pp. 73–105.
[27] A. S. Pérez-Martínez, S. A. Acevedo-Padilla, M. Bibbins-Martínez, J. Galván-Alonso, and S. Rosales-Mendoza, "A perspective on the use of Pleurotus for the development of convenient fungi-made oral subunit vaccines," *Vaccine*, vol. 33, no. 1, pp. 25–33, 2015.
[28] R. Castanera, A. Omarini, F. Santoyo, G. Pérez, A. G. Pisabarro, and L. Ramírez, "Non-Additive Transcriptional Profiles Underlie Dikaryotic Superiority in Pleurotus ostreatus Laccase Activity," vol. 8, no. 9, 2013.
[29] R. A. Ohm, R. Riley, A. Salamov, B. Min, I. G. Choi, and I. V. Grigoriev, "Genomics of wood-degrading fungi," *Fungal Genet. Biol.*, vol. 72, pp. 82–90, 2014.
[30] E. Fernández-Fueyo *et al.*, "A secretomic view of woody and nonwoody lignocellulose degradation by Pleurotus ostreatus," *Biotechnol. Biofuels*, vol. 9, no. 1, pp. 1–18, 2016.
[31] L. M. Larraya *et al.*, "Molecular karyotype of the white rot fungus *Pleurotus ostreatus*," *Appl. Environ. Microbiol.*, vol. 65, no. 8, pp. 3413–3417, 1999.

[32] F. J. Ruiz-Dueñas, E. Fernández, M. J. Martínez, and A. T. Martínez, "Pleurotus ostreatus heme peroxidases: An in silico analysis from the genome sequence to the enzyme molecular structure," *Comptes Rendus - Biol.*, vol. 334, no. 11, pp. 795–805, 2011.
[33] E. Fernández-fueyo *et al.*, "Ligninolytic peroxidase gene expression by Pleurotus ostreatus : Differential regulation in lignocellulose medium and effect of temperature and pH," vol. 72, pp. 150–161, 2014.
[34] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, and G. Sannia, "Laccases: A never-ending story," *Cell. Mol. Life Sci.*, vol. 67, no. 3, pp. 369–385, 2010.
[35] D. Sirim, F. Wagner, L. Wang, R. D. Schmid, and J. Pleiss, "The Laccase Engineering Database: A classification and analysis system for laccases and related multicopper oxidases," *Database*, vol. 2011, pp. 1–7, 2011.
[36] M. D. Asemoloye, M. A. Marchisio, V. K. Gupta, and L. Pecoraro, "Genome-based engineering of ligninolytic enzymes in fungi," *Microb. Cell Fact.*, vol. 20, no. 1, pp. 1–18, 2021.
[37] P. Baldrian, "Fungal laccases-occurrence and properties," *FEMS Microbiol. Rev.*, vol. 30, no. 2, pp. 215–242, 2006.
[38] I. Bassanini, E. E. Ferrandi, S. Riva, and D. Monti, "Biocatalysis with laccases: An updated overview," *Catalysts*, vol. 11, no. 1, pp. 1–30, 2021.
[39] L. Munk, A. K. Sitarz, D. C. Kalyani, J. D. Mikkelsen, and A. S. Meyer, "Can laccases catalyze bond cleavage in lignin?," *Biotechnol. Adv.*, vol. 33, no. 1, pp. 13–24, 2015.
[40] D. W. S. Wong, "Structure and Action Mechanism of Ligninolytic Enzymes," *Appl. Biochem. Biotechnol.*, vol. 157, no. 2, pp. 174–209, 2009.
[41] S. Kawai, T. Umezawa, M. Shimada, and T. Higuchi, "Aromatic ring cleavage of 4,6-di(tert-butyl)guaiacol, a phenolic lignin model compound, by laccase of Coriolus versicolor," *FEBS Lett.*, vol. 236, no. 2, pp. 309–311, 1988.
[42] C. Weng, X. Peng, and Y. Han, "Depolymerization and conversion of lignin to value-added bioproducts by microbial and enzymatic catalysis," *Biotechnol. Biofuels*, vol. 14, no. 1, pp. 1–22, 2021.
[43] A. Rico, J. Rencoret, J. C. Del Río, A. T. Martínez, and A. Gutiérrez, "Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock," *Biotechnol. Biofuels*, vol. 7, no. 1, pp. 1–14, 2014.
[44] J. Rencoret, A. Pereira, J. C. del Río, A. T. Martínez, and A. Gutiérrez, "Laccase-Mediator Pretreatment of Wheat Straw Degrades Lignin and Improves Saccharification," *Bioenergy Res.*, vol. 9, no. 3, pp. 917–930, 2016.
[45] O. V. Morozova, G. P. Shumakovich, S. V. Shleev, and Y. I. Yaropolov, "Laccase-mediator systems and their applications: A review," *Appl. Biochem. Microbiol.*, vol. 43, no. 5, pp. 523–535, 2007.
[46] S. Kawai, M. Iwatsuki, M. Nakagawa, M. Inagaki, A. Hamabe, and H. Ohashi, "An alternative β-ether cleavage pathway for a non-phenolic β-O-4 lignin model dimer catalyzed by a laccase-mediator system," *Enzyme and Microbial Technology*, vol. 35, no. 2–3. pp. 154–160, 2004.
[47] W. Fungi *et al.*, "Biological treatment of Lignocelluloses with white-rot funghi and its applications: Review," *BioResources*, vol. 6, no. 4, pp. 5224–

5259, 2011.
[48] G. Díaz-Godínez, M. Téllez-Téllez, C. Sánchez, and R. Díaz, "Characterization of the Solid-State and Liquid Fermentation for the Production of Laccases of Pleurotus ostreatus," in *INTECH*, 2017, pp. 57-.
[49] M. Papagianni, "Fungal morphology and metabolite production in submerged mycelial processes," *Biotechnology Advances*, vol. 22, no. 3. pp. 189–259, 2004.
[50] D. M. Mate and M. Alcalde, "Laccase: a multi-purpose biocatalyst at the forefront of biotechnology," *Microb. Biotechnol.*, vol. 10, no. 6, pp. 1457–1467, 2017.
[51] K. Sun, S. Li, Y. Si, and Q. Huang, "Advances in laccase-triggered anabolism for biotechnology applications," *Crit. Rev. Biotechnol.*, 2021.
[52] C. Sánchez, "Cultivation of Pleurotus ostreatus and other edible mushrooms," *Appl. Microbiol. Biotechnol.*, vol. 85, no. 5, pp. 1321–1337, 2010.
[53] R. A. Ohm, R. Riley, A. Salamov, B. Min, I. G. Choi, and I. V. Grigoriev, "Genomics of wood-degrading fungi," *Fungal Genet. Biol.*, vol. 72, pp. 82–90, 2014.
[54] U. Kues and M. Ruhl, "Multiple Multi-Copper Oxidase Gene Families in Basidiomycetes – What for?," *Curr. Genomics*, vol. 12, no. 2, pp. 72–94, 2011.
[55] G. Pérez, J. Pangilinan, A. G. Pisabarro, and L. Ramírez, "Telomere organization in the ligninolytic basidiomycete pleurotus ostreatus," *Appl. Environ. Microbiol.*, vol. 75, no. 5, pp. 1427–1436, 2009.
[56] A. Piscitelli, P. Giardina, V. Lettera, C. Pezzella, G. Sannia, and V. Faraco, "Induction and transcriptional regulation of laccases in fungi.," *Curr. Genomics*, vol. 12, no. 2, pp. 104–112, 2011.
[57] C. Pezzella, F. Autore, P. Giardina, A. Piscitelli, G. Sannia, and V. Faraco, "The Pleurotus ostreatus laccase multi-gene family: Isolation and heterologous expression of new family members," *Curr. Genet.*, vol. 55, no. 1, pp. 45–57, 2009.
[58] X. Jiao *et al.*, "Systematic analysis of the pleurotus ostreatus laccase gene (PoLac) Family and functional characterization of PoLac2 involved in the degradation of cotton-straw lignin," *Molecules*, vol. 23, no. 4, p. 880, 2018.
[59] R. Castanera *et al.*, "Transcriptional and enzymatic profiling of pleurotus ostreatus laccase genes in submerged and solid-state fermentation cultures," *Appl. Environ. Microbiol.*, vol. 78, no. 11, pp. 4037–4045, 2012.
[60] C. Pezzella, V. Lettera, A. Piscitelli, P. Giardina, and G. Sannia, "Transcriptional analysis of Pleurotus ostreatus laccase genes," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 2, pp. 705–717, 2013.
[61] P. Giardina *et al.*, "Structural characterization of heterodimeric laccases from Pleurotus ostreatus," pp. 1293–1300, 2007.
[62] V. Faraco, C. Ercole, G. Festa, P. Giardina, A. Piscitelli, and G. Sannia, "Heterologous expression of heterodimeric laccase from Pleurotus ostreatus in Kluyveromyces lactis," *Appl. Microbiol. Biotechnol.*, vol. 77, no. 6, pp. 1329–1335, 2008.
[63] P. Giardina *et al.*, "Protein and gene structure of a blue laccase from Pleurotus ostreatus," *Biochem. J.*, vol. 341, no. 3, pp. 655–663, 1999.
[64] G. Macellaro *et al.*, "Effective mutations in a high redox potential laccase

from Pleurotus ostreatus," pp. 4949–4961, 2014.
[65] P. Aza, F. De Salas, G. Molpeceres, D. Rodríguez-Escribano, I. De La Fuente, and S. Camarero, "Protein engineering approaches to enhance fungal laccase production in S. cerevisiae," *Int. J. Mol. Sci.*, vol. 22, no. 3, pp. 1–19, 2021.
[66] M. Maestre-Reyna *et al.*, "Structural and functional roles of glycosylation in fungal laccase from lentinus sp.," *PLoS One*, vol. 10, no. 4, pp. 1–28, 2015.
[67] A. I. Grandes-Blanco, G. Díaz-Godínez, M. Téllez-Téllez, R. J. Delgado-Macuil, M. Rojas-López, and M. D. Bibbins-Martínez, "Ligninolytic activity patterns of pleurotus ostreatus obtained by submerged fermentation in presence of 2,6-dimethoxyphenol and remazol brilliant blue R dye," *Prep. Biochem. Biotechnol.*, vol. 43, no. 5, pp. 468–480, 2013.
[68] R. Díaz, M. Téllez-Téllez, C. Sánchez, M. D. Bibbins-Martínez, G. Díaz-Godínez, and J. Soriano-Santos, "Influence of initial pH of the growing medium on the activity, production and genes expression profiles of laccase of Pleurotus ostreatus in submerged fermentations," *Electron. J. Biotechnol.*, vol. 16, no. 4, 2013.
[69] M. Téllez-Téllez, G. Díaz-Godínez, M. B. Aguilar, C. Sánchez, and F. J. Fernández, "Description of a laccase gene from Pleurotus ostreatus expressed under submerged fermentation conditions," *BioResources*, vol. 7, no. 2, pp. 2038–2050, 2012.
[70] M. Téllez-Téllez, F. J. Fernández, A. M. Montiel-González, C. Sánchez, and G. Díaz-Godínez, "Growth and laccase production by Pleurotus ostreatus in submerged and solid-state fermentation," *Appl. Microbiol. Biotechnol.*, vol. 81, no. 4, pp. 675–679, Dec. 2008.
[71] S. Tlecuitl-Beristain, C. Sánchez, O. Loera, G. D. Robson, and G. Díaz-Godínez, "Laccases of Pleurotus ostreatus observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform," *Mycol. Res.*, vol. 112, no. 9, pp. 1080–1084, 2008.
[72] V. Lettera, C. Del Vecchio, A. Piscitelli, and G. Sannia, "Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: The case of laccase in Pleurotus ostreatus," *Comptes Rendus - Biol.*, vol. 334, no. 11, pp. 781–788, 2011.
[73] R. Tinoco-Valencia, C. Gómez-Cruz, E. Galindo, and L. Serrano-Carreón, "Toward an understanding of the effects of agitation and aeration on growth and laccases production by Pleurotus ostreatus," *J. Biotechnol.*, vol. 177, no. 1, pp. 67–73, 2014.
[74] A. Parenti *et al.*, "Induction of laccase activity in the white rot fungus Pleurotus ostreatus using water polluted with wheat straw extracts," *Bioresour. Technol.*, vol. 133, pp. 142–149, 2013.
[75] N. Mikiashvili, S. P. Wasser, E. Nevo, and V. Elisashvili, "Effects of carbon and nitrogen sources on Pleurotus ostreatus ligninolytic enzyme activity," *World J. Microbiol. Biotechnol.*, vol. 22, no. 9, pp. 999–1002, 2006.
[76] R. Tinoco, A. Acevedo, E. Galindo, and L. Serrano-Carreón, "Increasing Pleurotus ostreatus laccase production by culture medium optimization and copper/lignin synergistic induction," *J. Ind. Microbiol. Biotechnol.*, vol. 38, no. 4, pp. 531–540, 2011.
[77] M. Stajić *et al.*, "Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected Pleurotus species," *Enzyme Microb.*

Technol., vol. 38, no. 1–2, pp. 65–73, 2006.
[78] A. Efeyan, W. C. Comb, and D. M. Sabatini, "Nutrient-sensing mechanisms and pathways," *Nature*, vol. 517, no. 7534, pp. 302–310, 2015.
[79] N. A. Brown, L. N. Ries, and G. H. Goldman, "How nutritional status signalling coordinates metabolism and lignocellulolytic enzyme secretion," *Fungal Genet. Biol.*, vol. 72, pp. 48–63, 2014.
[80] M. Adnan *et al.*, "Carbon catabolite repression in filamentous Fungi," *Int. J. Mol. Sci.*, vol. 19, no. 1, pp. 1–24, 2018.
[81] L. B. Huberman, J. Liu, L. Qin, and N. L. Glass, "Regulation of the lignocellulolytic response in filamentous fungi," *Fungal Biol. Rev.*, vol. 30, no. 3, pp. 101–111, 2016.
[82] L. J. de Assis *et al.*, "Carbon catabolite repression in filamentous fungi is regulated by phosphorylation of the transcription factor crea," *MBio*, vol. 12, no. 1, pp. 1–21, 2021.
[83] M. A. Davis and K. H. Wong, "Nitrogen Metabolism in Filamentous Fungi," *Cell. Mol. Biol. Filamentous Fungi*, pp. 325–338, 2010.
[84] B. Tudzynski, "Nitrogen regulation of fungal secondary metabolism in fungi," *Front. Microbiol.*, vol. 5, no. NOV, pp. 1–15, 2014.
[85] X. Han *et al.*, "Functional Analysis of the Nitrogen Metabolite Repression Regulator Gene nmrA in Aspergillus flavus," vol. 7, no. November, pp. 1–12, 2016.
[86] R. B. Todd, J. A. Fraser, K. H. Wong, M. A. Davis, and M. J. Hynes, "Nuclear Accumulation of the GATA Factor AreA in Response to Complete Nitrogen Starvation by Regulation of Nuclear Export," vol. 4, no. 10, pp. 1646–1653, 2005.
[87] K. H. Wong, M. J. Hynes, R. B. Todd, and M. A. Davis, "Transcriptional control of nmrA by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in Aspergillus nidulans," vol. 66, no. September, pp. 534–551, 2007.
[88] J. Fernandez, J. D. Wright, D. Hartline, C. F. Quispe, N. Madayiputhiya, and R. A. Wilson, "Principles of Carbon Catabolite Repression in the Rice Blast Fungus : Tps1 , Nmr1-3 , and a MATE – Family Pump Regulate Glucose Metabolism during Infection," *PLos Genet.*, vol. 8, no. 5, 2012.
[89] E. D. Harris, "Cellular Copper Transport and Metabolism," *Annu. Rev. Nutr.*, vol. 20, pp. 291–310, 2000.
[90] J. Kardos, L. Héja, Á. Simon, I. Jablonkai, R. Kovács, and K. Jemnitz, "Copper signalling: causes and consequences," *Cell Commun. Signal.*, vol. 16, no. 1, pp. 1–22, 2018.
[91] M. J. Petris, "The SLC31 (Ctr) copper transporter family," pp. 752–755, 2004.
[92] A. D. Smith, B. L. Logeman, and D. J. Thiele, "Copper Acquisition and Utilization in Fungi," *Annual Review of Microbiology*, vol. 71, no. 1. pp. 597–623, 2017.
[93] M. Choi and V. L. Davidson, "Cupredoxins - A study of how proteins may evolve to use metals for bioenergetic processes," *Metallomics*, vol. 3, no. 2, pp. 140–151, 2011.
[94] Y. Lu, "Electron Transfer: Cupredoxins," *Compr. Coord. Chem. II*, vol. 8, pp. 91–122, Jan. 2003.

[95] C. Galhaup, H. Wagner, B. Hinterstoisser, and D. Haltrich, "Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens," *Enzyme Microb. Technol.*, vol. 30, no. 4, pp. 529–536, 2002.
[96] M. GL., "Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar," *Anal. Chem.*, vol. 31, pp. 426–428, 1959.
[97] M. M. Bradford, "A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," vol. 254, pp. 248–254, 1976.
[98] V. Lettera, A. Piscitelli, G. Leo, L. Birolo, C. Pezzella, and G. Sannia, "Identification of a new member of Pleurotus ostreatus laccase family from mature fruiting body," *Fungal Biol.*, vol. 114, no. 9, pp. 724–730, 2010.
[99] R. Castanera, L. López-Varas, A. G. Pisabarro, and L. Ramíre, "Validation of reference genes for transcriptional analyses in Pleurotus ostreatus by using reverse transcription-quantitative PCR," *Appl. Environ. Microbiol.*, vol. 81, no. 12, pp. 4120–4129, 2015.
[100] R. Castanera, A. Omarini, F. Santoyo, G. Pérez, A. G. Pisabarro, and L. Ramírez, "Non-Additive Transcriptional Profiles Underlie Dikaryotic Superiority in Pleurotus ostreatus Laccase Activity," *PLoS One*, vol. 8, no. 9, 2013.
[101] A. Dobin *et al.*, "STAR: ultrafast universal RNA-seq aligner.," *Bioinformatics*, vol. 29, no. 1, pp. 15–21, Jan. 2013.
[102] L. M. Larraya *et al.*, "Molecular Karyotype of the White Rot Fungus Pleurotus ostreatus," *Appl. Envir. Microbiol.*, vol. 65, no. 8, pp. 3413–3417, Aug. 1999.
[103] R. Riley *et al.*, "Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 27, pp. 9923–8, Jul. 2014.
[104] I. V. Grigoriev *et al.*, "MycoCosm portal: Gearing up for 1000 fungal genomes," *Nucleic Acids Res.*, vol. 42, no. D1, pp. 699–704, 2014.
[105] Z. Zhang, S. Schwartz, L. Wagner, and W. Miller, "A greedy algorithm for aligning DNA sequences," *Journal of Computational Biology*, vol. 7, no. 1–2. J Comput Biol, pp. 203–214, 2000.
[106] A. Morgulis, G. Coulouris, Y. Raytselis, T. L. Madden, R. Agarwala, and A. A. Schäffer, "Database indexing for production MegaBLAST searches," *Bioinforma. Orig. Pap.*, vol. 24, no. 16, pp. 1757–1764, 2008.
[107] E. Kachlishvili, M. J. Penninckx, N. Tsiklauri, and V. Elisashvili, "Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation," *World J. Microbiol. Biotechnol.*, vol. 22, no. 4, pp. 391–397, 2006.
[108] M. Stajić *et al.*, "Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected Pleurotus species," *Enzyme Microb. Technol.*, vol. 38, no. 1–2, pp. 65–73, 2006.
[109] É. C. D'Agostini, T. R. D. Mantovani, J. S. Do Valle, L. D. Paccola-Meirelles, N. B. Colauto, and G. A. Linde, "Low carbon/nitrogen ratio increases laccase production from basidiomycetes in solid substrate cultivation," *Sci. Agric.*, vol. 68, no. 3, pp. 295–300, 2011.
[110] C. Zhu, G. Bao, and S. Huang, "Optimization of laccase production in the white-rot fungus Pleurotus ostreatus (ACCC 52857) induced through yeast

extract and copper," *Biotechnol. Biotechnol. Equip.*, vol. 30, no. 2, pp. 270–276, 2016.
[111] S. G. Karp *et al.*, "Statistical Optimization of Laccase Production and Delignification of Sugarcane Bagasse by Pleurotus ostreatus in Solid-State Fermentation," vol. 2015, 2015.
[112] C. J. Barelle *et al.*, "Asynchronous cell cycle and asymmetric vacuolar inheritance in true hyphae of Candida albicans," *Eukaryot. Cell*, vol. 2, no. 3, pp. 398–410, 2003.
[113] R. Periasamy and T. Palvannan, "Optimization of laccase production by Pleurotus ostreatus IMI 395545 using the Taguchi DOE methodology," *J. Basic Microbiol.*, vol. 50, no. 6, pp. 548–556, 2010.
[114] J. Yang, G. Wang, T. B. Ng, J. Lin, and X. Ye, "Laccase production and differential transcription of laccase genes in cerrena sp. in response to metal ions, aromatic compounds, and nutrients," *Front. Microbiol.*, vol. 6, no. JAN, pp. 1–11, 2016.
[115] M. Hazuchová, D. Chmelová, and M. Ondrejovič, "The optimization of propagation medium for the increase of laccase production by the white-rot fungus Pleurotus ostreatus," *Nov. Biotechnol. Chim.*, vol. 16, no. 2, pp. 113–123, 2017.
[116] P. Baldrian and J. Gabriel, "Copper and cadmium increase laccase activity in Pleurotus ostreatus," *FEMS Microbiol. Lett.*, vol. 206, no. 1, pp. 69–74, 2002.
[117] R. Kannaiyan, V. Kostenko, and R. J. Martinuzzi, "Nutrient media optimization for simultaneous enhancement of the laccase and peroxidases production by coculture of Dichomitus squalens and Ceriporiopsis subvermispora," pp. 173–185.
[118] R. Kannaiyan, N. Mahinpey, T. Mani, R. J. Martinuzzi, and V. Kostenko, "Enhancement of Dichomitus squalens tolerance to copper and copper-associated laccase activity by carbon and nitrogen sources," vol. 67, pp. 140–147, 2012.
[119] A. Chenthamarakshan, N. Parambayil, N. Miziriya, P. S. Soumya, and M. S. K. Lakshmi, "Optimization of laccase production from Marasmiellus palmivorus LA1 by Taguchi method of Design of experiments," *BMC Biotechnol.*, vol. 17, no. 12, pp. 1–10, 2017.
[120] B. H.M., J. H.P., and S. R.Z., "Statistical optimization for enhanced production of extracellular laccase from Aspergillus sp . HB _ RZ4 isolated from bark scrapping," *Environ. Sustain.*, vol. 1, pp. 159–166, 2018.
[121] G. Palmieri, P. Giardina, C. Bianco, B. Fontanella, and G. Sannia, "Copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus," *Appl. Environ. Microbiol.*, vol. 66, no. 3, pp. 920–924, 2000.
[122] G. Palmieri, G. Cennamo, V. Faraco, A. Amoresano, G. Sannia, and P. Giardina, "Atypical laccase isoenzymes from copper supplemented Pleurotus ostreatus cultures □," vol. 33, pp. 220–230, 2003.
[123] M. M. Peñas, G. Azparren, Á. Domínguez, H. Sommer, L. Ramírez, and A. G. Pisabarro, "Identification and functional characterisation of ctr1, a Pleurotus ostreatus gene coding for a copper transporter," *Mol. Genet. Genomics*, vol. 274, no. 4, pp. 402–409, 2005.
[124] A. Abbas and S. Ansumali, "Global Potential of Rice Husk as a Renewable Feedstock for Ethanol Biofuel Production," *Bioenergy Res.*, vol. 3, no. 4, pp.

328–334, 2010.
[125] A. Valverde, B. Sarria, and J. Monteagudo, “Análisis comparativo de las características fisicoquímicas de la cascarilla de arroz.,” *Sci. Tech.*, no. 37, p. 6, 2007.
[126] R. Sindhu, P. Binod, and A. Pandey, “Biological pretreatment of lignocellulosic biomass – An overview,” *Bioresour. Technol.*, vol. 199, pp. 76–82, 2016.
[127] V. K. Gupta *et al.*, “Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass,” *Trends Biochem. Sci.*, vol. 41, no. 7, pp. 633–645, 2016.
[128] P. Baldrian and V. Valášková, “Degradation of cellulose by basidiomycetous fungi,” *FEMS Microbiol. Rev.*, vol. 32, no. 3, pp. 501–521, 2008.
[129] S. M. Cragg *et al.*, “Lignocellulose degradation mechanisms across the Tree of Life,” *Curr. Opin. Chem. Biol.*, vol. 29, pp. 108–119, 2015.
[130] A. P. Singh, Byung-Dae Park, S. G. Wi, K. H. Lee, Y. Tae-Ho, and Y. Soo Kim, “Light and electron microscopic characterization of rice husk from Korean rice,” *Korean J. Plant. Res.*, vol. 5, no. 2, pp. 95–103, 2002.
[131] Z. Chen, Y. Xu, and S. Shivkumar, “Microstructure and tensile properties of various varieties of rice husk,” *J. Sci. Food Agric.*, vol. 98, no. 3, pp. 1061–1070, 2018.
[132] S. Yoshida, Y. Ohnishi, and K. Kitagishi, “Histochemistry of Silicon in Rice Plant,” *Soil Sci. Plant Nutr.*, vol. 8, no. 2, pp. 1–5, 1962.
[133] B. de Lhoneux, L. Gerlache, A. Clemente, M. L. Roda-Santos, J. A. G. Menaia, and T. H. Fernandes, “Ultrastructural characterization of rice husk submitted to different pretreatments to optimize its fermentation,” *Biol. Wastes*, vol. 23, no. 3, pp. 163–180, 1988.
[134] L. P. Devendra, M. Kiran Kumar, and A. Pandey, “Evaluation of hydrotropic pretreatment on lignocellulosic biomass,” *Bioresour. Technol.*, vol. 213, pp. 350–358, 2016.
[135] E. M. Podgorbunskikh, A. L. Bychkov, and O. I. Lomovskii, “Pretreatment of rice husk in a pilot scale mill for further enzymatic hydrolysis,” *Catal. Ind.*, vol. 8, no. 3, pp. 274–279, 2016.
[136] T. N. Ang, G. C. Ngoh, and A. S. M. Chua, “Comparative study of various pretreatment reagents on rice husk and structural changes assessment of the optimized pretreated rice husk,” *Bioresour. Technol.*, vol. 135, pp. 116–119, 2013.
[137] R. Potumarthi, R. R. Baadhe, P. Nayak, and A. Jetty, “Simultaneous pretreatment and sacchariffication of rice husk by Phanerochete chrysosporium for improved production of reducing sugars,” *Bioresour. Technol.*, vol. 128, pp. 113–117, 2013.
[138] a. Sluiter *et al.*, “NREL/TP-510-42618 analytical procedure - Determination of structural carbohydrates and lignin in Biomass,” *Lab. Anal. Proced.*, no. April 2008, p. 17, 2012.
[139] A. B. D. Nandiyanto, R. Oktiani, and R. Ragadhita, “How to read and interpret ftir spectroscope of organic material,” *Indones. J. Sci. Technol.*, vol. 4, no. 1, pp. 97–118, 2019.
[140] S. B. Daffalla, H. Mukhtar, and M. S. Shaharun, “Preparation and characterization of rice husk adsorbents for phenol removal from aqueous systems,” *PLoS One*, vol. 15, no. 12 December, 2020.

[141] G. Bekiaris, G. Koutrotsios, P. A. Tarantilis, C. S. Pappas, and G. I. Zervakis, "FTIR assessment of compositional changes in lignocellulosic wastes during cultivation of Cyclocybe cylindracea mushrooms and use of chemometric models to predict production performance," *J. Mater. Cycles Waste Manag.*, vol. 22, no. 4, pp. 1027–1035, 2020.
[142] H. Yang, R. Yan, H. Chen, D. H. Lee, and C. Zheng, "Characteristics of hemicellulose, cellulose and lignin pyrolysis," *Fuel*, vol. 86, no. 12–13. pp. 1781–1788, 2007.
[143] M. H. Shahrokh Abadi, A. Delbari, Z. Fakoor, and J. Baedi, "Effects of annealing temperature on infrared spectra of SiO2 extracted from rice husk," *J. Ceram. Sci. Technol.*, vol. 6, no. 1, pp. 41–45, 2015.
[144] S. B. Daffalla, H. Mukhtar, and M. S. Shaharun, "Characterization of Adsorbent Developed from Rice Husk: Effect of Surface Functional Group on Phenol Adsorption," *J. Appl. Sci.*, vol. 10, no. 12, pp. 1060–1067, 2010.
[145] F. Xu, J. Yu, T. Tesso, F. Dowell, and D. Wang, "Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: A mini-review," *Appl. Energy*, vol. 104, pp. 801–809, 2013.
[146] D. Durán-Aranguren *et al.*, "Effect of bioactive compounds extracted from Cordyceps nidus ANDES-F1080 on laccase activity of Pleurotus ostreatus ANDES-F515," *Biotechnology Reports*, vol. 26. 2020.
[147] H. Hou, J. Zhou, J. Wang, C. Du, and B. Yan, "Enhancement of laccase production by Pleurotus ostreatus and its use for the decolorization of anthraquinone dye," *Process Biochem.*, vol. 39, no. 11, pp. 1415–1419, 2004.
[148] R. Zhuo, P. Yuan, Y. Yang, S. Zhang, F. Ma, and X. Zhang, "Induction of laccase by metal ions and aromatic compounds in Pleurotus ostreatus HAUCC 162 and decolorization of different synthetic dyes by the extracellular laccase," *Biochem. Eng. J.*, vol. 117, pp. 62–72, 2017.
[149] C. Pezzella, V. Lettera, A. Piscitelli, P. Giardina, and G. Sannia, "Transcriptional analysis of Pleurotus ostreatus laccase genes," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 2, pp. 705–717, 2013.
[150] Q. An, H.-F. Ma, M.-L. Han, J. Si, and Y.-C. Dai, "Laccase of Pleurotus ostreatus," *BioResources*, vol. 13, no. 1, pp. 1143–1156, 2018.
[151] A. Gutiérrez *et al.*, "Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks," *Bioresour. Technol.*, vol. 119, pp. 114–122, 2012.
[152] W. D. H. Schneider *et al.*, "Lignin degradation and detoxification of eucalyptus wastes by on-site manufacturing fungal enzymes to enhance second-generation ethanol yield," *Appl. Energy*, vol. 262, no. January, 2020.
[153] J. Rencoret, A. Pereira, J. C. Del Río, Á. T. Martínez, and A. Gutiérrez, "Delignification and Saccharification Enhancement of Sugarcane Byproducts by a Laccase-Based Pretreatment," *ACS Sustain. Chem. Eng.*, vol. 5, no. 8, pp. 7145–7154, 2017.
[154] Y. Sun, X. Qiu, and Y. Liu, "Chemical reactivity of alkali lignin modified with laccase," *Biomass and Bioenergy*, vol. 55, pp. 198–204, 2013.
[155] X. Li, Y. Wei, J. Xu, N. Xu, and Y. He, "Quantitative visualization of lignocellulose components in transverse sections of moso bamboo based on ftir macro- and micro-spectroscopy coupled with chemometrics," *Biotechnology for Biofuels*, vol. 11, no. 1. pp. 1–16, 2018.

[156] T. Rashid, C. F. Kait, and T. Murugesan, "A 'fourier Transformed Infrared' Compound Study of Lignin Recovered from a Formic Acid Process," *Procedia Eng.*, vol. 148, pp. 1312–1319, 2016.
[157] V. Hospodarova, E. Singovszka, and N. Stevulova, "Characterization of Cellulosic Fibers by FTIR Spectroscopy for Their Further Implementation to Building Materials," *Am. J. Anal. Chem.*, vol. 09, no. 06, pp. 303–310, 2018.
[158] M. Cespugli *et al.*, "Rice husk as an inexpensive renewable immobilization carrier for biocatalysts employed in the food, cosmetic and polymer sectors," *Catalysts*, vol. 8, no. 10. 2018.
[159] H. Hage and M. N. Rosso, "Evolution of fungal carbohydrate-active enzyme portfolios and adaptation to plant cell-wall polymers," *J. Fungi*, vol. 7, no. 3, pp. 1–16, 2021.
[160] D. C. Eastwood, "The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi (Science (762))," *Science*, vol. 333, no. 6051. p. 1825, 2011.
[161] S. Miyauchi, D. Navarro, S. Grisel, D. Chevret, J. G. Berrin, and M. N. Rosso, "The integrative omics of white-rot fungus Pycnoporus coccineus reveals co-regulated CAZymes for orchestrated lignocellulose breakdown," *PLoS One*, vol. 12, no. 4, pp. 1–17, 2017.
[162] G. Paës *et al.*, "Biotechnology for Biofuels Tracking of enzymatic biomass deconstruction by fungal secretomes highlights markers of lignocellulose recalcitrance," pp. 1–15, 2019.
[163] M. Andlar, T. Rezić, N. Marđetko, D. Kracher, R. Ludwig, and B. Šantek, "Lignocellulose degradation: An overview of fungi and fungal enzymes involved in lignocellulose degradation," *Eng. Life Sci.*, vol. 18, no. 11, pp. 768–778, 2018.
[164] F. J. Contesini, R. J. N. Frandsen, and A. Damasio, "Editorial: CAZymes in Biorefinery: From Genes to Application Fabiano," 2021.
[165] V. Lombard, H. G. Ramulu, E. Drula, P. M. Coutinho, and B. Henrissat, "The carbohydrate-active enzymes database (CAZy) in 2013," *Nucleic Acids Res.*, vol. 42, no. November 2013, pp. 490–495, 2014.
[166] M. Peng *et al.*, "Comparative analysis of basidiomycete transcriptomes reveals a core set of expressed genes encoding plant biomass degrading enzymes," *Fungal Genet. Biol.*, vol. 112, no. August 2017, pp. 40–46, 2018.
[167] Y. Liu *et al.*, "Biotechnology for Biofuels Lignin degradation potential and draft genome sequence of Trametes trogii S0301," *Biotechnol. Biofuels*, pp. 1–13, 2019.
[168] A. O. Oghenekaro, T. Raffaello, A. Kovalchuk, and F. O. Asiegbu, "De novo transcriptomic assembly and profiling of Rigidoporus microporus during saprotrophic growth on rubber wood," *BMC Genomics*, vol. 17, no. 1, pp. 1–17, 2016.
[169] F. Valadares *et al.*, "The secretome of two representative lignocellulose-decay basidiomycetes growing on sugarcane bagasse solid-state cultures," *Enzyme Microb. Technol.*, vol. 130, no. July, pp. 1–10, 2019.
[170] M. Di Falco, M. Zhou, M. V. Aguilar-pontes, O. Sieti, A. Tsang, and R. P. De Vries, "The molecular response of the white-rot fungus Dichomitus squalens to wood and non-woody biomass as examined by transcriptome and exoproteome analyses," vol. 19, pp. 1237–1250, 2017.

[171] M. Couturier *et al.*, "Enhanced degradation of softwood versus hardwood by the white-rot fungus Pycnoporus coccineus," *Biotechnol. Biofuels*, vol. 8, no. 1, pp. 1–16, 2015.
[172] D. V. Vasina, A. R. Pavlov, and O. V. Koroleva, "Extracellular proteins of Trametes hirsuta st. 072 induced by copper ions and a lignocellulose substrate," *BMC Microbiol.*, vol. 16, no. 1, pp. 1–15, 2016.
[173] Q. Zhang *et al.*, "Comparative transcriptomics and transcriptional regulation analysis of enhanced laccase production induced by co-culture of Pleurotus eryngii var. ferulae with Rhodotorula mucilaginosa," *Appl. Microbiol. Biotechnol.*, 2019.
[174] K. K. Jain, A. Kumar, A. Shankar, D. Pandey, B. Chaudhary, and K. K. Sharma, "De novo transcriptome assembly and protein profiling of copper-induced lignocellulolytic fungus Ganoderma lucidum MDU-7 reveals genes involved in lignocellulose degradation and terpenoid biosynthetic pathways," *Genomics*, vol. 112, no. 1, pp. 184–198, 2020.
[175] Y. Li *et al.*, "De novo transcriptome analysis of Pleurotus djamor to identify genes encoding CAZymes related to the decomposition of corn stalk lignocellulose," *J. Biosci. Bioeng.*, vol. 128, no. 5, pp. 529–536, 2019.
[176] de J. M. G. and G. T. B. Davinia Salvachúa, Rui Katahira, Nicholas S. Cleveland, Payal Khanna, Michael G. Resch, Brenna A. Black, Samuel O. Purvine, Erika M. Zink, Alicia Prieto, María J. Martínez, Angel T. Martínez, Blake A. Simmons, "Lignin depolymerization by fungal secretomes and a microbial sink," *Green Chem.*, vol. 18, no. 22, 2016.
[177] S. Il Yoo *et al.*, "Comparative transcriptome analysis identified candidate genes involved in mycelium browning in Lentinula edodes," *BMC Genomics*, vol. 20, no. 1, pp. 1–13, 2019.
[178] P. Kersten and D. Cullen, "Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes," *Fungal Genet. Biol.*, vol. 72, pp. 124–130, 2014.
[179] L. Wastes *et al.*, "Comparative Study on Laccase Activity of White Rot Fungi under Submerged Fermentation with Different," vol. 15, no. 4, pp. 9166–9179, 2020.
[180] T. Yuliana, N. Z. Putri, D. Z. Komara, E. Mardawati, I. Lanti, and S. Rahimah, "Study of Ganoderma lucidum in laccase production using corncob and paddies straw substrates on submerged fermentation system," *Pakistan J. Biol. Sci.*, vol. 23, no. 8, pp. 1060–1065, 2020.
[181] E. M. Rodrigues, S. G. Karp, L. C. Malucelli, C. V. Helm, and T. M. Alvarez, "Evaluation of laccase production by Ganoderma lucidumin submerged and solid-state fermentation usingdifferent inducers.pdf," *J. Basic Microbiol.*, vol. 59, pp. 784–791, 2019.
[182] S. Bakkiyaraj, R. Aravindan, S. Arrivukkarasan, and T. Viruthagiri, "Enhanced laccase production by Trametes hirusta using wheat bran under submerged fermentation," *Int. J. ChemTech Res.*, vol. 5, no. 3, pp. 1224–1238, 2013.
[183] G. Songulashvili, D. Spindler, G. A. Jimenéz-Tobón, C. Jaspers, G. Kerns, and M. J. Penninckx, "Production of a high level of laccase by submerged fermentation at 120-L scale of Cerrena unicolor C-139 grown on wheat bran," *Comptes Rendus - Biol.*, vol. 338, no. 2, pp. 121–125, 2015.

[184] L. H. Zhao, W. Chen, L. L. Wang, H. J. Sun, and Z. Zhu, "Improvement of laccase production by pleurotus ostreatus by means of agroindustrial waste and fermentation kinetics," *Mycosphere*, vol. 8, no. 1, pp. 147–161, 2017.
[185] A. M. Mustafa, T. G. Poulsen, and K. Sheng, "Fungal pretreatment of rice straw with Pleurotus ostreatus and Trichoderma reesei to enhance methane production under solid-state anaerobic digestion," *Appl. Energy*, vol. 180, pp. 661–671, 2016.
[186] M. Taniguchi *et al.*, "Effect of steam explosion pretreatment on treatment with Pleurotus ostreatus for the enzymatic hydrolysis of rice straw," *J. Biosci. Bioeng.*, vol. 110, no. 4, pp. 449–452, 2010.
[187] J. Yu, J. Zhang, J. He, Z. Liu, and Z. Yu, "Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull," *Bioresour. Technol.*, vol. 100, no. 2, pp. 903–908, 2009.
[188] C. Mamimin, S. Chanthong, C. Leamdum, S. O-Thong, and P. Prasertsan, "Improvement of empty palm fruit bunches biodegradability and biogas production by integrating the straw mushroom cultivation as a pretreatment in the solid-state anaerobic digestion," *Bioresour. Technol.*, vol. 319, no. October 2020, p. 124227, 2021.
[189] H. S. Hafid, A. S. Baharuddin, M. N. Mokhtar, F. N. Omar, M. A. P. Mohammed, and M. Wakisaka, "Enhanced laccase production for oil palm biomass delignification using biological pretreatment and its estimation at biorefinary scale," *Biomass and Bioenergy*, vol. 144, no. November 2020, p. 105904, 2021.
[190] Z. K. Bagewadi, S. I. Mulla, and H. Z. Ninnekar, "Optimization of laccase production and its application in delignification of biomass," *International Journal of Recycling of Organic Waste in Agriculture*, vol. 6, no. 4. pp. 351–365, 2017.
[191] S. Dinant *et al.*, "Synchrotron FTIR and Raman spectroscopy provide unique spectral fingerprints for Arabidopsis floral stem vascular tissues," *J. Exp. Bot.*, vol. 70, no. 3, pp. 937–948, 2019.
[192] M. Alfaro, A. Majcherczyk, U. Kües, L. Ramírez, and A. G. Pisabarro, "Glucose counteracts wood-dependent induction of lignocellulolytic enzyme secretion in monokaryon and dikaryon submerged cultures of the white-rot basidiomycete Pleurotus ostreatus," *Sci. Rep.*, vol. 10, no. 1, pp. 1–10, 2020.
[193] S. Yoav *et al.*, "Effects of cre1 modification in the white-rot fungus Pleurotus ostreatus PC9: Altering substrate preference during biological pretreatment," *Biotechnol. Biofuels*, vol. 11, no. 1, pp. 1–16, 2018.
[194] Y.-S. Bahn, C. Xue, A. Idnurm, J. C. Rutherford, J. Heitman, and M. E. Cardenas, "Sensing the environment: lessons from fungi.," *Nat. Rev. Microbiol.*, vol. 5, no. 1, pp. 57–69, 2007.
[195] C. Xue, Y. P. Hsueh, and J. Heitman, "Magnificent seven: Roles of G protein-coupled receptors in extracellular sensing in fungi," *FEMS Microbiol. Rev.*, vol. 32, no. 6, pp. 1010–1032, 2008.
[196] F. Bianchi, J. S. van't Klooster, S. J. Ruiz, and B. Poolman, "Regulation of Amino Acid Transport in Saccharomyces cerevisiae," *Microbiol. Mol. Biol. Rev.*, vol. 83, no. 4, pp. 1–38, 2019.
[197] J. R. Robinson, O. S. Isikhuemhen, and F. N. Anike, "Fungal–metal interactions: A review of toxicity and homeostasis," *J. Fungi*, vol. 7, no. 3,

2021.
[198] C. Xue, Y. P. Hsueh, and J. Heitman, “Magnificent seven: Roles of G protein-coupled receptors in extracellular sensing in fungi,” *FEMS Microbiol. Rev.*, vol. 32, no. 6, pp. 1010–1032, 2008.
[199] L. Li, S. J. Wright, S. Krystofova, G. Park, and K. A. Borkovich, “Heterotrimeric G Protein Signaling in Filamentous Fungi,” *Annu. Rev. Microbiol.*, vol. 61, no. 1, pp. 423–452, 2007.
[200] S. J. Pirt, “Form of Pellets in Submerged Culture A Theory of the Mode of Growth of Fungi in the,” *Proc. R. Soc. Lond. B*, pp. 369–373, 1966.
[201] M. J. García-Soto, E. Botello-Álvarez, H. Jiménez-Islas, J. Navarrete-Bolaños, E. Barajas-Conde, and R. Rico-Martínez, “Growth morphology and hydrodynamics of filamentous fungi in submerged cultures,” *Adv. Agric. Food Biotechnol.*, vol. 661, no. 2, pp. 17–34, 2006.
[202] T. Lu, Q. Zhang, S. Yao, and B. Engineering, “Mycelial pellet formation of marine-derived fungus: new formation pathway directly from hyphae,” *Res. Rev. J. Microbiol. Biotechnol. Pathw. directly from hyphae*, vol. 4, no. 2, pp. 18–25, 2015.
[203] E. J. Espinosa-Ortiz, E. R. Rene, K. Pakshirajan, E. D. van Hullebusch, and P. N. L. L. Lens, “Fungal pelleted reactors in wastewater treatment: Applications and perspectives,” *Chem. Eng. J.*, vol. 283, pp. 553–571, 2016.
[204] P. a Gibbs, R. J. Seviour, and F. Schmid, “Growth of filamentous fungi in submerged culture: Problems and possible solutions,” *Critical Reviews in Biotechnology*, vol. 20, no. 1. pp. 17–48, 2000.
[205] D. Pei, J. Xu, Q. Zhuang, H.-F. Tse, and M. a Esteban, “The Taming of the Shrew - Controlling the Morphology of Filamentous Eukaryotic and Prokaryotic Microorganisms,” *Adv. Biochem. Eng. Biotechnol.*, vol. 123, no. July 2015, pp. 127–141, 2010.
[206] H. A. Raja, A. N. Miller, C. J. Pearce, and N. H. Oberlies, “Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community,” *J. Nat. Prod.*, vol. 80, no. 3, pp. 756–770, 2017.
[207] F. A. Avin, S. Bhassu, Y. S. Tan, P. Shahbazi, and S. Vikineswary, “Molecular divergence and species delimitation of the cultivated oyster mushrooms: Integration of IGS1 and ITS,” *Sci. World J.*, vol. 2014, 2014.
[208] Y. M. Kim and H. G. Song, “Effect of fungal pellet morphology on enzyme activities involved in phthalate degradation,” *J. Microbiol.*, vol. 47, no. 4, pp. 420–424, 2009.
[209] A. Ahamed and P. Vermette, “Effect of culture medium composition on Trichoderma reesei’s morphology and cellulase production,” *Bioresour. Technol.*, vol. 100, no. 23, pp. 5979–5987, 2009.
[210] A. Peciulyte, G. E. Anasontzis, K. Karlström, P. T. Larsson, and L. Olsson, “Morphology and enzyme production of Trichoderma reesei Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates,” *Fungal Genet. Biol.*, vol. 72, pp. 64–72, 2014.
[211] W. D. H. Schneider, L. Dos Reis, M. Camassola, and A. J. P. Dillon, “Morphogenesis and production of enzymes by penicillium echinulatum in response to different carbon sources,” *Biomed Res. Int.*, vol. 2014, pp. 1–10, 2014.
[212] F. C., J. Michel, E. A. Grulke, and C. A. Reddy, “Determination of the

Respiration Kinetics for Mycelial Pellets of Phanerochaete chrysosporium," *Appl. Environ. Microbiol.*, vol. 58, no. 5, pp. 1740–1745, 1992.
[213] F. C. Michel, E. A. Grulke, and C. A. Reddy, "A kinetic model for the fungal pellet lifecycle," *AIChE J.*, vol. 38, no. 9, pp. 1449–1460, 1992.
[214] K. I. Fernández-Alejandre *et al.*, "Diffusional and transcriptional mechanisms involved in laccases production by Pleurotus ostreatus CP50," *J. Biotechnol.*, vol. 223, pp. 42–49, 2016.
[215] F. Sbrana *et al.*, "Atomic force microscopy images suggest aggregation mechanism in cerato-platanin," *Eur. Biophys. J.*, vol. 36, no. 7, pp. 727–732, 2007.
[216] D. Xu, Y. Wang, A. A. Keerio, and A. Ma, "Identification of hydrophobin genes and their physiological functions related to growth and development in Pleurotus ostreatus," *Microbiol. Res.*, vol. 247, no. June 2020, p. 126723, 2021.
[217] S. Longobardi *et al.*, "Environmental conditions modulate the switch among different states of the hydrophobin Vmh2 from Pleurotus ostreatus," *Biomacromolecules*, vol. 13, no. 3, pp. 743–750, 2012.
[218] A. Armenante *et al.*, "The Pleurotus ostreatus hydrophobin Vmh2 and its interaction with glucans," *Glycobiology*, vol. 20, no. 5, pp. 594–602, 2010.
[219] L. Lav and A. G. Pisabarro, "Comparative and transcriptional analysis of the predicted secretome in the lignocellulose-degrading basidiomycete fungus Pleurotus ostreatus," vol. 18, pp. 4710–4726, 2016.
[220] L. Grifoll-Romero, S. Pascual, H. Aragunde, X. Biarnés, and A. Planas, "Chitin deacetylases: Structures, specificities, and biotech applications," *Polymers (Basel).*, vol. 10, no. 4, pp. 1–29, 2018.
[221] N. Pareek, V. Vivekanand, S. Saroj, A. K. Sharma, and R. P. Singh, "Purification and characterization of chitin deacetylase from Penicillium oxalicum SAE M-51," *Carbohydrate Polymers*, vol. 87, no. 2. pp. 1091–1097, 2012.
[222] Q. Xiao *et al.*, "Differential Proteomic Profiles of Pleurotus ostreatus in Response to Lignocellulosic Components Provide Insights into Divergent Adaptive Mechanisms," vol. 8, no. March, 2017.
[223] C. Toyokawa *et al.*, "Effects of overexpression of PKAc genes on expressions of lignin-modifying enzymes by Pleurotus ostreatus," *Biosci. Biotechnol. Biochem.*, vol. 80, no. 9, pp. 1759–1767, 2016.
[224] Y. Magae and J. Magae, "Effect of staurosporine on growth and hyphal morphology of Pleurotus ostreatus," *J. Gen. Microbiol.*, vol. 139, no. 1, pp. 161–164, 1993.
[225] Y. YAO *et al.*, "The White-Rot Fungus Pleurotus ostreatus Transformant Overproduced Intracellular cAMP and Laccase," *Biosci. Biotechnol. Biochem.*, vol. 77, no. 11, pp. 2309–2311, 2013.
[226] I. Khemiri, F. Tebbji, and A. Sellam, "Transcriptome Analysis Uncovers a Link Between Copper Metabolism, and Both Fungal Fitness and Antifungal Sensitivity in the Opportunistic Yeast Candida albicans," *Front. Microbiol.*, vol. 11, no. May, pp. 1–13, 2020.
[227] C. Bosch, Z. Bhana, B. Toplis, H. Volschenk, and A. Botha, "Transcriptomic response of Cryptococcus neoformans to ecologically relevant nitrogen concentrations," *FEMS Yeast Res.*, vol. 21, no. 4, pp. 1–13, 2021.

[228] V. Faraco, P. Giardina, and G. Sannia, “Metal-responsive elements in Pleurotus ostreatus laccase gene promoters,” *Microbiology*, vol. 149, no. 8, pp. 2155–2162, 2003.
[229] A. Amore, Y. Honda, and V. Faraco, “Copper induction of enhanced green fluorescent protein expression in Pleurotus ostreatus driven by laccase poxa1b promoter,” *FEMS Microbiol. Lett.*, vol. 337, no. 2, pp. 155–163, 2012.
[230] Y. Qi, C. Liu, X. Sun, L. Qiu, and J. Shen, “The identification of transcriptional regulation related gene of laccase poxc through yeast one-hybrid screening from Pleurotus ostreatus,” *Fungal Biol.*, vol. 121, no. 11, pp. 905–910, 2017.
[231] S. Mazumder, S. K. Basu, and M. Mukherjee, “Laccase production in solid-state and submerged fermentation by Pleurotus ostreatus,” *Eng. Life Sci.*, vol. 9, no. 1, pp. 45–52, 2009.
[232] G. Palmieri *et al.*, “Purification, Characterization, and Functional Role of a Novel Extracellular Protease from Pleurotus ostreatus,” *Appl. Environ. Microbiol.*, vol. 67, no. 6, pp. 2754–2759, 2001.
[233] P. J. Hoegger, S. Kilaru, T. Y. James, J. R. Thacker, and U. Kües, “Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences,” *FEBS J.*, vol. 273, no. 10, pp. 2308–2326, 2006.

Annexes

List of acronyms and abbreviations

A

AA ..... Auxiliary activities
AA1_1 ..... Auxiliary Activity Family 1 / Subf 1
AA1_2 ..... Auxiliary Activity Family 1 / Subf 2
AA293 ..... predicted metallo-protease
AA5_1 ..... Auxiliary Activity Family 5 / Subf 1
AA6 ..... Auxiliary Activity Family 6
AA7 ..... Auxiliary Activity Family 7
AA9 ..... Auxiliary Activity Family 9
ABTS ..... 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonate)
ACP ..... 3-oxoacyl-[acyl-carrier-protein] reductase
ALDH2 ..... Aldehyde Dehydrogenase (NAD+)
ARE ..... antioxidant responsive elements

B

BLAST ..... Basic Local Alignment Search Tool

C

C + Cu^{2+} ..... CMC plus Copper Sulfate
CN ..... Carbon-Nitrogen ratio
cAMP ..... Cyclic adenosine monophosphate
CAZymes, CAZy ..... Carbohydrate-Active EnZymes
CBM13 ..... Carbohydrate-Binding Module Family 13
CBMs ..... Carbohydrate-Binding Modules
CCD ..... Central Composite Design
CCR ..... Carbon Catabolite Repression
CDAs ..... Chitin deacetylase
CE ..... carbohydrate esterases
CE4 ..... Carbohydrate Esterase Family 4
Chts ..... Chitinase
CMC, C ..... carboxymethylcellulose
CP ..... Cerato-platanin like
CRE ..... catabolic responsive elements
CsbD-like ..... general stress response protein
ctr1 ..... predicted Copper transporter gene
Ctr1p ..... High-affinity copper transporter
CTR6/Cr1p ..... Cu+ transporting (copper detoxification) ATPase
Cup ..... Cupredoxin
CVNH ..... CyanoVirin-N Homology domain
CX + Cu^{2+} ..... CMC-Xylose plus copper sulfate
CX ..... CMC and Xylose
Cα ..... alpha carbon

D

DAO ..... D-aspartate oxidase
DEG ..... differentially expressed genes
*dk*N001 ..... dikaryotic parental N001, a commercial strain of *P. ostreatus*
DNS ..... 3,5-dinitrosalicylic acid

E

E3 ..... E3 ubiquitin ligase

F

FDR ..... False Discovery Rate
FOGE3 ..... FOG Predicted E3 ubiquitin ligase
Fre1 ..... Plasma membrane Fe3+ and Cu2+ reductase, *Véase*
FTIR ..... Fourier Transform Infrared Spectrometry

G

G ..... guaiacyl
GH ..... Glycoside Hydrolases
GH152 ..... Glycoside Hydrolase Family 152
GH18 ..... Glycoside Hydrolase Family 18, *Véase*
GH45 ..... Glycoside Hydrolase Family 45
GH79 ..... Glycoside Hydrolase Family 79
GOOX ..... Glucooligosaccharide Oxidase
GPCR ..... G protein-coupled receptor
GST ..... Glutathione S-transferase
GT ..... glucosyltransferases
GY+Cu^{2+} ..... Glucose-Yeast Extract and Copper Sulfate
GY2415 ..... Glucose and Yeast-extract at 24 and 15 g/L, respectively
GY3008 ..... Glucose and Yeast-extract at 30 and 8 g/L, respectively
GY3023 ..... Glucose and Yeast-extract at 30 and 23 g/L, respectively
GY4504 ..... Glucose and Yeast-extract at 45 and 4 g/L, repectively
GY4515 ..... Glucose, and Yeast-extract at 45, and 15 g/L, respectively
GY4515 + Cu^{2+} ..... GY4515 plus Copper sulfate
GY4526 ..... Glucose, and Yeast-extract at 45, and 26 g/L, respectively, *Véase*
GY6008 ..... Glucose and Yeast-extract at 60 and 8 g/L, respectively
GY6023 ..... Glucose and Yeast-extract at 60 and 23 g/L, respectively
GY6215 ..... Glucose and Yeast extract at 62 and 15 g/L, repectively
GYR ..... Glucose, Yeast-extract and Rice husks
GYR+Cu^{2+} ..... Glucose, Yeast-extract and Rice husk plus Copper Sulfate
GYR051580 + Cu^{2+} ..... GYR051580 plus copper sulfate
GYR051580 ..... Glucose, Yeast-extract, and Rice husks at 05, 15, and 80 g/L, repectively
GYR101080 ..... Glucose, Yeast- extract, and Rice husk at 10, 10, and 80 g/L, respectively
GYR150580 ..... Glucose, Yeast- extract, and Rice husk at 15, 05, and 80 g/L, respectively
GYR161271 ..... Glucose, Yeast- extract, and Rice husk at 16, 12, and 71 g/L, respectively
GYR210871 ..... Glucose, Yeast- extract, and Rice husk at 21, 08, and 71 g/L, respectively
GYR251560 ..... Glucose, Yeast- extract, and Rice husks at 25, 15, and 60 g/L, respectively
GYR281063 ..... Glucose, Yeast- extract, and Rice husk at 28, 10, and 63 g/L, respectively
GYR300565 ..... Glucose, Yeast-extract, and Rice husks at 30, 5, and 65 g/L, repectively
GYR360856 ..... Glucose, Yeast- extract, and Rice husk at 36, 08, and 56 g/L, respectively
GYR361351 ..... Glucose, Yeast- extract, and Rice husk at 36, 13, and 51 g/L, respectively
GYR450550 ..... Glucose, Yeast- extract, and Rice husk at 45, 05, and 50 g/L, respectively
GYR451045 ..... Glucose, Yeast-extract, and Rice husks at 45, 10, and 45 g/L, repectively
GYR451540 + Cu^{2+} ..... GYR451540 plus copper sulfate
GYR451540 ..... Glucose, Yeast- extract, and Rice husk at 45, 15, and 40 g/L, respectively

H

H p-hydroxyphenyl
HAA ..... 3-hydroxyanthranilic acid
HBT 1-hydroxybenzotriazole
HSE ..... heat shock elements
HSMT ..... Homocysteine S-methyltransferase

I

ICMT ..... Isoprenylcysteine carboxyl methyltransferase

J

JGI.....Joint Genome Institute

K

KCNAB.....Voltage-gated shaker-like K+ channel, subunit beta

L

L + Cu^{2+}.....Lignin plus Copper Sulfate
L .....Lignin-alkali,
lacc1.....Laccase 1 gene from *P. ostreatus*
lacc10.....Laccase 10 gene from *P. ostreatus*
LACC10, Lacc10 .....Multicopper oxidases, laccase LACC10=POXC
lacc11.....Laccase 11 gene from *P. ostreatus*
lacc12.....Laccase 12 gene from *P. ostreatus*
lacc2.....Laccase 2 gene from *P. ostreatus*
LACC2, Lacc2 ..... Multicopper oxidases, laccase LACC2
lacc3.....Laccase 3 gene from *P. ostreatus*
lacc4.....Laccase 4 gene from *P. ostreatus*
LACC5, Lacc5 ..... Multicopper oxidases, laccase LACC5
lacc5.....Laccase 5 gene from *P. ostreatus*
lacc6.....Laccase 6 gene from *P. ostreatus*
LACC6, Lacc6 .....Multicopper oxidases, laccase LACC6=POXA1B
lacc7.....Laccase 7 gene from *P. ostreatus*
lacc8.....Laccase 8 gene from *P. ostreatus*
lacc9.....Laccase 9 gene from *P. ostreatus*
LC + Cu^{2+}..... Lignin-CMC plus Copper Sulfate
LC ..... Lignin and CMX
LCX + Cu^{2+}..... Lignin,CMC and Xylose plus copper sulfate
LCX..... Lignin, CMC and Xylose
LDCs ..... Lignocellulose Derived Compounds
LiP.....Lignin Peroxidase
LMEs..... Lignin-Modifying Enzymes
LPMOs..... Lytic Polysaccharide-Monooxygenases
LX + Cu^{2+}..... Lignin, Xylose and Copper Sulfate
LX ..... Lignin and Xylose

M

Mch5 .....High affinity facilitated diffusion, riboflavin-regulated riboflavin uptake system
mkPC15..... monokaryotic protoclone 15 from *dk*N001 *P. ostreatus* strain
*mk*PC19 .....monokaryotic protoclone 9 from *dk*N001 *P. ostreatus* strain,
MLA..... maximum laccase activity
MnP.....manganese peroxidase
MRE.....metal responsive elements

N

NIT.....nitrogen binding site
NMO .....Nitronate monooxygenase
NMR .....Nitrogen Metabolite Repression
NmrA-like, *nmr1*.....Nitrogen metabolite repression in fungi
Nup98/Cu^{2+} .....Nuclear pore complex, Nup98 component, and Cupredoxin domain

P

P450 .....Cytochrome P450
PC.....Polyketide cyclase
PCA..... principal component analysis
PCC Ca^{2+} ..... Ca^{2+}-modulated nonselective cation channel polycystin
PC-Cu..... Blue (type 1) copper domain

PDY ..... Potato-Dextrose-Yeast-extract
PKA ..... cAMP-dependent Protein Kinase
PL ..... Polysaccharide Lyases
pox2 ..... Phenol oxidase 2 gene
POXA3a ..... Small subunit of laccase POXA3a
PTK ..... Protein tyrosine kinase

R

RH ..... Rice husks
RH + CX + Cu^{2+} ..... Rice husks plus CX and copper sulfate extract
RH + CX ..... Rice husks plus CX extract
RH + GY + Cu^{2+} ..... Rice husk plus GY and Copper sulfate extract
RH + GY ..... Rice husks plus GY extract, Rice husks plus GY extract
RH + L ..... Rice husk plus L- extract
RH + L + Cu^{2+} ..... Rice husks plus L and Copper sulfate extract
RH ..... rice husks
RPKM ..... reads per kilobase of transcript per million mapped reads

S

S ..... syringyl
S33 ..... Peptidase S33, prolyl aminopeptidase
S33.UPW ..... Peptidase S33, prolyl aminopeptidase
SEM ..... scanning electron microscopy
SLF ..... static liquid fermentation
SmF ..... submerged fermentation
Smf3p ..... Intracellular heavy metal transporter
SSF ..... solid-state fermentation
SSF ..... solid fermentation
STRE ..... stress-responsive elements, stress-responsive elements

T

T1 ..... Type 1 copper site
T2 ..... Type 2 copper site
T3 ..... Type 1 copper site
TCDB ..... Transporter Classification Database
Thau ..... Thaumatin

V

VMH2 ..... Specific vegetative mycelium hydrophobin 2
VP ..... versatile peroxidase
VPS1 ..... Vacuolar sorting protein 1

W

WAP ..... Whey Acidic Proteins

X

X ..... Xylose
XRE ..... xenobiotic responsive elements

Culture media names abbreviations and compositions

Chapter 2: Effect of Nutritional Factors and Copper on the Regulation of Laccase Enzymes Production in *Pleurotus ostreatus*

GY3008: culture medium contains 30.0 gL^{-1} of glucose and 7.5 gL^{-1} of yeast extract
GY6008: culture medium contains 60.0 gL^{-1} of glucose and 7.5 gL^{-1} of yeast extract
GY3023: culture medium contains 30.0 gL^{-1} of glucose and 22.5 gL^{-1} of yeast extract
GY6023: culture medium contains 60.0 gL^{-1} of glucose and 22.5 gL^{-1} of yeast extract
GY2415: culture medium contains 23.8 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract
GY6615: culture medium contains 66.2 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract
GY4504: culture medium contains 45.0 gL^{-1} of glucose and 4.30 gL^{-1} of yeast extract
GY4526: culture medium contains 45.0 gL^{-1} of glucose and 25.6 gL^{-1} of yeast extract
GY4515: culture medium contains 45.0 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract
GY3008 + Cu^{2+}: culture medium contains 30.0 gL^{-1} of glucose, 7.5 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate.
GY6008 + Cu^{2+}: culture medium contains 60.0 gL^{-1} of glucose, 7.5 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate.
GY3023 + Cu^{2+}: culture medium contains 30.0 gL^{-1} of glucose, 22.5 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate.
GY6023+ Cu^{2+}: culture medium contains 60.0 gL^{-1} of glucose, 22.5 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate.
GY2415 + Cu^{2+}: culture medium contains 23.8 gL^{-1} of glucose, 15.0 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate.
GY6615 + Cu^{2+}: culture medium contains 66.2 gL^{-1} of glucose, 15.0 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate
GY4504 + Cu^{2+}: culture medium contains 45.0 gL^{-1} of glucose, 4.30 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate
GY4526 + Cu^{2+}: culture medium contains 45.0 gL^{-1} of glucose, 25.6 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate
GY4515 + Cu^{2+}: culture medium contains 45.0 gL^{-1} of glucose, 15.0 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate

Other culture name abbreviations

GY4515-12: sample was taken in 12^{th} day of culture from culture medium with initial 45.0 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract concentrations
GY4515-21: sample was taken in 21^{th} day of culture from culture medium with initial 45.0 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract concentrations
GY0515: sample was taken in 12th day of culture from culture medium with initial 5.0 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract concentrations
GY4504: sample was taken in 12th day of culture from culture medium with initial 45.0 gL^{-1} of glucose and 4.0 gL^{-1} of yeast extract concentrations

Chapter 3: Role of copper-induced laccases and lignocellulose derived compounds on the rice husks modifications

LCX + Cu^{2+} : 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 5 gL^{-1} of CMC, 5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
LCX: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 5 gL^{-1} of CMC, 5 gL^{-1}, and of xylose.

CX + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of CMC, 5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
CX: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of CMC, and 5 gL^{-1} of xylose.
LX + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 0.5 gL^{-1} of CMC, 5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
LX: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 0.5 gL^{-1} of CMC, and 5 gL^{-1} of xylose.
X + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 0.5 gL^{-1} of CMC, 5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
X: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 0.5 gL^{-1} of CMC, and 5 gL^{-1} of xylose.
LC + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 5 gL^{-1} of CMC, 0.5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
LC: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 5 gL^{-1} of CMC, and 5 gL^{-1} of xylose.
C + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of CMC, 0.5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
C: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of CMC, and 0.5 gL^{-1} of xylose.
L + Cu^{2+}: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 0.5 gL^{-1} of CMC, 0.5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
L: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 5 gL^{-1} of lignin, 0.5 gL^{-1} of CMC, and 0.5 gL^{-1} of xylose.
GY+Cu^{2+} : 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 0.5 gL^{-1} of CMC, 0.5 gL^{-1} of xylose, and 0.25 gL^{-1} of copper sulfate.
GY: 40 gL^{-1} of glucose, and 20 gL^{-1} of yeast extract in the Glucose-Yeast-extract (GY) culture-based medium was supplemented with 0.5 gL^{-1} of CMC, and 0.5 gL^{-1} of xylose.

Other culture name abbreviations

RH + GY: Rice husks treated with GY extract
RH + GY + Cu^{2+}: Rice husks treated with GY + Cu^{2+} extract
RH + CX: Rice husks treated with CX extract
RH + CX + Cu^{2+}: Rice husks treated with GY + Cu^{2+} extract
RH + L: Rice husks treated with L- extract
RH + L + Cu^{2+}: Rice husks treated with L + Cu^{2+} extract

Chapter 4 : Laccases and other Lignocellulolytic Enzymes in the Transcriptome of *Pleurotus ostreatus* used for Biological Pretreatment Systems of Rice Husks in SmF

GYR161271: culture medium contains 16, 12, and 71 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR251560: culture medium contains 25, 15, and 60 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR281063: culture medium contains 28, 10, and 63 gL^{-1} of glucose, yeast extract, and rice husks, respectively.

GYR450550: culture medium contains 45, 5, and 50 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR361351: culture medium contains 36, 13, and 51 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR451540: culture medium contains 45, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR150580: culture medium contains 45, 5, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR101080: culture medium contains 10, 10, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR360856: culture medium contains 36, 8, and 56 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR210871: culture medium contains 21, 8, and 71 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR051580: culture medium contains 5, 15, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR451045: culture medium contains 45, 10, and 45 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR300565: culture medium contains 30, 05, and 65 gL^{-1} of glucose, yeast extract, and rice husks, respectively.

Other culture name abbreviations

GYR =GYR451540: culture medium contains 40, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR + Cu^{2+}= GYR451540 + Cu^{2+}: culture medium contains 40, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks, respectively, and copper sulfate at 0.25 gL^{-1}.
GYR051580 + Cu^{2+}: culture medium contains 5, 15, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively, and copper sulfate at 0.25 gL^{-1}.
GYR451540-14: GYR051580-14: Rice husks samples from culture medium in 14th day culture day. Culture medium initial contains 45, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR451540-21: GYR051580-14: Rice husks samples from culture medium in 21th day culture day. Culture medium initial contains 45, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR051580-14: Rice husks samples from culture medium in 14th day culture day. Culture medium initial contains 5, 15, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GYR051580-21: Rice husks samples from culture medium in 14th day culture day. Culture medium initial contains 5, 15, and 80 gL^{-1} of glucose, yeast extract, and rice husks, respectively.
GY = GY4515: culture medium contains 45.0 gL^{-1} of glucose and 15.0 gL^{-1} of yeast extract
GY + Cu^{2+} = GY4515 + Cu^{2+}: culture medium contains 45.0 gL^{-1} of glucose, 15.0 gL^{-1} of yeast extract, and 0.25 gL^{-1} of copper sulfate

Chapter 5: Comparative analysis of the *Pleurotus ostreatus* transcriptome in synthetic culture media, with rice husks and copper sulfate in SmF

GY: synthetic culture media contains 45, and 15 gL^{-1} of glucose and yeast extract.
GY + Cu^{2+}: synthetic culture media contains 45, and 15 gL^{-1} of glucose, yeast extract and 0.25 gL^{-1} of copper sulfate
GYR: modified culture media contains 45, 15, and 40 gL^{-1} of glucose, yeast extract, and rice husks.
GYR + Cu^{2+}: modified culture media contains 45, 15, and 40 gL^{-1} of glucose, yeast extract, and 0.25 gL^{-1} of copper sulfate.