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A COMBINED BIOLOGICAL PRETREATMENT OF WHEAT STRAW USING NATIVE WOOD-ROTTING FUNGI FOR IMPROVING ITS BIODEGRADABILITY

DOCTORAL THESIS IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR IN NATURAL RESOURCES SCIENCES

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A Dios,

A mi esposa – Lily,

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Summary and thesis outline

Lignocellulosic biomass represents a highly abundant feedstock for the production of chemical compounds (organic acids, xylitol, furfural, cresols, catechol, among others) and biofuels (bioethanol, biomethane, biohydrogen, among others). Lignocellulosic biomass is basically composed of cellulose, hemicellulose, and lignin. Cellulose is a major fraction (35-50% dry mass) of lignocellulosic biomass, which can be converted into glucose through enzymatic hydrolysis, and can subsequently be fermented into ethanol. However, lignocellulosic biomass is highly recalcitrant to enzymatic hydrolysis, because cellulose is sheathed by lignin and hemicellulose; this physical barrier hinders enzyme access to cellulose during hydrolysis. In addition, cellulose fibers show highly resistant crystalline regions. Therefore, a feasible process for obtaining cellulose-derived products from lignocellulosic biomass requires a pretreatment step to increase cellulose accessibility and biodegradability. In biological pretreatment, white-rot fungi have been used for their ability to selectively degrade lignin, whereas brown-rot fungi have been used for their ability to cause extensive degradation and depolymerization of cellulose and hemicellulose via Fenton reactions, with minimal assimilation of degradation products in early decay stages. For that reason, the main objective of this thesis was to evaluate a wheat straw pretreatment, comprising the combined action of white-rot fungus, *Ganoderma lobatum*, and brown-rot fungus, *Gloeophyllum trabeum*, for improving its biodegradability. These fungi were selected in a preliminary screening of 14 Chilean strains of wood-rotting fungi. In a first step, the effects of metal ions, Fe^{2+} and Mn^{2+}, as enzyme inducers and Fenton reactants, and NO_3^- as additional nitrogen source, were evaluated in order to find optimal culture conditions to promote an adequate wheat straw degradation by the application of a fungal pretreatment. In general, Mn^{2+} had the strongest positive effect on lignin degradation by *G. lobatum*, whereas Fe^{2+} had the strongest positive effect on decreasing total crystallinity index of wheat straw by *G. trabeum*. NO_3^- decreased the weight loss caused by both tested fungi during wheat straw degradation.

Afterwards, the effect of pretreatment by single-fungus-culture under optimal culture conditions was evaluated on enzymatic hydrolysis of wheat straw. The highest glucose yields from enzymatic hydrolysis were detected in pretreated wheat straw, by *G. lobatum* and *G. trabeum* single-cultures for 20 and 10 days of incubation, which increased the yields by 43.6% and 26.1% compared to untreated wheat straw, respectively. Based on these results, the effect of fungal co-culture was evaluated using different inoculation strategies, including co-inoculation and sequential inoculation. Co-culture using co-inoculation, both fungi were simultaneously inoculated in wheat straw and incubated for 20 days, resulted in higher glucose yield (70% higher than untreated wheat straw) when compared with single cultures, but no synergistic effect between fungi was observed. In contrast, the sequential inoculation of *G. lobatum*, incubated for 10 days, followed by *G. trabeum*, incubated for 10 more days, showed a synergic effect on enzymatic hydrolysis. The synergistic effect between *G. lobatum* and *G. trabeum* observed in co-culture using sequential inoculation was mainly related to their abilities to synergistically degrade lignin and modify the crystalline regions in the wheat straw lignocellulosic matrix, respectively. This co-culture showed the highest glucose yield (191.5 mg glucose g^{-1} wheat straw), which was 2.8-fold higher than untreated wheat straw. Although the glucose yield was higher than other biological pretreatments previously reported, it is still not comparable with the currently physicochemical methods. Nevertheless, new studies have been focusing on the use of biological pretreatments as a complementary step to physicochemical pretreatments in order to increase its efficiency. In this sense, a biological pretreatment with high glucose yield is a good alternative as a complementary method.

Table of contents

CHAPTER I
General introduction

General introduction

1.1 Introduction

Lignocellulosic biomass from agricultural, industrial and forest practices is the most abundant feedstock available worldwide, which can be used for the production of second-generation biofuels. Regardless of its origin, lignocellulosic biomass is composed of three major components, lignin, cellulose, and hemicellulose. Cellulose is the major fraction (35 to 50% of dry weight) of lignocellulosic biomass, which can be enzymatically hydrolyzed into glucose and subsequently fermented into biofuels (Silveira et al. 2015). However, in the native state of lignocellulosic biomass, the cellulosic fraction is sheathed by lignin and hemicellulose which implies a physical barrier that prevents the access of cellulase enzymes during hydrolysis (Mosier et al. 2005). Besides, cellulose fibers present intrachain and interchain hydrogen bonds forming highly resistant crystalline regions. Therefore, a feasible process for obtaining cellulose-derived biofuels from lignocellulosic biomass requires a pretreatment step to increase its availability and biodegradability. In nature, wood-rotting fungi including white-rot, brown-rot, and soft-rot fungi, degrade plant cell wall polymers through enzymatic and non-enzymatic mechanisms. In this context, white-rot fungi have been studied for their ability to selectively degrade lignin in wood and non-wood biomass, which results in greater cellulose availability to subsequent enzymatic hydrolysis (Saha et al. 2016; Wan and Li 2010). Whereas, brown-rot fungi have been used for their ability to cause extensive and fast degradation and depolymerization of cellulose and hemicellulose through non-enzymatic mechanisms, without a significant polysaccharide consumption by fungus in early decay stages. However, very few studies have evaluated non-wood biomass degradation by brown-rot fungi (Lee et al. 2008; Machado and Ferraz 2017; Rasmussen et al. 2010). The effectiveness of biological pretreatment varies between used species and even between strains of the same species (Machado and Ferraz 2017; Saha et al. 2016; Salvachúa et al. 2011).

Consequently, a preliminary screening of fungi is required in order to select fungi with high potential to carry out an effective biological pretreatment. Besides, as lignocellulosic biomass are often rich in carbon, but low in nutrients (nitrogen, phosphorus and trace elements) and water content, culture conditions as moisture and addition of nutrients, are other important factors that affect the effectiveness of biological pretreatment (Baldrian et al. 2005; Salvachúa et al. 2013; Shrivastava et al. 2012).

So far, biological pretreatment of lignocellulosic biomass has been performed using single-culture of fungi. However, in nature, degradation process of lignocellulosic biomass (mainly wood biomass) is carried out by the different wood-rotting fungi interaction. Therefore, the use fungal co-culture that mimics this type of interaction could shorten pretreatment time and improve its efficiency. Studies about wood-rotting fungi co-culture in lignocellulosic biomass have evaluated ligninolytic enzymes production or partial lignin degradation to facilitate the pulping process. These studies have evaluated co-cultures of white-rot fungal strains using simultaneous inoculation (Chi et al. 2007; Iakovlev and Stenlid 2000; Kannaiyan et al. 2015), whereas the effect of white-rot and brown-rot fungi co-culture or the sequential inoculation of fungi has been less studied.

1.2 Hypothesis

Considering the abilities of white-rot fungi to selectively degrade lignin, and the abilities of brown-rot fungi to modify cellulose and hemicellulose structure in lignocellulosic biomass with a minimal polysaccharide loss in early decay stages, we proposed the following hypothesis:

A biological pretreatment of wheat straw by the combined action of a selective lignin-degrading white-rot fungus and a brown-rot fungus will result in greater accessibility and degradability of cellulose in wheat straw for subsequent enzymatic hydrolysis process.

1.3 General objective

To evaluate biological pretreatment of wheat straw by the combined action of white-rot and brown-rot fungi isolated from forests ecosystems of southern Chile for improving the biodegradability of this substrate.

1.4 Specific objectives

(1) To select at least one strain of white-rot and brown-rot fungi with high lignocellulolytic potential.

(2) To evaluate the effect of metal ions (Fe^{2+} and Mn^{2+}) as enzyme inducers or Fenton reactants and nitrate (NO_3^-) as nitrogen source on wheat straw degradation by the selected white-rot and brown-rot fungi.

(3) To evaluate the simultaneous and sequential action of the selected white-rot and brown-rot fungi on the pretreatment and enzymatic hydrolysis of wheat straw.

1.5 Theoretical framework

1.5.1 Lignocellulosic biomass composition

Lignocellulosic biomass, as wheat straw, sugarcane bagasse, corn stalks, rye straw, rice straw, and barley straw, among others, is an important renewable carbon source. The cell wall represents more than 80% dry weight of lignocellulosic biomass; therefore their main constituents are cellulose, hemicellulose and lignin (Sánchez 2009a). Cellulose constitutes the major proportion of lignocellulosic biomass, it is a linear homopolymer of (1–4)-linked β-D-glucose and represent the real potential for obtaining bioethanol from lignocellulosic biomass. The β 1-4 bonds can be only cleaved by microbial enzymes. Cellulose can reach 2,000-6000 and 14,000 residues polymerization degrees in the primary and secondary cell wall, respectively (Chandel and Singh 2011). Cellulose fibers present intra-chain and inter-chain

hydrogen bonds forming highly resistant crystalline regions (Brett 2000). Hemicellulose represents about 25% dry weight of lignocellulosic biomass and it is composed by a lineal backbone with branched chains formed by 5 and 6 carbons sugar compounds (L-arabinose, D-glucose, D-mannose, D-xylose, L-galactose). Based on their sugar composition, hemicellulose can be characterized as xylan, xyloglucan and galactomannans (Hayashi and Kaida 2011). Hemicellulose structure and composition varies from different taxonomic orders and even in a tissue-specific manner (Hoffman et al. 2005). Xylan is the major hemicellulose present in cereals and hardwood biomass (Kubicek 2012). Hemicellulose is a bonding agent between lignin and cellulose and can be hydrolyzed much faster than cellulose because it is an amorphous polymer (Ballesteros 2010). However, hemicellulose is the most affected plant cell wall component by lignification; therefore, its degradation is not maximized (Graminha et al. 2008). Lignin is a complex aromatic macromolecule form by the oxidative coupling of three primary hydroxycinnamyl alcohols; p-coumaryl, coniferyl, and sinapyl alcohol (Ralph et al. 2004). Based on the methoxy substitution on aromatic rings their corresponding phenylpropanoid units are p-hydrophenyl, guaiacyl and syringyl. The phenylpropanoid units are arranged randomly in an irregular three-dimensional network that provides its strength structure. Lignin interacts with cellulose forming covalent cross-links and with hemicelluloses forming diferulic acid bridges. Although lignin minor constituent of lignocellulosic biomass (18-24% dry weight), its aromatic structure and strong association with cellulose and hemicellulose greater limits the effective utilization of cellulose. Lignocellulosic biomass presents low protein, pectin, soluble carbohydrate, vitamin and mineral content (Bisaria 1998; Jonathan and Adeoyo 2011).

1.5.2 Bioethanol production from lignocellulosic biomass

Second-generation bioethanol (2G bioethanol) can be obtained from lignocellulosic biomass feedstocks such as, wood, bagasse, straw and grass biomass. The 2G bioethanol production

comprises pre-treatment, polysaccharide hydrolysis, fermentation and distillation in order to obtain an anhydrous ethanol as the end product (Aditiya et al. 2016). Pretreatment changes lignocellulosic biomass composition and microstructure. In this process, lignin and hemicellulose are degraded, and cellulose crystalline regions are modified (Chen et al. 2017; Da Silva et al. 2010). Pretreatment goal is to increase the availability and biodegradability of cellulose fraction, promoting enzyme-substrate interaction to enhance sugar hydrolysis (Silveira et al. 2015). Besides, pretreatment attempts to (1) prevent polysaccharide loss, (2) avoid the production of enzymatic hydrolysis inhibitors, (3) decrease the enzymatic loading require for efficient sugar hydrolysis (4) avoid the use of chemical reagents, (5) reduce the solid residues generation, (6) control process costs derived from energy consumption and (7) allow recovering lignin and other compounds for further obtaining added-value coproducts (Amin et al. 2017; Chen et al. 2017). A suitable pretreatment technology is the first prerequisite for carrying out feasible and efficient ethanol production from lignocellulosic biomass. Different pretreatment methods have been investigated in order to enhance the cellulose accessibility in lignocellulosic biomass. The pretreatments methods nowadays available can be summarized as: (1) physical pretreatments (steam explosion, liquid hot water hydrothermolysis, ball milling and irradiation), (2) chemical pretreatments (acid, alkaline and organosolv pretreatment, catalyzed steam explosion) and (3) biological pretreatments (white-rot fungi, brown-rot fungi, bacterial consortium and anaerobic fungus from digestive system of termites). In general, lignocellulosic biomass pretreatments modify, solubilize or degrade lignin and hemicellulose, increase accessible surface area, reduce crystallinity and polymerization degree of cellulose (Da Silva et al. 2010; Mosier et al. 2005; Taherzadeh and Karimi 2008). Some economic and technical drawbacks of physicochemical pretreatments are the high energy consumption, require a pressure and corrosiveness resistant reactor, and production of enzymatic hydrolysis and fermentation inhibitors during pretreatment.

After pretreatment, polysaccharide hydrolysis releases monomeric sugars from cellulose and hemicellulose chains and is mainly catalyzed by microbial enzymes (also known as enzymatic hydrolysis), but also by acid (Aditiya et al. 2016). This process is required due to the fact that microorganisms employed in the fermentation process are only able to digest monomeric sugars. Therefore, polysaccharide hydrolysis performance and hydrolysate quality (sugar syrup) are critical in 2G bioethanol production. Hydrolysate Fermentation is carried out by metabolic activity of yeast (*Saccharomyces cerevisiae*, *Candida shehatae*) or bacteria (*Zymomonas mobilis*, *Clostridium thermocellum*, *Thermoanaerobacter mathranii*) (Aditiya et al. 2016; Nichols et al. 2014). Theoretically, each kg of glucose and xylose can produce 0.49 kg carbon dioxide with 0.51 kg ethanol (Hamelinck et al. 2005). The fermentation performance depends on the supporting conditions such as temperature and pH. Finally, ethanol solution from fermentation process is distilled in order to remove water content, giving anhydrous ethanol. Distillation utilizes the difference in boiling points of the component to separate ethanol from the solution. The boiling temperature of ethanol is 78.2 °C; therefore, when the mixture is heated to this temperature, ethanol in the solution is vaporized and separated from other components. Strictly, anhydrous ethanol cannot contain more than 0.5% (v/v) water content. Other techniques for obtain anhydrous ethanol can be summarized as; adsorption process, azeotropic distillation, chemical dehydration, diffusion distillation, extractive distillation, membrane process, and vacuum distillation.

1.5.3 Biological pretreatment

Biological pretreatment attempts to control lignin or hemicellulose degradation in lignocellulosic biomass caused by microorganisms (mainly wood-rotting fungi), preventing the inevitable glucose loss associated to microorganism consumption for self-growth and metabolism as much as possible. Biological pretreatments are a low cost and environmentally friendly method but it requires long microorganism exposure time (from weeks to months).

Nevertheless, post-harvest, the lignocellulosic biomass is stored on farm for months, therefore, the fungal pretreatment as an application during storage is an alternative that could solve the long exposure time required by fungi (Wan and Li, 2010). However, to integrate storage and pretreatment in Chile, the normal conservation of lignocellulosic biomass (crop residues) by dry-storage should be replaced by wet-storage. The physical conditions of wet-storage on farm would facilitate the fungal growth on the biological pretreatment of lignocellulosic biomass. Besides, biomass storage will always be required due to the fact that industrial biofuel production needs constant feedstock flow, whereas biomass production process is seasonal. Therefore, the integration of storage and lignocellulosic biomass pretreatment could successfully provide year-round delignified biomass to biofuel production plant.

1.5.4 Lignocellulose degradation mechanisms of white-rot fungi

Based on chemical composition of lignocellulosic biomass, the wood rotting fungi are the most suitable microorganisms for carrying out biological pretreatment processes. White-rot fungi are able to degrade lignin, cellulose and hemicellulose through a complex enzymatic machinery (Martínez et al. 2005). Lignin degradation by white-rot fungi is carried out by extracellular lignin-modifying enzymes, also known as ligninolytic enzymes, which include a copper-containing phenoloxidase, laccase (Lac), and three heme-containing peroxidases; lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (MiP) (Figure 1) (Pointing 2001). A versatile peroxidase (VP) is also involved in the lignin degradation through aromatic rings oxidation. VP has been particularly reported in *Bjerkandera* and *Pleurotus* genres (Hatakka 1994). Nevertheless, as these peroxidases require hydrogen peroxide as an oxidizing substrate, their activity depends on an accessory enzyme, glyoxal oxidase, that provide it. The hydrogen peroxide is a short half-life (nanoseconds) compound and is the most powerful oxidizing agent of living cells (Eastwood

et al. 2011). Lignin degradation by white-rot fungi has been widely reported in several reviews (Hatakka 2005; Pointing 2001; Sánchez 2009a).

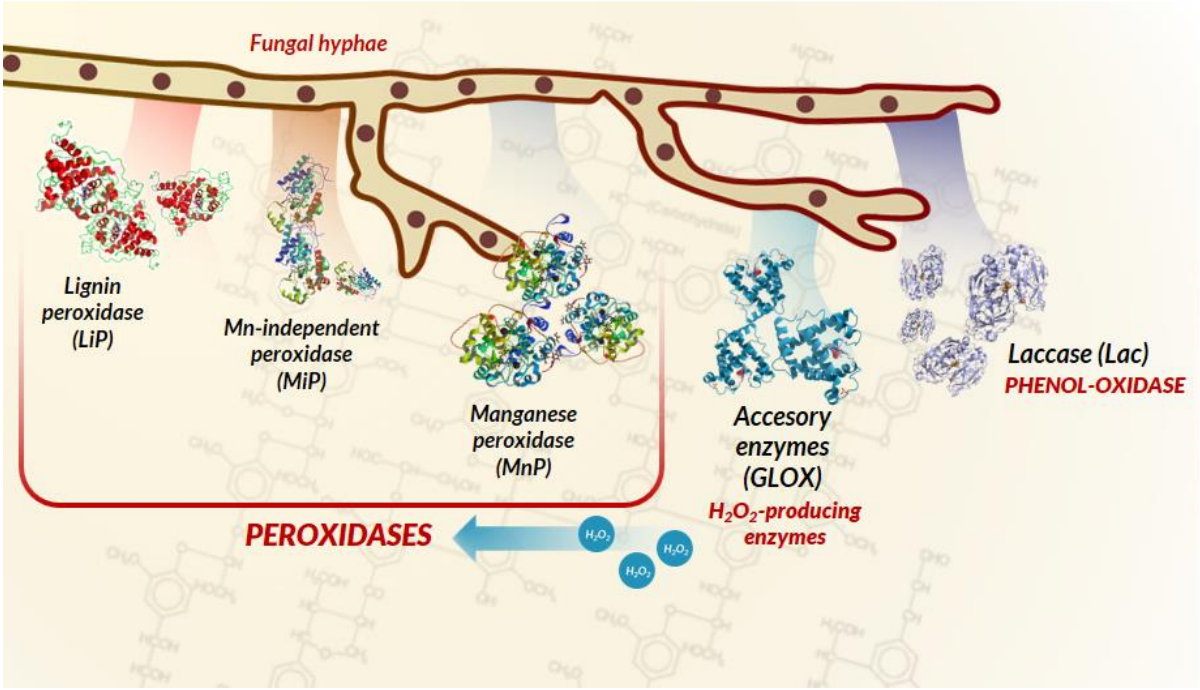

Figure 1 Extracellular enzymes involved in lignin degradation by white-rot fungi.

Cellulose degradation by white-rot fungi is carried out by extracellular a multienzymatic system composed of several hydrolytic enzymes. Briefly, endoglucanase randomly cleaves accessible β-bonds on cellulose chain, which creates more chain-ends for the cellobiohydrolases action. Cellobiohydrolase hydrolyzes cellulose chain-ends into cellobiose. Finally, exoglucanase (β-glucosidase) cleaves cellobiose into two glucose units (Baldrian and Valásková 2008). Hemicellulose degradation requires of a hydrolytic enzyme complex called hemicellulases. Enzymes required vary depending on hemicellulose origin. Enzymatic complex for xylan degradation includes, endoxylanase, generates oligosaccharides from xylan backbone, and β-xylosidase, that cleaves oligosaccharides into xylose units (Martínez et al. 2005). Besides, accessory enzymes that help to breakdown hemicellulose branch-chains such as; xylan esterases, ferulic and p-coumaric esterases, α-arabinofuranosidases, α-

glucuronosidases (Jeffries 1994). Xyloglucan degradation requires of the action of endoxylanase, acetyl esterase, α-glucuronidase and β-xylosidase. Whereas, galactomannans degradation requires of endomannase that breaks mannan backbone into oligosaccharides, β-mannosidase and β-glycosidase that cleavage the oligosaccharides into mannose, and acetylglucomannan esterase and α-galactosidase that remove acetyl groups and galactose residues, respectively (Eastwood et al. 2011). Although the above-mentioned enzymes are directly involved in the degradation of lignocellulose, many other enzymes are also secreted by fungi during lignocellulosic biomass pretreatment, which could be involved in the transformation and degradation.

In recent LC-MS/MS secretome analysis, Adav et al. (2012) reported that the lignocellulolytic enzyme expression and regulation of *Phanerochaete chrysosporium* cultivated in lignocellulosic biomass (corn stover, sawdust, sugarcane bagasse, wheat bran and wood chips) depend on nature and complexity of lignocellulosic biomass as well as biomass physical size. Moreover, the ligninolytic enzymes, cellulolytic and hemicellulolytic enzymes correspond to 10.0%, 36.6% and 15.6% of total secreted proteins, respectively. The remaining proteins (about 37.8% proteins) secreted by fungus correspond to peptidases, proteases, chitinases, lipases, transport-proteins and unknown proteins. In a similar study, ligninolytic, cellulolytic and hemicellulolytic enzymes secreted by *Ganoderma lucidum* cultivated in sugarcane bagasse, represent 24%, 34% and 5% of total proteins (Manavalan et al. 2012). Based on their lignocellulose degradation pattern, white-rot fungi can be classified into two groups. Simultaneous pattern (non-selective lignin degradation), cellulose, lignin, and hemicellulose are degraded at the same time. This degradation pattern has been shown in lignocellulosic biomass degraded by *Trametes versicolor, P. chrysosporium, Heterobasidion annosum,* and *Phlebia radiate* (Dashtban et al. 2010). Whereas, selective lignin degradation pattern, lignin and hemicellulose are degraded before that cellulose. This pattern is specific of certain

basidiomycete species such as *Irpex lacteus*, *Ganoderma australe*, *Phlebia tremellosa*, *Ceriporiopsis subvermispora*, *Phellinus pini* and several *Pleurotus* species (Blanchette 1984; Martínez et al. 2005). Lignocellulosic pretreatment requires selecting selective-lignin degrading white-rot fungi, due to the fact that simultaneous degrading fungus generates high polysaccharide loss. Besides, the fungus consumes the easily accessible carbohydrates, leaving fungal pretreated lignocellulosic biomass less digestible and resistant to enzymatic hydrolysis (Sawada et al. 1995; Wan and Li 2012).

1.5.5 Lignocellulose degradation mechanisms of brown-rot fungi

Brown-rot fungi are able to efficiently degrade cellulose and hemicellulose. Its degradation mechanisms of these fungi are not completely clarified, but it has been suggested that it is due to both oxidative and hydrolytic attack. In early degradation stages, cellulose degradation by brown-rot fungi involves a non-enzymatic attack through extracellular generation of hydroxyl radicals (·OH) via the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {\cdot}OH + H_2O$) (Eastwood et al. 2011) (Figure 2). Hydrogen peroxide is generated by oxidase enzymes such as glyoxal oxidases and copper radical oxidases. Although, Fe^{2+} is readily oxides into Fe^{3+} under aerobic conditions, where the fungus is obligate, Fe^{3+} is reduced by phenolates (e.g. fungal-secreted metabolites or lignin demethylation by-products) synthesized by brown-rot fungi (Arantes et al. 2012b; Shimokawa et al. 2004). After cellulose structure is modified, arabinan and galactan side-chains in hemicellulose are removed, followed by xylan and mannan backbone. Finally, crystalline cellulose is hydrolyzed enzymatically by exoglucanases (Arantes et al. 2012b). In early degradation stages by brown-rot fungi, cellulose and hemicellulose are degraded faster than the degradation products are consumed by the fungus, which is favorable in its application for the lignocellulosic biomass pretreatment (Ray et al. 2010).

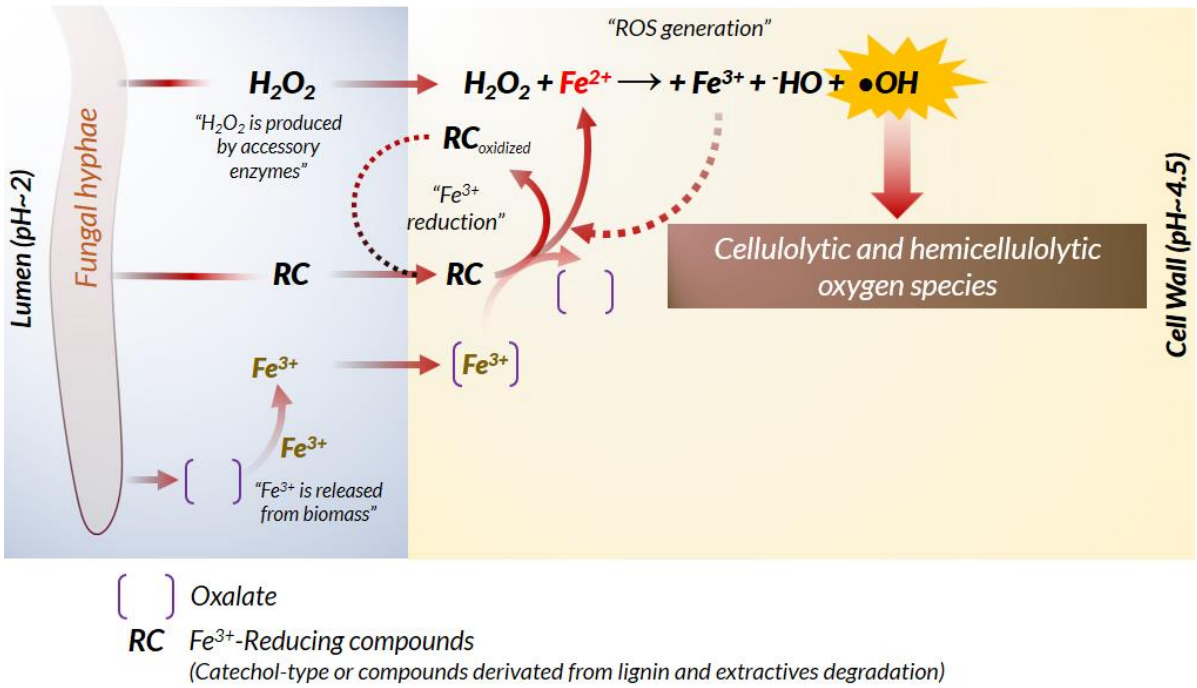

Figure 2 Fenton reaction system of brown-rot fungi (non-enzymatic degradation of cellulose and hemicellulose. Scheme adapted from Arantes et al. (2012).

Although brow-rot fungi are not able to degrade lignin, it generates lignin structural modifications. These modifications are produced through demethylation and hydroxylation reactions, but without breaking down aromatic rings (Arantes et al. 2012). These modifications could increase lignin susceptibility to chemical and other biological treatments. Brown-rot fungi have been lesser studied than white-rot fungi on lignocellulosic biomass pretreatment.

1.6 Wheat straw as a feedstock for bioethanol production in Chile

Chile is among the top 30 wheat producers worldwide with about 245,277 ha wheat crops with annual production of 1,200,000 tons of grain. About 42% of total surface corresponding to the Araucanía Region, contributing 626,000 tons of grain (ODEPA, 2017). It has been reported that each 1 kg wheat grain produced 1.1 kg of wheat straw (Bamaga et al. 2003).

Therefore, according to Bamaga et al. (2003) is estimated that 690,000 tons of wheat straw were produced by Araucanía Region in season 2015/16. Wheat straw is basically composed of cellulose (35 to 45%), hemicelluloses (26 to 32%), and lignin (16 to 22%) (Sánchez 2009b). Wheat straw has drawbacks to zero-tillage or direct seeding use, because its decomposition rate is usually low and increases C/N ratio, causing nitrogen starvation and soil acidity. Other application of wheat straw is as ruminant feed due to its high carbohydrate content, but the carbohydrate availability in intact biomass to hydrolytic system of ruminant is limited. In Chile, after harvest of wheat grain, the major fraction of straw produced is often burned (Hetz H. et al. 2006). This practice causes air pollution contributing to the global warming and reduction on the abundance of soil microorganisms (Zhang et al. 2008). Moreover, it is an unsustainable practice and it is expected to be banned in the next few years, as occurred in developed countries. Therefore, it is imperative the development of new technologies to take advantage of the potential of this raw material to produce added-value products.

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CHAPTER II

Screening of white-rot and brown-rot fungi with high lignocellulolytic potential for improving wheat straw biodegradability

Screening of white-rot and brown-rot fungi with high lignocellulolytic potential for improving wheat straw biodegradability

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Abstract

The fungal pretreatment of lignocellulosic biomass efficacy, residence period, lignin degradation and polysaccharide loss levels vary between different fungal strains and even between the same species ecotypes. Preliminary screening assays were carried out to qualitatively evaluate the lignocellulolytic potential of 14 Chilean native strains of wood-rotting fungi. This was performed in Petri dishes containing agar-solid medium with substrate indicators of ligninolytic (ABTS and RBBR), hemicellulolytic (Xylan from Birchwood), cellulolytic (CMC) and Fe^{3+}-reducing (Ferrozine) activity. Two strains of white-rot fungi, *Anthracophyllum discolor* and *Ganoderma lobatum* and two strains of brown-rot fungi, *Gloeophyllum trabeum* and *Daedalea* sp. showed high degradative potentials. These strains were inoculated on wheat straw under solid-state fermentation conditions and incubated for 10, 20, 30 and 40 days. Wheat straw degradation by fungi was evaluated in terms of lignin content, lignin degradation selectivity, weight loss, released reducing-sugars, and lignocellulolytic enzymes. White-rot fungus, *G. lobatum*, showed similar levels of lignin degradation (43%) compared with *A. discolor* (42%), but associated to lower weight loss (24% compared to 38%) after 40 days of incubation, resulting in a higher lignin degradation

selectivity. Manganese-dependent peroxidase and manganese-independent peroxidase were the most important ligninolytic enzymes detected during wheat straw degradation by both white-rot fungi. Brown-rot fungi showed lower wheat straw degradation than white-rot fungi. Brown-rot fungus, *G. trabeum* showed higher cellulase and xylanase activity and higher weight loss than *Daedalea* sp. after 40 days of incubation. Moreover, *Daedalea* sp. showed poor growth in wheat straw. Consequently, *G. lobatum* and *G. trabeum* were selected for further assays.

Keywords: Wood-rotting fungi; screening; wheat straw; pretreatment.

2.1 Introduction

Lignocellulosic biomass represents a renewable, abundant and still industrially untapped carbon source produced worldwide. The lignocellulosic biomass is basically composed by cellulose, hemicellulos and lignin. Cellulose contained in lignocellulosic biomass represents the greatest potential to produce high value products (e.g. chemical compounds and biofuel production) (Ajila et al. 2012). Particularly, biofuels (ethanol or biogas) are the most attractive products. Nevertheless, the lignocellulosic biomass conversion processes usually require multi-steps, which include: (1) pretreatment (to increase the cellulose accessibility and biodegradability), (2) saccharification or cellulose hydrolysis (to convert of cellulose into glucose units), (3) glucose fermentation (to convert glucose into ethanol or methane), and (4) separation and purification (to obtain pure products) (Dashtban et al. 2010; Sánchez 2009). The pretreatment is a key step on biofuel production from lignocellulosic biomass. The main lignocellulosic biomass characteristics that hinder the cellulose hydrolysis are: the presence of lignin and hemicellulose polymers that sheath cellulose, high cellulose crystallinity, pore volume and total surface area availability for enzymatic attack (Liew et al. 2012; Mosier et al. 2005).

Some microorganisms, especially wood-rotting fungi, white, brown and soft-rot fungi have the ability to degrade all plant cell wall polymers through enzymatic and non-enzymatic mechanisms in an environmentally friendly and energy-efficient manner (Wan and Li 2012). Currently, pretreatment of lignocellulosic biomass has been mainly studied in white-rot fungi (Machado and Ferraz 2017; Saha et al. 2016; Salvachúa et al. 2011), unlike brown-rot fungi that have been studied mainly on pretreatment of wood biomass (Schilling et al. 2012). White-rot fungi have been used for degrading lignin (Locci et al. 2008; Taniguchi et al. 2005; Wan and Li 2010), whereas brown-rot fungi have been used for their ability to degrade and modify cellulose and hemicellulose (Lee et al. 2008; Rasmussen et al. 2010). In this sense, an intensive biological pretreatment of lignocellulosic biomass by the combined action of white and brown-rot fungi on co-culture could be an interesting strategy for improving the access and biodegradability to cellulose in the lignocellulosic biomass. However, the efficacy, residence period, lignin degradation and polysaccharide loss levels of fungal pretreatment by lignocellulosic biomass vary between different fungal strains and even between the same species ecotypes (Machado and Ferraz 2017; Saha et al. 2016; Salvachúa et al. 2011). Therefore, it is necessary to select strains of white-rot fungi with a high selectivity for lignin degradation and brown-rot fungi that have mechanisms to degrade or modify cellulose and hemicellulose in early stages of the treatment. On the other hand, isolated strains of white-rot fungi and brown-rot fungi from forests ecosystems of southern Chile have shown high lignocellulolytic and hollocellulolytic potentials, respectively (Guillén et al. 2009; Tortella et al. 2008). The general goal of this work was to select at least one strain of white-rot and brown-rot fungi with high lignocellulolytic potential for improving the biodegradability of wheat straw.

2.2 Materials and methods

2.2.1 Fungal strains

Five strains of brown-rot fungi and three strains of white-rot fungi were collected from temperate forests of Antuco and Concepcion, Region del Bio-Bio, Chile (Table 1). The fungal strains were collected from trees such as *Nothofagus dombeyi* (Coigue), *Persea lingue* (Lingue), *Gevuina avellana* (Avellano) and *Pinus* sp. (Pino). All strains were deposited in the culture collection of the Environmental Biotechnology Laboratory at the Universidad de La Frontera, Chile. Other six strains of white-rot fungi previously isolated by Tortella et al. (2008) were also included in the screening (Table 1).

Table 1 Strains of wood-rotting fungi isolated from Chilean forest.

Code	Strain	Wood-rot type	Site
An1	*Schizophyllum commune*	White-rot	Antuco
An2	*Ganoderma lobatum*	White-rot	Antuco
An3	*Gloephyllum trabeum*	Brown-rot	Antuco
An4	*Trametes* sp.	White-rot	Antuco
An5	Unknown	Brown-rot	Antuco
An6	*Daedalea* sp.	Brown-rot	Antuco
Con1	Unknown	Brown-rot	Concepcion
Con2	Unknown	Brown-rot	Concepcion
Ru-104	*Stereum hirsutum*	White-rot	Rucamanque
Ru-107	*Trametes versicolor*	White-rot	Rucamanque
Sp1	*Stereum hirsutum*	White-rot	Lican-ray
Sp2	*Inonotus* sp.	White-rot	Lican-ray
Sp3	*Galerina patagónica*	White-rot	Lican-ray
Sp4	*Anthracophyllum discolor*	White-rot	Lican-ray

Fungal strains from Antuco and Concepcion were isolated in this report.
Fungal strains from Rucamanque and Lican-ray were previously isolated by Tortella *et al*. (2008)

2.2.2 Culture conditions and fungal identification

Fungal isolation was carried out by transferring small fragments of the fungi fruiting bodies, or fragments of decayed wood colonized by fungi, on glucose malt extract agar plates (15 g/L agar, 3.5 g/L malt extract, and 10 g/L glucose), and kept at 25°C. Fungus-pure cultures were

obtained under aseptic conditions. The DNA of each fungal strain was extracted using E.Z.N.A.® SP Fungal DNA Mini Kit D5524-01 and the sequence PCR amplification was carried out using universal primers ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS1 5'-TCCGTAGGTGAACCTGCGG-3' (White et al. 1990). The ITS-1 and ITS-4 DNA sequences were compared with the compilation available in the GenBank/EMBL/DDBJ database (Horisawa et al. 2013).

2.2.3 Qualitative detection of lignocellulolytic enzymes

Assays of qualitative detection of ligninolytic, cellulolytic and xylanolytic enzymatic activities were carried out in Petri dishes with lignin-modifying enzyme (LBM), cellulolysis (CBM) and xylanolysis (XBM) solid basal media, respectively (Pointing 1999). Synthetic dyes and substrates were added to the corresponding basal medium in order to determine the enzymatic activities of the tested fungi following the methodology described by Pointing (1999). Laccase (Lac) was tested using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), peroxidases with Remazol brilliant blue R (RBBR), endoxylanase and β-xylosidase with addition of xylan-birchwood and cellulases with carboxymethylcellulose (CMC). All media were inoculated with a fresh mycelia disc (6 mm diameter) of each fungus strain. All assays were kept in the dark at 25 °C ±1 for 2 weeks and were carried out in triplicate to check the reproducibility of the results. Uninoculated media were carried out as negative control. Reaction intensities were measured as the percentage of total area colored or discolored by enzymatic reaction.

2.2.4 Qualitative detection of Fe-reducing compounds

Ferrozine plate test was carried out to detect the production of Fe^{3+}-reducing compounds (Fe^{3+}RC) on solid media (Oviedo et al. 2003). Petri dishes containing PDA medium were inoculated with a fresh mycelia disc (6 mm diameter) of each fungus strain. The dishes were kept in the dark at 25 °C ±1 for 2 weeks and were carried out in triplicate to check the

reproducibility of the results. After incubation, the dishes were overlain with a solution containing: 0.2 mM $FeCl_3$ (prepared in 10 mM HCl), 0.4 mM trans-1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid; (CDTA, Sigma Aldrich), 50 mM acetate buffer, 2 mM ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine; Sigma] and 5 g agar l^{-1}. Uninoculated media were carried out as negative control. Reaction intensities were measured as the percentage of total area colored or discolored by reaction.

2.2.5 Inoculum preparation

For inoculum preparation, an Erlenmeyer flask (500 mL) containing 100 mL of modified Kirk medium (per liter: 10 g of glucose, 2 g of peptone, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 500 µM $MnSO_4xH_2O$, 2 mg of thiamine, and 10 mL of mineral salts) was autoclaved at 121°C for 15 min. Thereafter, the flask was inoculated with five agar disks (6-mm diameter) of active mycelia from a five-day-old culture on malt-extract agar cultivated in Petri dishes, and incubated at 30°C for 10 days. Then, the fungal culture was homogenized in a sterilized blender for 1 min and used as an inoculum (blended fungal mycelia) (Rubilar et al. 2007).

2.2.6 Wheat straw degradation by wood-rotting fungi

Biodegradation of wheat straw by *G. lobatum* was carried out in 250-mL Erlenmeyer flasks containing 5 g of wheat straw (particle size ~5 cm) and 25 mL of sterile water (Singh et al. 2011). All of the flasks were stoppered with hydrophobic cotton and gauze and autoclaved for 25 min at 121 °C. Afterwards, they were cooled and then inoculated with 0.5 mL of blended fungal mycelia (20 mg dried weight mycelia/mL). Finally, they were kept in the dark at 25 °C ±1 for 10, 20, 30 and 40 days. Flasks without a fungal inoculum were used as negative controls (untreated wheat straw).

2.2.7 Sample treatment

The extracts were obtained from untreated and fungal-treated wheat-straw samples after the addition of 25 mL of sterile water and incubation in a rotary shaker (150 rpm) at room temperature for 2 h. Then, the extracts were collected by filtration through Whatman Nº 1 filter paper (pore size 11 µm), and aliquots were used to quantify enzyme activities, total phenol content and reducing sugars. Wheat straw from each flask was washed with tap water to remove fungal mycelia and was dried at 105 °C until it reached a constant weight. This value was used to calculate the weight loss of the samples.

2.2.8 Determination of enzymatic activity

The activities of Lac, MiP and MnP were determined spectrophotometrically by the oxidation of 2,6-dimethoxyphenol (2,6-DMP) to coerulignone (molar extinction coefficient = 49,600 $M^{-1}cm^{-1}$ at 468 nm). The reaction mixture contained 200 µL of 50 mM sodium malonate buffer (pH: 4.5), 50 µL of 20 mM DMP, 50 µL of 20 mM $MnSO_4$, 100 µL of 4 mM H_2O_2 and 50 µL of aqueous extract. Lac activity was measured by replacing H_2O_2 and $MnSO_4$ with distilled water, and MiP activity was measured by adding 100 µL of 20 mM EDTA to the reaction mixture (Mester and Field 1998). One activity unit (U) was defined as the amount of enzyme that oxidizes 1 µmol of 2,6-DMP per minute at pH 4.5. MnP activity was corrected with Lac and MiP activities. LiP activity was determined by oxidation of veratryl alcohol at 310 nm (Kirk and Tien 1984).

Endoglucanase (CMCase) activity was measured by the release of reducing sugars from carboxymethylcellulose, according to Wood and Bhat (1988) with some modifications. The reaction mixture contained 125 µL of aqueous extract and 125 µL of 1% w/v CMC (carboxymethycellulose) in 250 mM citrate buffer (pH: 4.5). The reaction was incubated in a water bath at 37°C for 30 min and stopped by the addition of 750 µL of 3,5-dinitro salicylic acid (DNS) reagent and incubated in water bath at 100°C for 5 min. Finally, 4 mL of distilled

water were added, and the absorbance of the reaction mixture was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of glucose per min. Xylanase activity was measured in a reaction mixture containing 125 µL of enzyme extract and 125 µL of 1% w/v xylan-birchwood in 250 mM citrate buffer (pH: 4.5). The reaction was stopped by adding 750 µL of 3,5-dinitro salicylic acid (DNS) reagent. The mixture was incubated in a water bath at 100°C for 5 min, and then 4 mL of distilled water were added. The absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of xylose per min. The amount of reducing sugars released during CMCase and xylanase activities was calculated using calibration curves for glucose and xylose, respectively.

2.2.9 Chemical analysis

The reducing sugars were measured by the DNS method (Miller 1959). The lignin content of the wheat straw was determined as acid-insoluble Klason lignin (TAPPI T222-om02) and acid-soluble lignin (TAPPI UM 250) (Lin and Dence 1989). A 1-g sample was hydrolyzed for 2 h in 72% H_2SO_4 at 25 °C and then diluted to 3% H_2SO_4 with distilled water, and incubated in a water bath at 95°C for 4 hours. Acid-insoluble lignin was determined gravimetrically and expressed as a percentage of the original sample. The acid-soluble lignin was determined by UV-absorbance at 204 nm of supernatant from 3% H_2SO_4 hydrolysis using a molar extinction coefficient of 110 l $g^{-1}cm^{-1}$. Total lignin was calculated as the sum of acid-insoluble and acid-soluble lignin. The percentage of lignin degradation produced by fungus was calculated from the difference between initial lignin content in untreated wheat straw and lignin content in fungal-treated wheat straw adjusted by weight loss, according to the equation (1):

$$\% \text{ Lignin degradation} = (1 - W(L_f)/(W_o(L_o))) \times 100 \quad (1)$$

where L_0 is the lignin content (%) in untreated wheat straw; L_f is the lignin content (%) in wheat straw after fungal-treatment, W is dry weight of wheat straw (g) after fungal-treatment and Wo is the dry weight of wheat straw (g) before fungal-treatment.

The total lignin content and weight loss of wheat straw before and after treatment with *G. lobatum* were used to estimate the relative selectivity (Srel) of the fungus for lignin degradation (Fackler et al. 2007), according to the equation (2):

$$Srel = [(L_0 - L_f * (100\text{-}WL)/100] / WL \quad (2)$$

where L_0 is the initial lignin content (%) in wheat straw; L_f is the lignin content (%) in wheat straw after fungal treatment, WL percentage of weight loss after fungal-treatment. A Srel=1 indicates that the weight loss of wheat straw occurs exclusively due to degradation of lignin; Srel<1 indicates that the weight loss of wheat straw is due to the degradation of lignin and other components; and Srel=0 indicates that no weight loss is due to lignin degradation.

2.3 Results and discussion

2.3.1 Qualitative assay of fungal degradative potentials

A first qualitative screening of nine white-rot fungi and five brown-rot fungi was carried out to identify their different enzymatic and Fe^{3+}-reducing potentials. Six strains of white-rot fungi were previously isolated by Tortella et al. (2008) and included in the screening. Eight new strains of wood-rotting fungi were isolated from forests ecosystems of Antuco and Concepcion, Bio-Bio region, Chile (Table 1) and also included in the screening. Among these isolated strains, *Trametes* sp., *Ganoderma* sp., *Shizophyllum commune* and *Gloeophyllum trabeum*, are widely studied wood-rotting fungi (Aguiar et al. 2013; Borràs et al. 2011; Schilling et al. 2012; Shrivastava et al. 2012). The six tested strains of white-rot fungi showed

all tested enzymatic activities, whereas the five strains of brown-rot fungi showed activity only in CMC and Xylan media, cellulase and xylanase activities, respectively (Table 2). Similar results were shown by Tortella et al. (2008) for strains: *A. discolor*, *Galerina patagonica*, *Inonotus* sp., *Stereum hirsutum* and *Trametes versicolor*, which indicated that the fungal strains have maintained their enzymatic potentials after subcultures since they were isolated. *A. discolor* and *G. lobatum* were the selected white-rot fungi for further solid-state fermentation studies on wheat straw because they produced the highest reaction intensity with all enzymatic indicators in media tested, which indicates that they have enzymatic mechanisms to degrade all the lignocellulosic components of wheat straw (Table 2). On the other hand, the brown-rot fungi, *G. trabeum* and *Daedalea* sp., were selected because they showed higher reaction intensities in CMC, Xylan and Ferrozine media, which indicate that they have enzymatic and no-enzymatic mechanisms for degrading cellulose and hemicellulose (Table 2).

2.3.2 Wheat straw colonization by fungi

Despite the inoculum volume (500 µL) used in this assay was about a quarter in relation to other studies for the same wheat straw amount (Salvachúa et al. 2011; Saritha et al. 2012; Singh et al. 2011), an efficient wheat straw colonization was obtained. *G. trabeum*, *G. lobatum* and *A. discolor* covered the entire surface of wheat straw with mycelia after 10 days, whereas *Daedalea* sp. showed a poor wheat straw colonization. The fungal mycelium was observed inside and on the surface of wheat straw. Higher mycelia density was observed in wheat straw inoculated with white-rot fungi than with brown-rot fungi. Wan and Li (2012) explained that a minimum inoculum level is usually required for effective colonization and subsequent delignification. Moreover, an excessive inoculum level may only have a marginal effect on fungal colonization and delignification.

Table 2 Qualitative detection of enzymatic activity and Fe^{3+}-reducing compounds by native wood-rotting fungal strains in solid medium.

Fungus	Strain code	Type	Laccase (ABTS)	Peroxidase (RBBR)	Cellulase (CMC)	Xylanase (Xylan)	Fe^{3+}-RC (Ferrozine)
Anthracophyllum discolor	Sp4	WR	+++	++++	+++	+++	-
Daedalea sp.	An6	BR	-	-	++	+++	+++
Galerina patagónica	Sp3	WR	-	-	++	+++	-
Ganoderma lobatum	An2	WR	++++	++++	+++	+++	+
Gloephyllum trabeum	An3	BR	-	-	+++	+++	++++
Inonotus sp.	Sp2	WR	-	+++	+++	+++	-
Schyzophyllum commune	An1	WR	-	-	+	+	-
Stereum hirsutum	Sp1	WR	+	+++	+	+	-
Stereum hirsutum	Ru-104	WR	++	+++	+	++	-
Trametes versicolor	Ru-107	WR	++++	++	++	++	-
Trametes sp.	An4	WR	++++	+++	++	+	-
Unknown	Con1	BR	-	-	+	+	++
Unknown	An5	BR	-	-	++	++	++
Unknown	Con2	BR	-	-	+	+	++

Reaction intensity +, (25%); ++, (25>50%),+++,(>50%); ++++,(100%) signs denote whether visible reaction occurred.- denotes no visible reaction. WR= white-rot fungus and BR= Brown-rot fungus.

2.3.3 Enzymatic activities

Fungal production of lignocellulolytic enzymes was used as an indicator of wheat straw degradation. Brown-rot fungus, *G. trabeum*, produced the highest CMCase (25 U g^{-1} wheat straw) and xylanase (27 U g^{-1}) activities after 10 days of incubation (Figure 2a and 2c). In the case of *G. lobatum*, CMCase and xylanase increased significantly from 10 days to 30 days of incubation, reaching the highest activities after 30 days. *A. discolor* Sp4 produced the lowest CMCase and xylanase activity after all incubation times (Figure 2a and 2b). The CMCase and xylanase activities detected by *G. trabeum* were higher than previously showed by Aguiar et al. (2013) in aqueous extracts obtained from culture of *G. trabeum* growing on *Pinus taeda*

wood chips after periods of 7, 14 and 28 days. The ligninolytic enzymes, MnP and MiP, were secreted by both white-rot fungi during wheat straw pretreatment. However, *G. lobatum* produced significantly higher MnP activity than *A. discolor* after 10, 20 and 40 days (Figure 2c) and there were no significant differences in MiP activity after 10 and 30 days, except after 20 days, where MiP activity of *G. lobatum* was significantly higher (Figure 2d). The MiP activity was not detected after 40 days in both fungi. LiP was not detected under our study conditions. Similarly, in other studies previously performed, LiP was not detected in extracts from cultures of *Ganoderma* species on lignocellulosic biomass (Dinis et al. 2009; Manavalan et al. 2012; Salvachúa et al. 2011). LiP activity was detected in liquid cultures of *A. discolor* (Acevedo et al. 2011), therefore, this activity could be inhibited under solid state culture conditions. Very low activities of Lac were detected, with a maximum of 0.001 IU g^{-1} and 0.0007 IU g^{-1} by *A. discolor* and *G. lobatum* only after 20 days, respectively. Previous studies have reported that the fungi secretion of Lac varies with physico-chemical characteristics of lignocellulosic biomass, namely particle size and chemical composition (Knežević et al. 2013). MnP and MiP have been showed as the most important enzymes involved in lignin degradation in wheat straw by white-rot fungi, but the MnP and MiP activities in the present study were lower than other studies (Dias et al. 2010; Knežević et al. 2013; Salvachúa et al. 2011). However, these studies showed that lignin degradation is not necessarily correlated with the enzyme activity level.

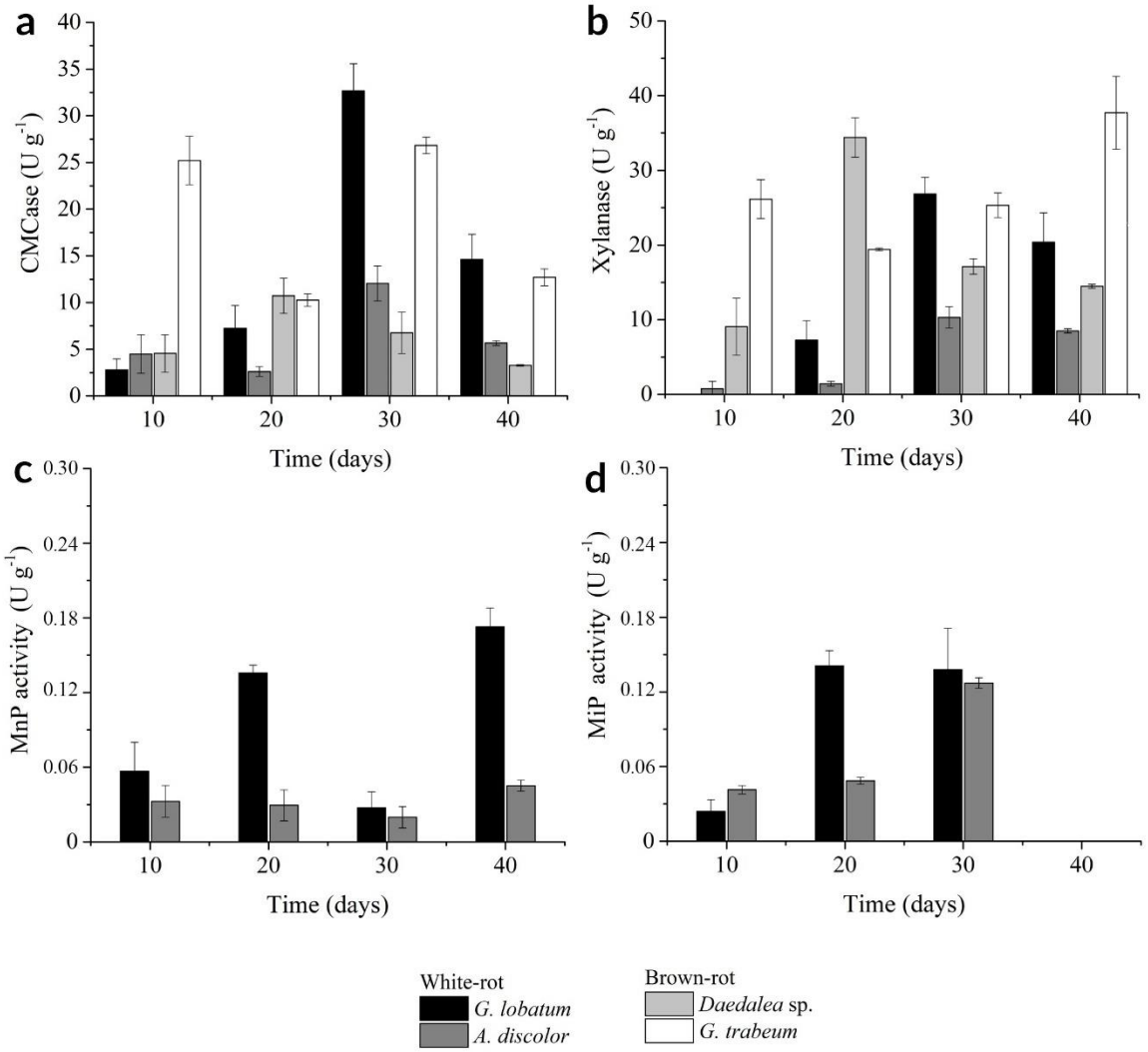

Figure 1 a) CMCase, b) xylanase, c) MnP and d) MiP activities measured in aqueous extracts from fungal-pretreated wheat straw by white-rot and brown-rot fungi**.** Data shown are expressed as means ±sd.

2.3.4 Reducing sugars from wheat straw

Reducing-sugars detected in aqueous extracts are soluble sugars (2% dry weight), which are contained in wheat straw and/or soluble products from fungal cellulose or hemicellulose degradation. The content of reducing-sugars detected in aqueous extracts from fungal-pretreated wheat straw were significant lower than the ones detected in aqueous extracts from untreated wheat straw, except in wheat straw pretreated by *G. lobatum* after 30 and 40 days (Figure 2). Reducing-sugars decreased after 10 days due to the fact that the soluble sugars in

untreated wheat straw provides available carbon and energy source for initial fungal colonization. However, the content of reducing sugars detected in aqueous extracts represents only <2% of dry weight of wheat straw. It could suggest that cellulose and hemicellulose were not extensively degraded by the fungi or that the fungi quickly consumed the soluble sugars after they were released from cellulose or hemicellulose, preventing their accumulation on wheat straw. Nevertheless, after the pretreatment step, cellulose hydrolysis using commercial enzyme is required to obtain soluble and fermentable sugars from cellulose or hemicellulose fraction.

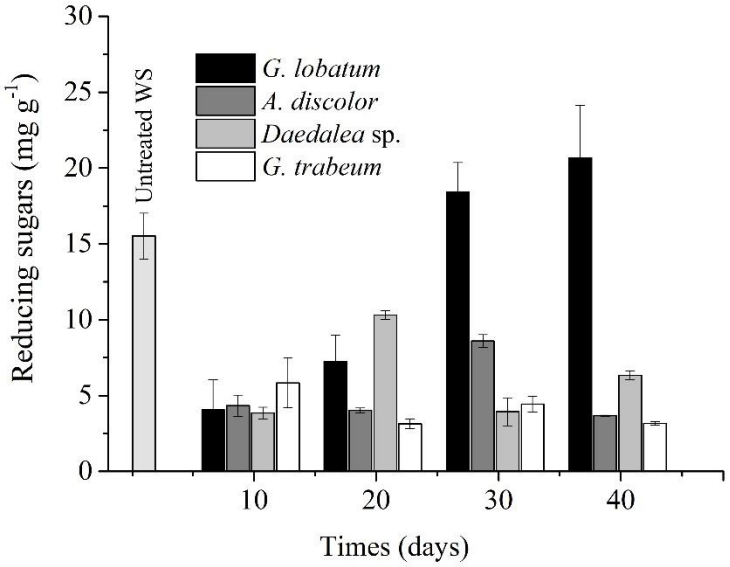

Figure 2 Reducing sugars detected in aqueous extract from fungal-pretreated and untreated wheat straw. Data shown are expressed as means ±sd.

2.3.5 Weight losses from wheat straw

Weight loss from treated wheat straw was in mean 6% and no significant differences were found in all fungal pretreatment after 10 days. A progressive increase of weight loss was observed in pretreated wheat straw by the white-rot fungi, *A. discolor* and *G. lobatum* after 20 days. *A. discolor* showed weight losses two-fold higher than *G. lobatum* after 30 days. The

maximum recorded weight losses were 38% and 24% by *A. discolor* and *G. lobatum* after 40 days, respectively (Figure 3). *G. lobatum* showed lower weight loss than reported in other studies (Dinis et al. 2009; Salvachúa et al. 2011; Shrivastava et al. 2011). On the other hand, the brown-rot fungi showed weight losses lower than 10% after 40 days. Since brown rot fungi have been evaluated in wood biomass, it is likely that the different physicochemical characteristics of wheat straw inhibit the growth of fungi. Therefore, in further assays it is necessary to evaluate culture conditions to improve the colonization and degradative abilities of the brown-rot fungi.

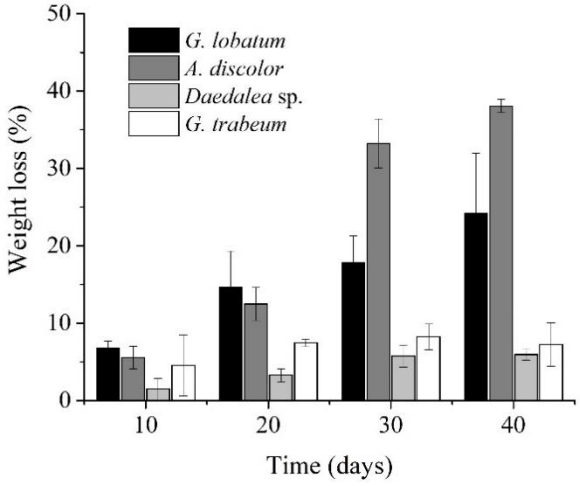

Figure 3 Weight losses from wheat straw colonized by white-rot and brown-rot fungi. Data shown are expressed as means ±sd.

2.3.6 Lignin degradation in wheat straw

Untreated wheat straw contained 22.1% lignin (19.9% acid-insoluble lignin and 2.2% acid-soluble lignin). No significant changes were detected in lignin content in wheat straw pretreated with *G. trabeum* and *Daedalea* sp. It has been widely reported that brown-rot fungi do not degrade lignin to an appreciable extent, they can generate some alterations to lignin structure (demethylation and hydroxylation reactions), but without breaking down aromatic rings (Arantes et al. 2012; Martínez et al. 2005). On the other hand, quick lignin degradation by white-rot fungi was achieved until 20 days (Table 3). Wheat straw pretreated with *G. lobatum* and *A. discolor* showed similar lignin degradation levels during all incubation times, reaching a maximum of 43% and 41% after 40 days, respectively. Both *G. lobatum* and *A. discolor* showed high values of relative selectivity index (Srel) (0.84 and 0.74) after 10 days, respectively. In concordance with weight loss results, Srel values decreased after 20 days. However, Srel values of *A. discolor* decreased <0.40 after 30 days, whereas *G. lobatum* exhibited lower decrease after 30 days (Srel = 0.58) with a slight increase toward 40 days (Srel = 0.62).

The selective degradation of lignin by white-rot fungi decreased when the fungi generated high weight loss in wheat straw (Figure 3 and Table 3), which produced cellulose and hemicellulose degradation and assimilation by the fungi. This behavior was most evident by *A. discolor* because its Srel values showed a strong decrease after 20 days, due to the fact that weight loss increased progressively after 20 days without a significant increase in lignin degradation. On the other hand, *G. lobatum* showed higher selectivity for lignin degradation until 40 days, with a maximum of 43% lignin degradation and 17.2% weight loss. Studies with other selective lignin-degrading fungi showed similar levels of lignin degradation compared with G. lobatum; however, their results showed high weight loss. In this way, *P. ostreatus* HP-1 showed 40% lignin degradation with 40% weight loss after 32 days of

incubation (Thakur et al. 2012), *I. lacteus* showed 45.9% lignin degradation with 38% weight loss after 21 days (Salvachua et al., 2011) and *C. subvermispora* showed 39.2% lignin degradation with 18.8% weight loss after 42 days (Wan and Li, 2010). Therefore, *G. lobatum* could be a good strain to develop a biological pretreatment. It is important to highlight that *Ganoderma* species have been reported as white-rot fungi with selective mechanisms for lignin degradation (Martínez et al. 2005; Otjen and Blanchette 1986), whereas the degradation mechanism of *A. discolor* has not been reported yet. It has also been reported that the selectivity of lignin degradation of white-rot fungi is not constant during the degradation process. Lignin is initially degraded by white-rot fungi because the polysaccharides present in plant cell walls are less accessible; therefore hemicellulose and cellulose degradation occur later (Fackler et al. 2007). Fackler et al. (2007) and Knežević et al. (2013) also showed that the selective degradation of lignin degradation under solid-state fermentation conditions occurred in the primary fungal growth phase (first two weeks).

Table 3 Lignin degradation (%) and relative selectivity index of lignin degradation (Srel value) after wheat straw pretreatment with white-rot fungi *A. discolor* and *G. lobatum*.

Incubation time	*G. lobatum*		*A. discolor*	
	Lignin degradation (%)	Srel value	Lignin degradation (%)	Srel value
10 days	25.9 ± 6.1a	0.84	21.2 ± 1.4a	0.74
20 days	38.7 ± 6.4a	0.70	37.7 ± 3.1a	0.64
30 days	42.3 ± 6.8a	0.53	41.4 ± 7.5a	0.36
40 days	43.2 + 6.0a	0.62	42.3 ± 6.9a	0.32

22.1% total lignin content (19.9% acid-insoluble lignin and 2.2% acid-soluble lignin) in untreated wheat straw. Data shown are expressed as means ±sd.

This report contributes to better understanding the behavior of enzymatically active strains of white-rot and brown-rot fungi on the degradation of wheat straw. These results allowed to select a strain of each type of wood-rotting fungi, *G. trabeum* (brow-rot fungus) and *G. lobatum* (white-rot fungus). White-rot fungus, *G. lobatum*, showed similar levels of lignin

degradation (43%) compared with *A. discolor* (42%), but associated to lower weight loss (24% compared to 38%) after 40 days of incubation, resulting in a higher lignin degradation selectivity. Brown-rot fungus, *G. trabeum* showed higher cellulase and xylanase activity and higher weight loss than *Daedalea* sp. after 40 days of incubation. Moreover, *Daedalea* sp. showed poor growth in wheat straw. Consequently, *G. lobatum* and *G. trabeum* were selected for further assays.

2.4 Conclusions

The screening of adequate fungal strains is key step to develop a feasible biological pretreatment. The qualitative assays allow to select enzymatically active fungi (*G. trabeum*, *Daedalea* sp., *G. lobatum* and *A. discolor*). White-rot fungi, *G. lobatum* and *A. discolor*, exhibited similar lignin degradation in wheat straw, but *G. lobatum* maintained high selective lignin degradation during all incubation times, which could indicate that it preserves polysaccharide fraction of wheat straw. Brown-rot fungus, *G. trabeum*, was selected for further assays due to the fact that it showed better colonization and higher enzymatic activities than *Daedalea* sp. However, the brown-rot fungi showed lower potential to colonize and degrade wheat straw than the white-rot fungi. Therefore, in further assays it is necessary to evaluate culture conditions to improve selective lignin degradation by *G. lobatum* and to improve the colonization and degradative abilities of *G. trabeum*.

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CHAPTER III

Combined effect of enzyme inducers and nitrate on selective lignin degradation in wheat straw by *Ganoderma lobatum*

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Combined effect of enzyme inducers and nitrate on selective lignin degradation in wheat straw by *Ganoderma lobatum*

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Abstract

Lignin is one of the main barriers to obtaining added-value products from cellulosic fraction of lignocellulosic biomass due to its random aromatic structure and strong association with cellulose and hemicellulose. Inorganic and organic compounds have been used as enzyme inducers to increase the ligninolytic potential of white-rot fungi, without considering their effect on the selectivity of degradation. In this study, the selective lignin degradation in wheat straw by *Ganoderma lobatum* was optimized using a central composite design to evaluate the combined effect of Fe^{2+} and Mn^{2+} as inducers of ligninolytic enzymes and NO_3^- as an additional nitrogen source. Selective lignin degradation was promoted to maximize lignin degradation and minimize weight losses. The optimal conditions were 0.18 M NO_3^-, 0.73 mM Fe^{2+} and 1 mM $Mn^{2+,}$ which resulted in 50.0% lignin degradation and 18.5% weight loss after 40 days of fungal-treatment. A decrease in absorbance at 1505 cm^{-1} and 900 cm^{-1} in fungal-treated samples was observed in the FTIR spectra, indicating lignin and cellulose degradation in fungal-treated wheat straw, respectively. The main ligninolytic enzymes detected during

lignin degradation were manganese-dependent and manganese-independent peroxidases. Additionally, confocal laser-scanning microscopy revealed that lignin degradation in wheat straw by *G. lobatum* resulted in higher cellulose accessibility. We concluded that the addition of enzyme inducers and NO_3^- promotes selective lignin degradation in wheat straw by *G. lobatum*.

Keywords selective lignin degradation, lignocellulosic biomass, white-rot fungus, pretreatment.

3.1 Introduction

Every year, close to 1000 gigatons (Gt) of lignocellulosic biomass is generated worldwide, including wheat straw, sugarcane bagasse, corn stalks, rye straw, rice straw, and barley straw, among others. This biomass has great potential to be converted into added-value products derived from the polysaccharide fraction, including carbohydrate-enriched ruminant feed, chemical compounds, and biofuels (Wan and Li, 2012; Parenti et al., 2013; van Kuijk et al., 2016, Rouches et al., 2016). However, the random aromatic structure of lignin and its strong association with cellulose and hemicellulose are major barriers to obtaining these added-value products. White-rot fungi are the most efficient lignin-degrading microorganisms in nature, and their use in the pretreatment of lignocellulosic biomass has been studied by several authors (Salvachúa et al., 2011; Kim et al., 2014; Saha et al., 2016). Additionally, some white-rot species have become a major focus of research due to their ability to remove lignin without causing a significant loss of polysaccharides in lignocellulosic biomass, the so-called selective lignin degradation pattern (Martínez et al., 2011). This ability is specific to certain basidiomycete species such as *Bjerkandera adusta*, *Ceriporiopsis subvemispora*, *Dichomytus squalens*, *Ganoderma australe*, *Ganoderma lobatum*, *Inonotus dryophilus*, *Irpex lacteus* and

Pleourotus ostreatus (Blanchette, 1984; Kuhad and Singh, 2007; Salvachua et al., 2013; Martinez et al., 2011). Selective lignin degradation favors the conversion of lignocellulosic biomass because the cellulosic fraction is largely unaffected and becomes more available (Gupta et al., 2011; Shrivastava et al., 2011). Lignin degradation by white-rot fungi is mainly attributed to a ligninolytic complex of non-specific substrate enzymes, including lignin peroxidases (LiP), manganese peroxidases (MnP), manganese-independent peroxidases (MiP) and laccases (Lac) (Gianfreda and Rao, 2004; Rouches et al., 2016).

To increase the ligninolytic potential of white-rot fungi, Mn^{2+}, Cu^{2+}, Fe^{2+}, unsaturated fatty acids and aromatic compounds have been used as inducers or mediators of enzymes on submerged and solid-state fermentation cultures (Couto et al., 1998; Acevedo et al., 2011; van Kuijk et al., 2016). Likewise, the addition of exogenous Mn^{2+} and Fe^{2+} increased the activities of MnP and Lac and lignin degradation (45.9% after 21 days) in wheat straw by the white-rot fungus *Irpex lacteus* in solid-state culture (Salvachúa et al, 2013). Knežević et al. (2014) showed that the addition of 0.5 mM Fe^{2+} promoted the lignin degradation in wheat straw by *Trametes gibbosa*. On the other hand, lignocellulosic biomass has a low nitrogen content (less than 1%), which is below the required level for efficient fungal growth; therefore, nitrogen supplementation is necessary for fungal colonization in the initial stage (Bisaria et al., 1997; Mane et al., 2007; Jonathan et al., 2011). So far, the effect of enzyme inducers and exogenous nitrogen on lignin degradation in lignocellulosic biomass by white-rot fungi has been evaluated without considering their effect on the selectivity of degradation. The main objective of this study was to evaluate the combined effect of different enzyme inducers and nitrate as a nitrogen source to optimize conditions for selective lignin degradation in wheat straw by the white-rot fungus *Ganoderma lobatum*.

3.2 Material and methods

3.2.1 Organism

The fungal strain *Ganoderma lobatum* An2 was collected from a temperate forest of Antuco, located in Region of Bio-Bio, Chile (latitude, 38° 39'S; longitude, 72° 35'W). The fungal isolate was obtained by placing small fragments of the fungi fruiting bodies on glucose malt-extract agar plates (per liter: 15 g of agar, 3.5 g of malt extract, and 10 g of glucose), which were kept at 25°C. The pure fungus culture was kept in slant culture tubes with malt-extract agar medium at 4 °C and periodically sub-cultured. *Ganoderma lobatum* An2 was identified molecularly and deposited in Colección Chilena de Cultivos Tipo (code CCCT16.03), Scientific and Technological Bioresource Nucleus at Universidad de La Frontera (Chile).

For inoculum preparation, an Erlenmeyer flask (500 mL) containing 100 mL of modified Kirk medium (per liter: 10 g of glucose, 2 g of peptone, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 500 µM $MnSO_4xH_2O$, 2 mg of thiamine, and 10 mL of mineral salts) was autoclaved at 121°C for 15 min. Thereafter, the flask was inoculated with five agar disks (6-mm diameter) of active mycelia from a five-day-old culture on malt-extract agar cultivated in Petri dishes and incubated at 30°C for 10 days. Then, the fungal culture was homogenized in a sterilized blender for 1 min and used as an inoculum (blended fungal mycelia) (Rubilar et al., 2007) and the number of mycelial fragments in liquid inoculum was determined by counting with a Neubauer hemocytometer according to Lestan et al. (1998).

3.2.2 Solid-state fermentation

Biodegradation of wheat straw by *G. lobatum* was carried out in 250-mL Erlenmeyer flasks containing 5 g of wheat straw (particle size ~5 cm) and 25 mL of sterile water supplemented with NO_3^-, Fe^{2+}, and Mn^{2+} at different concentrations according to the experimental design (Table 1). All of the flasks were stoppered with hydrophobic cotton and gauze and autoclaved for 25 min at 121 °C. Afterwards, they were cooled and then inoculated with 0.5 mL of

blended fungal mycelia (20 mg dried weight mycelia/mL = $5x10^5$ mycelial fragments/mL). Finally, they were kept in the dark at 25 °C ±1 for 10, 20, 30 and 40 days. Flasks without a fungal inoculum were used as negative controls (untreated wheat straw).

3.2.3 Experimental design

The effect of NO_3^{-1} (X_1), Fe^{2+} (X_2) and Mn^{2+} (X_3) on the biodegradation of wheat straw by *G. lobatum* was evaluated according to a response-surface methodology (RSM) of central composite design (CCD). All of the factors were varied at three levels in 17 experiments (Table 1). All experiments were carried out in triplicate, and the means of each value were taken as the dependent variables. The evaluated responses were weight loss, lignin degradation, phenol content, reducing sugars and the enzymatic activities of MnP, MiP, cellulase and xylanase. The Design Expert software, v.8.0 trial-version (https://www.legacy.statease.com/dx8trial.html) was used to build the experimental design, evaluate the statistical significance of effects and model the above-mentioned responses as a function of factor levels based on numerical optimizations. Where required, the obtained quadratic, polynomial and cubic models were reduced by incorporating all of the individual effects as well as the most significant interactions to simplify the model and increase its statistical significance.

The software allows the simultaneous optimization of multiple responses. Hence, to promotes the selective lignin degradation by *G. lobatum*, a multiple optimization using the models of lignin degradation and weight loss was carried out to find the levels of NO_3^-, Fe^{2+} and Mn^{2+} which resulted in both minimum weight loss and maximum lignin degradation in wheat straw after 40 days of fungal-treatment.

3.2.4 Sample treatment

The extracts were obtained from untreated and fungal-treated wheat-straw samples after the addition of 25 mL of sterile water and incubation in a rotary shaker (150 rpm) at room

temperature for 2 h. Then, the extracts were collected by filtration through Whatman N° 1 filter paper (pore size 11 µm), and aliquots were used to quantify enzyme activities, the total phenol content and reducing sugars. Wheat straw from each flask was washed with tap water to remove fungal mycelia and was dried at 105 °C until it reached a constant weight. This value was used to calculate the weight loss of the samples.

Table 1 Experimental values versus predicted values of lignin degradation, weight loss and accumulated lignocellulolytic activity of wheat straw treated with *G. lobatum* after 40 days of incubation.

Trial no.	Coded levels			Real levels			Responses									
	X_1	X_2	X_3	X_1	X_2	X_3	Y_1 Lignin degradation (%)		Y_2 Weight loss (%)		Y_3 MnP Activity (UI L^{-1})		Y_4 MiP Activity (UI L^{-1})		Y_5 CMCase Activity (UI g^{-1})		Y_6 Xylanase Activity (UI g^{-1})	
	$NaNO_3$ (M)	$FeSO_4$ (mM)	$MnSO_4$ (mM)	$NaNO_3$ (M)	$FeSO_4$ (mM)	$MnSO_4$ (mM)	Exp. Value	Pred. Value	Exp. Value	Pred. Value	Exp. Value	Pred. Value	Exp. Value	Pred. Value	Exp. Value	Pred. Value	Exp. Value	Pred. Value
1	-1	-1	-1	0.06	0.25	0.25	31.12	42.45	25.25	23.04	108.36	123.03	24.73	26.50	19.87	20.51	18.93	20.29
2	+1	-1	-1	0.24	0.25	0.25	42.45	37.83	19.81	18.46	61.09	71.83	13.09	12.83	21.30	18.19	20.15	21.15
3	-1	+1	-1	0.06	1.00	0.25	33.07	32.28	19.37	20.48	119.27	142.23	18.91	23.30	18.37	19.63	21.00	21.62
4	+1	+1	-1	0.24	1.00	0.25	22.88	27.65	16.23	15.90	104.73	91.03	8.73	9.63	17.57	17.31	23.08	22.48
5	-1	-1	+1	0.06	0.25	1.00	43.18	36.65	24.46	23.27	197.09	149.65	23.27	24.17	17.87	18.69	18.34	20.69
6	+1	-1	+1	0.24	0.25	1.00	24.99	32.02	20.27	18.69	64.727	98.45	13.09	10.50	17.69	16.37	24.86	21.56
7	-1	+1	+1	0.06	1.00	1.00	50.17	50.82	21.90	20.71	152.00	168.85	23.27	20.97	22.48	17.82	24.98	22.02
8	+1	+1	+1	0.24	1.00	1.00	49.07	46.19	17.14	16.13	114.18	117.65	5.82	7.30	12.84	15.50	18.35	22.88
9	-1	0	0	0.06	0.63	0.63	36.67	35.02	21.34	21.86	152.00	145.94	29.09	23.73	18.51	19.16	20.38	21.15
10	+1	0	0	0.24	0.63	0.63	31.70	30.40	15.98	17.28	128.00	94.74	10.18	10.06	16.08	16.84	21.50	22.02
11	0	-1	0	0.15	0.25	0.63	30.01	22.76	18.12	20.87	103.27	110.74	18.18	18.50	16.50	18.44	20.20	20.92
12	0	+1	0	0.15	1.00	0.63	26.54	24.92	20.50	18.31	140.36	129.94	19.64	15.30	17.59	17.56	21.69	22.25
13	0	0	-1	0.15	0.63	0.25	54.60	43.84	20.65	19.45	122.18	107.03	25.45	18.06	20.70	18.91	24.13	21.38
14	0	0	+1	0.15	0.63	1.00	48.55	50.37	18.71	19.68	120.73	133.65	13.82	15.73	17.85	17.09	22.77	21.79
15	0	0	0	0.15	0.63	0.63	24.69	32.71	18.18	19.57	102.54	120.34	15.27	16.90	16.86	18.00	21.48	21.59
16	0	0	0	0.15	0.63	0.63	31.47	32.71	17.74	19.57	130.91	120.34	18.18	16.90	17.33	18.00	23.35	21.59
17	0	0	0	0.15	0.63	0.63	29.77	32.71	17.30	19.57	124.32	120.34	16.55	16.90	16.60	18.00	21.77	21.59

3.2.5 Determination of enzymatic activity

The activities of Lac, MiP and MnP were determined spectrophotometrically by the oxidation of 2,6-dimethoxyphenol (2,6-DMP) to coerulignone (molar extinction coefficient = 49,600 $M^{-1}cm^{-1}$ at 468 nm). The reaction mixture contained 200 µL of 50 mM sodium malonate buffer (pH: 4.5), 50 µL of 20 mM DMP, 50 µL of 20 mM $MnSO_4$, 100 µL of 4 mM H_2O_2 and 50 µL of enzyme extract. The Lac activity was measured by replacing H_2O_2 and $MnSO_4$ with distilled water, and the MiP activity was measured by adding 100 µL of 20 mM EDTA to the reaction mixture (Mester and Field, 1998). One activity unit (U) was defined as the amount of enzyme that oxidizes 1 µmol of 2,6-DMP per minute at pH 4.5. The MnP activity was corrected by the Lac and MIP activities. The LiP activity was determined by oxidation of veratryl alcohol at 310 nm according to Tien and Kirk (1983).

Endoglucanase (CMCase) activity was measured by the release of reducing sugars from carboxymethylcellulose according to Wood and Bhat (1998) with some modifications. The reaction mixture contained 125 µL of enzyme extract and 125 µL of 1% w/v CMC (carboxymethycellulose) in 250 mM citrate buffer (pH: 4.5). The reaction was incubated in a water bath at 37°C for 30 min and stopped by the addition of 750 µL of 3,5-dinitro salicylic acid (DNS) reagent and incubated in water bath at 100°C for 5 min. Finally, 4 mL of distilled water was added, and the absorbance of the reaction mixture was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of glucose per min.

Xylanase activity was measured in a reaction mixture containing 125 µL of enzyme extract and 125 µL of 1% w/v xylan-birchwood in 250 mM citrate buffer (pH: 4.5). The reaction was stopped by adding 750 µL of 3,5-dinitro salicylic acid (DNS) reagent. The mixture was incubated in a water bath at 100°C for 5 min, and then 4 mL of distilled water was added. The absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of xylose per min. The amount of reducing sugars released during CMCase

and xylanase activities was calculated using calibration curves for glucose and xylose, respectively.

3.2.6 Chemical analysis

The reducing sugars were measured by the DNS method, according to Miller (1959). The total phenol content was measured by the Folin-Ciocalteu method (Singleton and Rossi, 1965). The lignin content of the wheat straw was determined as acid-insoluble Klason lignin (TAPPI T222-om02) and acid-soluble lignin (TAPPI UM 250) according to Lin and Dence (1992). A 1-g sample was hydrolyzed for 2 h in 72% H_2SO_4 at 25 °C and then diluted to 3% H_2SO_4 with distilled water and incubated in a water bath at 95°C for 4 hours. Acid-insoluble lignin was determined gravimetrically and expressed as a percentage of the original sample. The acid-soluble lignin was determined by UV-absorbance at 204 nm of supernatant from 3% H_2SO_4 hydrolysis using a molar extinction coefficient of 110 l $g^{-1}cm^{-1}$. Total lignin was calculated as the sum of acid-insoluble and acid-soluble lignin. The percentage of lignin degradation produced by fungus was calculated from the difference between initial lignin content in untreated wheat straw and lignin content in fungal-treated wheat straw adjusted by weight loss according to the equation (1):

$$\% \text{ Lignin degradation} = (1 - W(L_f)/(W_o(L_o))) \times 100 \quad (1)$$

where L_0 is the lignin content (%) in untreated wheat straw; L_f is the lignin content (%) in wheat straw after fungal-treatment, W is dry weight of wheat straw (g) after fungal-treatment and Wo is the dry weight of wheat straw (g) before fungal-treatment.

The total lignin content and weight loss of wheat straw before and after treatment with *G. lobatum* were used to estimate the relative selectivity (Srel) of the fungus for lignin degradation (Fackler et al. 2007) according to the equation (2):

$$Srel = [(L_0 - L_f * (100\text{-}WL)/100] / WL \quad (2)$$

where L_0 is the initial lignin content (%) in wheat straw; L_f is the lignin content (%) in wheat straw after fungal treatment, WL percentage of weight loss after fungal-treatment. A Srel=1 indicates that weight loss of wheat straw occurs exclusively due to degradation of lignin; Srel<1 indicates that weight loss of wheat straw is due to the degradation of lignin and another component; and Srel=0 indicates that no weight loss is due to lignin degradation.

3.2.7 Fourier-transform-infrared spectroscopy (FTIR) analysis

The solid samples dried in an oven at 105°C for 24 h were milled into a fine powder and homogenized before FTIR analysis. The infrared spectra were collected at room temperature in the 4000-600 cm^{-1} range at a resolution of 4 cm^{-1} with an average of 32 scans per sample using a Cary-630 spectrometer equipped with an ATR accessory. Spectra were obtained using Agilent Resolution Pro software (Agilent Technologies Inc., Santa Clara, CA).

3.2.8 Confocal laser scanning microscopy (CLSM)

The CLSM was used to evaluate the lignin degradation, cellulose accessibility and fungal colonization in fungal-treated wheat straw by applying three different dyes. Untreated and fungal-treated wheat straw samples were stained sequentially with dyes 0.1% safranin O (Bond et al., 2008), 1% Congo red (Verbelen and Kerstens, 2000) and 0.1% Calcofluor white (Monheit et al., 1984) to visualize differentially lignin, cellulose and fungal mycelia, respectively. Samples were stained in each dye for 20 min and then rinsed with warm distilled water at 30°C. Stained samples were visualized in a Fluoview FV1000 Confocal Laser Scanning Microscope (Olympus, Japan) at $\lambda_{ex}/\lambda_{em}$ 546/590, 635/690 and 405/450 nm, and the images were captured with FV10 ver. 2.0c software. The fluorescence intensity of CLSM images was analyzed using Sigmascan Pro software version 5.0 (Systat, Inc., San Jose, CA).

3.3 Results and Discussion

3.3.1 Production of lignocellulolytic enzymes

The production of lignocellulolytic enzymes by *G. lobatum* was used as indicator of degradation process of wheat straw. The MnP, MIP, CMCase and xylanase activities detected in aqueous extracts from fungal-treated wheat straw along incubation time are shown in Figure 1a and 1b. Very low Lac activity was detected and no LiP activity was detected in the enzymatic extracts. Therefore, Lac activity was not considered in the surface-response analysis. The production peak of MnP activity was detected at day 20 of incubation, with a 34% decrease until the 40-day mark and the highest MiP activity was detected at day 40 of incubation (Figure 1a). The levels of MnP and MiP activities showed in the present study were lower than those reported by Dias et al. (2010) and Salvachúa et al. (2011). Nevertheless, Knežević et al. (2014) reported that the lignin degradation is not necessarily correlated with the level of enzyme activity and Salvachúa et al. (2011) reported that *B. adusta* and *Coriolopsis rigida* produced high level of lignin degradation (37% and 34%, respectively) associated to very low ligninolytic activities after 21 days.

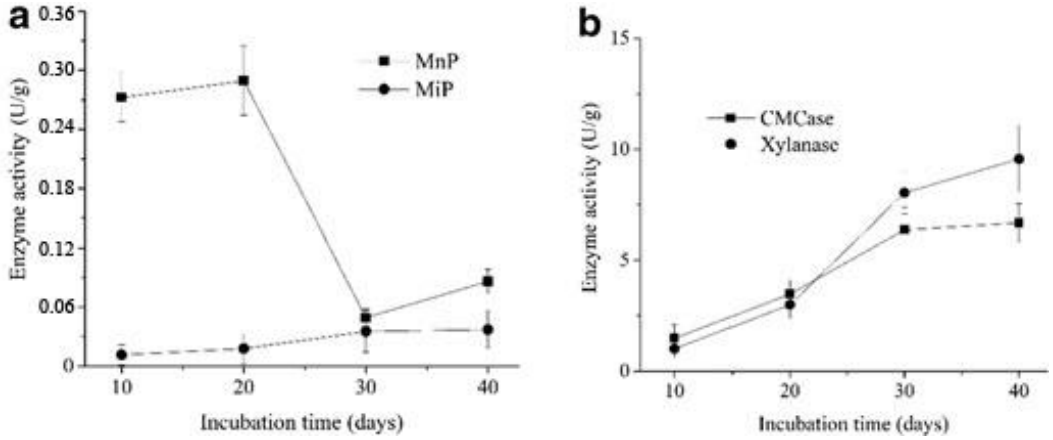

Figure 1 Enzymatic activity of MnP and MiP (a), and CMCase and xylanase (b) in aqueous extracts from wheat straw treated with *Ganoderma lobatum* along cultivation time under the different culture conditions tested. Data are expressed as the means ± standard error from 17 trials.

To better assess the factors affecting lignocellulolytic enzymes, the accumulated activity after 40 days of incubation values were included as independent variables. The response-surface plots for accumulated MnP, MIP, CMCase and xylanase activities during the degradation of wheat straw by *G. lobatum* are shown in Figure 2 (a - d), respectively. The experimental and predicted values of accumulated above-mentioned enzymatic activities after 40 days of incubation are presented in Table 1. MnP was the main ligninolytic enzyme produced by *G. lobatum* during incubation, with a maximum accumulated activity of 197.1 ± 31.8 U L^{-1} after 40 days of incubation, at the highest concentration of Mn^{2+} (1 mM) and lowest concentration of Fe^{2+} (0.25 mM) and NO_3^- (0.06 M). In addition, the positive effect of Mn^{2+} on MnP activity decreased strongly when the concentration of NO_3^- increased (Figure 2a). The response of MiP activity was similar to that of MnP to Fe^{2+} and NO_3^-; nevertheless, an increase in the concentration of Mn^{2+} produced a slight decrease in the accumulated activity of MiP. Therefore, the maximum accumulated activity of MiP (24.1 U L^{-1}) after 40 days of incubation was detected at the lowest concentration of Mn^{2+}, Fe^{2+} and NO_3^-. These results revealed that under our conditions of solid-state fermentation, the addition of NO_3^- as an exogenous nitrogen source decreased the ligninolytic activity of MnP and MiP during biodegradation of wheat straw by *G. lobatum*. This behavior has been widely reported to be due to the enhanced expression of genes encoding ligninolytic enzymes caused by white-rot fungi under nitrogen limitation during secondary metabolism (Kersten and Cullen, 2007; Wu and Zhang, 2010). Nevertheless, Kannaiyan et al. (2014) showed that in a submerged co-culture of *D. squalens* and *C. subvermispora*, two white-rot fungi, 0.02 M NO_3^- enhanced the activities of Lac and MnP. Acevedo et al. (2011) showed that Mn^{2+} at 0.25 mM induces MnP activity under submerged conditions. The positive effect of Fe^{2+} and Mn^{2+}, both at 0.3 mM, on MnP activity in the white-rot fungus *I. lacteus* was previously reported by Salvachúa et al. (2013). In several studies, cellulase and xylanase activities were not monitored during the degradation of lignocellulosic

biomass by white-rot fungi, but only ligninolytic activities were monitored. In this study CMCase and xylanase activities were measured to assess the effect of enzyme inducers and nitrate in the production of enzyme involved in cellulose and hemicellulose degradation, which are directly related to the selectivity of lignin degradation by the fungus. The activity CMCase and xylanase showed a progressive increase after 20 days of incubation Figure 1b. The accumulated activity of CMCase was fitted to a polynomial model, which revealed that it was negatively affected by the combination of the three factors tested (Figure 2c). However, individually, the highest concentration of Mn^{2+} (1 mM) had a positive effect on CMCase activity. The negative effect of NO_3^- on CMCase activity was promoted at 1.00 mM Fe^{2+}. By contrast, Gahda et al. (2009) showed that the CMCase production by *Aspergillus terreus* increased by the addition of $NaNO_3$ 5 gL^{-1}. Furthermore, Fe^{2+} had no significant effect on CMCase activity at the higher concentration of NO_3^- (0.24 M). NO_3^-, Fe^{2+} and Mn^{2+} had no significant effect on xylanase activity (Figure 2d). To promote selective lignin degradation, it is necessary to decrease the degradation of cellulose and hemicellulose. According to the fitted model, the minimum CMCase activity occurs at 0.24 M NO_3^-, 1 mM Fe^{2+} and 1 mM Mn^{2+}, 38% lower than the maximum CMCase activity (22.4 U g^{-1} at 0.06 M NO_3^-, 1 mM Fe^{2+} and 1 mM Mn^{2+}). Knežević et al. (2014) reported that Fe^{2+} at 0.5 mM decreased the degradation of cellulose and hemicellulose and increased that of lignin by 3.9%, 6.1% and 6.4%, respectively, in wheat straw.

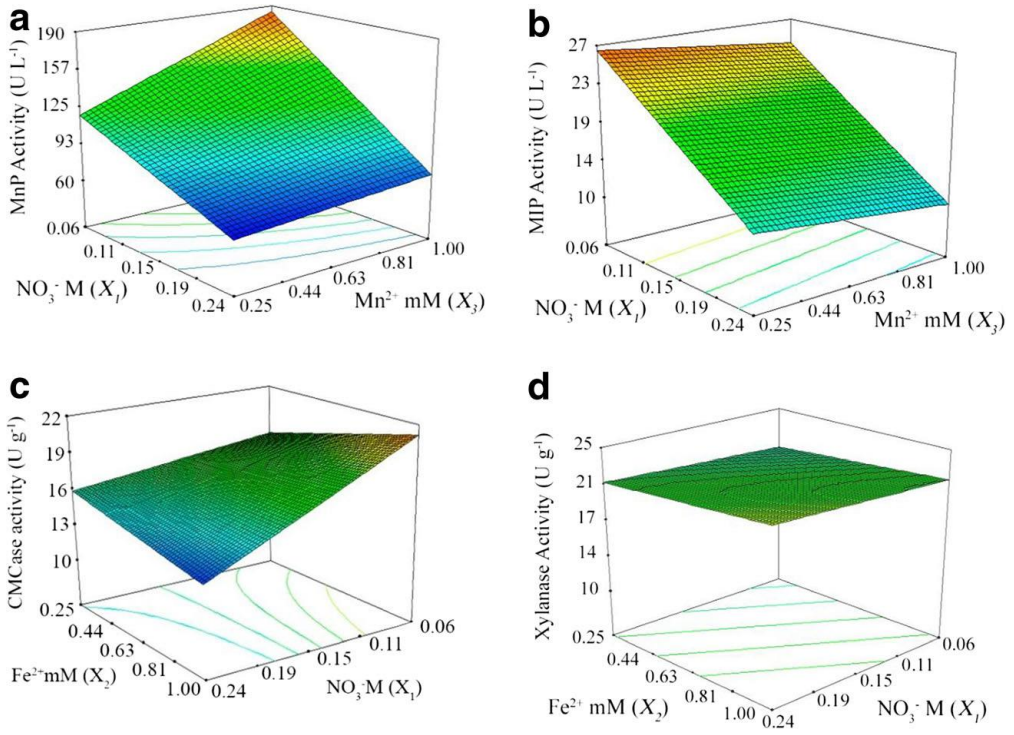

Figure 2 3D surface plots of accumulated lignocellulolytic enzyme activities produced by *G. lobatum* after 40 days of incubation. Plots of MnP (a) and MiP (b) activities as functions of NO_3^- (X_1) and Mn^{2+} (X_3) with 0.25 mM Fe^{2+} (X_2). Plot of CMCase (c) and Xylanase (d) activity as a function of NO_3^- (X_1) and Fe^{2+} (X_2) with 1 mM Mn^{2+} (X_3).

3.3.2 Weight loss and release of phenols and reducing sugars

Weight loss gives an estimate of the extent of the degradation of lignocellulosic components of wheat straw. Hence, a suitable treatment of wheat straw using white-rot fungi should present very high lignin degradation associated to minimal weight loss. During the incubation of wheat straw with *G. lobatum*, weight losses were, on average, 4.3%, 9.4%, 16.5% and 19.6% after 10, 20, 30 and 40 days, respectively. Weight loss was adjusted to the linear models (Table 2). Figure 3 shows the surface plots of the fitted models of weight loss after 10, 20, 30 and 40 days of incubation. In general, the increase in the NO_3^- concentration was associated with a slight decrease in weight loss at all of the incubation times, whereas an increase in the Fe^{2+} concentration only showed a negative effect on weight loss after 30 and 40 days of incubation. On the other hand, the highest degrees of weight loss were observed at 1.00 mM Mn^{2+} at all incubation times (Figure 3e, f, g and h). No effect of interactions between the tested factors on

weight loss was detected. *G. lobatum* was previously found to produce 38% weight loss after 40 days without the addition of any nitrogen source or metal ions (unpublished results). Another strain of *Ganoderma* sp. rckk02 produced 16.48% weight loss on wheat straw after 15 days of incubation, with 34.95% lignin degradation (Shrivastava et al., 2012). By contrast, Salvachúa et al. (2011) reported up to 35% weight loss after 21 days of biodegradation of wheat straw by white-rot fungi. In another study, white-rot fungi produced decreases of 19 - 46% in the dry weight of wheat straw after 10 weeks (Cianchetta et al., 2014).

Table 2 Fitted-model equations for each response.

Response		Model equation in terms of coded factors	R^2	P value	A.P.
	10 days	$+14.82 - 2.76\ X_1* + 0.082\ X_2 + 1.8\ X_3* - 3.23\ X_1^2 - 3.26\ X_2^2*$	0.83	<0.001	10.35
Y_1	20 days	$+23.48 - 4.55\ X_1* + 0.73\ X_2 + 1.22\ X_3 - 5.13\ X_2^2 + 7.29\ X_3^2*$	0.67	0.017	5.49
Lignin degradation	30 days	$+27.89 - 1.79\ X_1 - 1.01\ X_2 + 1.59\ X_3 + 3.8\ X_2X_3 - 7.24\ X_2^2* + 11.48\ X_3^2*$	0.66	0.044	6.15
	40 days	$+32.65 - 2.31\ X_1 + 1.00\ X_2 + 3.18\ X_3 + 6.09\ X_2X_3* - 8.86\ X_2^2 + 14.44\ X_3^2*$	0.68	0.039	6.03
	10 days	$+4.31 - 1.28\ X_1* - 0.035\ X_2 + 0.82\ X_3$	0.51	0.023	7.51
Y_2	20 days	$+13.66 - 0.029\ X_1* - 1.67\ X_2 + 0.60\ X_3$	0.65	0.003	8.44
Weight loss	30 days	$+13.66 - 0.029\ X_1* - 1.67\ X_2* + 0.60\ X_3$	0.67	0.002	11.27
	40 days	$+19.59 - 2.29\ X_1* - 1.28\ X_2* + 0.12\ X_3$	0.62	0.004	8.48

A P value <0.05 indicates that the fit of the model is statistically significant. A.P = Adequate precision ratio; if A.P. is >4, the model is adequate and can be used to navigate the design space. Factors or interactions followed by * have significant effects in the variable model according to ANOVA ($p<0.05$). The model equation in terms of coded factors indicates how each factor changes the response Y.

The content of phenol and reducing sugar in aqueous extracts was monitored to estimate lignin and cellulose or hemicellulose degradation. NO_3^-, Fe^{2+} and Mn^{2+} had no significant effect on

both variables, phenol content and reducing sugars. The concentration of release of phenol compounds in aqueous extracts from wheat straw fluctuated during incubation, increasing until day 20, strongly decreasing at 30 days, and then increasing at 40 days, with average concentrations of 16.4, 36.9, 7.1 and 64.4 mg eq gallic acid kg^{-1} dry weight of wheat straw, respectively (Figure 4). Considering that the free phenol content in the extracts increases with lignin degradation and that white-rot fungi can mineralize lignin; the significant decrease after 30 days could be the result of the mineralization of accumulated phenols by the fungal degradation of wheat straw.

Reducing sugars are released from wheat straw by cellulose or hemicellulose degradation by the fungus. Independent of any other factor, the reducing sugar content in the extracts was lower than 2 mg g^{-1} dry weight of wheat straw until day 20 of incubation and increased after day 20 (Figure 4). The maximum content of reducing sugars detected in aqueous extracts was 10.5 mg g^{-1} dry weight of wheat straw, with 0.06 M NO_3^-, 0.63 mM Fe^{2+} and 0.63 mM Mn^{2+} after 40 days. Although, phenolic compounds and reducing sugars were detected in the aqueous extracts from the fungal-treated wheat straw, their concentrations were very low, consequently these could inadequately represent the degradation of lignin and degradation of cellulose and hemicellulose by *G. lobatum*. Nevertheless, these low concentrations may be due to sugars released by cellulose and hemicellulose degradation are easily assimilable carbon sources for the fungus and as was mentioned before, white-rot fungi can also mineralize phenol compounds, which prevents their accumulation in the medium.

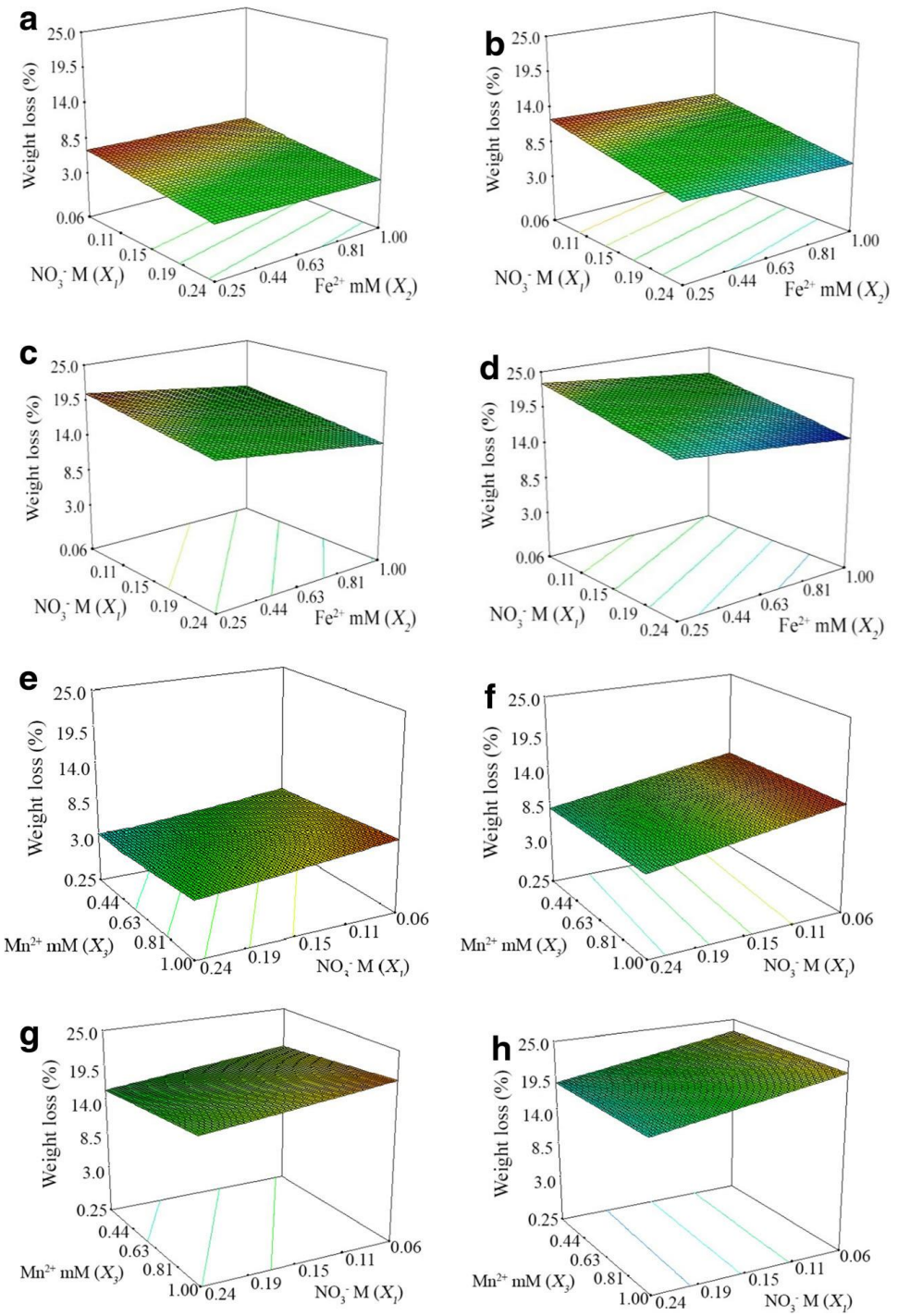

Figure 3 3D Plots of the modeled weight loss (%) as functions of NO_3^- (X_1) and Fe^{2+} (X_2) after 10 (a), 20 (b), 30 (c) and 40 days of incubation (d) with 1.00 mM Mn^{2+}, and the modeled weight loss (%) as functions of NO_3^- (X_1) and Mn^{2+} (X_3) after 10 (e), 20 (f), 30 (g) and 40 days of incubation (h) with 0.25 mM Fe^{2+}.

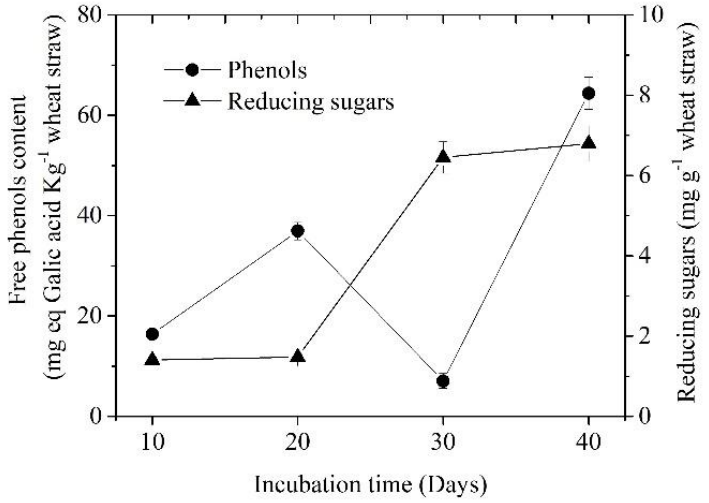

Figure 4 Free phenols and reducing sugars in aqueous extracts from wheat straw treated with *Ganoderma lobatum* along cultivation time under the different culture conditions tested. Data are expressed as the means ± standard error from 17 trials.

3.3.3 Lignin degradation in wheat straw

Lignin degradation was fitted to quadratic models (Table 2). As for the above-described independent variables, lignin degradation was negatively influenced by NO_3^-. The Mn^{2+} had a positive effect on lignin degradation at all of the incubation times and had the strongest influence over the experimental equations (Table 2). The highest level of lignin degradation (54%) after 40 days was obtained with the lowest concentration of NO_3^- (0.06 M), highest concentration of Mn^{2+} (1 mM) and 0.69 mM of Fe^{2+} (Figure 5h). Lignin degradation decreased at lower and higher concentrations of Fe^{2+}. Van Kuijk et al. (2016) showed that the addition of Mn^{2+} at 150 μg g^{-1} wheat straw increased lignin degradation by *C. subvermispora* by 10%. Tijani et al. (2011) also showed that Mn^{2+} had positive influence in lignin degradation. Figure 5 shows that the positive effect of Mn^{2+} and Fe^{2+} increased over time, whereas no significant effect of NO_3^- on lignin degradation was found after 30 days, suggesting that the fungus consumed NO_3^- only in the initial colonization of wheat straw. In agreement with this result, Jonathan *et al*. (2011) also reported that an additional nitrogen source increased initial fungal

colonization of lignocellulosic biomass. The selectivity for lignin degradation in wheat straw by *G. lobatum* was not constant during incubation time. Independent of the tested factors, lignin degradation and weight loss produced by *G. lobatum* showed a high positive correlation (r^2 = 0.867) until 20 days. Between 30 and 40 days, the correlation decreased sharply (r^2= 0.156) (Figure 6a). In accordance with this result, a strong positive correlation was also found between MnP activity and lignin degradation and between MnP activity and weight loss until 20 days (Figure 6b). These results indicated that *G. lobatum* had high selectivity for lignin degradation on wheat straw until 20 days, with MnP as the main ligninolytic enzyme. Fackler et al. (2007) and Knežević et al. (2014), showed that the significant and quick induction of the lignin degradation associated to low weight loss of lignocellulosic biomass occurred in the primary fungal growth phase (first two weeks).

To promote selective lignin degradation by *G. lobatum* until 40 days of incubation, a multiple and simultaneous optimization using the experimental equations of lignin degradation and weight loss was carried out with the goals of maximizing lignin degradation and minimizing weight loss at all incubation times. The optimum solution was 0.18 M NO_3^-, 0.73 mM Fe^{2+} and 1 mM Mn^{2+}, with desirability factors of 0.78, 0.55 and 0.68, for lignin degradation, weight loss and the combined variables, respectively. The predicted values of lignin degradation and weight loss after 40 days from the optimization are shown in Table 3. Additionally, the index of the relative selectivity of lignin degradation (Srel) was estimated to compare the highest experimental values of lignin degradation at different incubation times to the maximum values predicted by the numerical optimization (Table 3). According to the optimized solution, the highest value of the index of relative selectivity of lignin degradation should be obtained at day 20, when 90% of weight loss would result from lignin degradation. At optimal concentrations of Mn^{2+} and Fe^{2+} are correlated with ligninolytic enzyme activity, and Fe^{2+} has also a negative effect on cellulose activity. Although the maximum production of the ligninolytic enzymes

occurred at the lowest concentration of NO_3^-, 0.18 M NO_3^- controlled cellulase activity and was associated with weight loss, which might be caused by cellulose degradation without considerably decreasing lignin degradation. In this scenario, the selectivity of lignin degradation was increased or unaltered over the course of the incubation period reaching a maximum of 50.0% lignin degradation with 18.5% weight loss (Srel = 0.6) at 40 days of incubation. Studies with other selective lignin-degrading fungi showed similar levels of lignin degradation compared with *G. lobatum*; however, their results showed a high weight loss. In this way, *P. ostreatus* HP-1 showed 40% lignin degradation with 40% weight loss after 32 days of incubation (Thakur et al., 2012), *I. lacteus* showed 45.9% lignin degradation with 38% weight loss after 21 days (Salvachua et al., 2011) and *C. subvermispora* showed 39.2% lignin degradation with 18.8% weight loss after 42 days (Wan and Li, 2010).

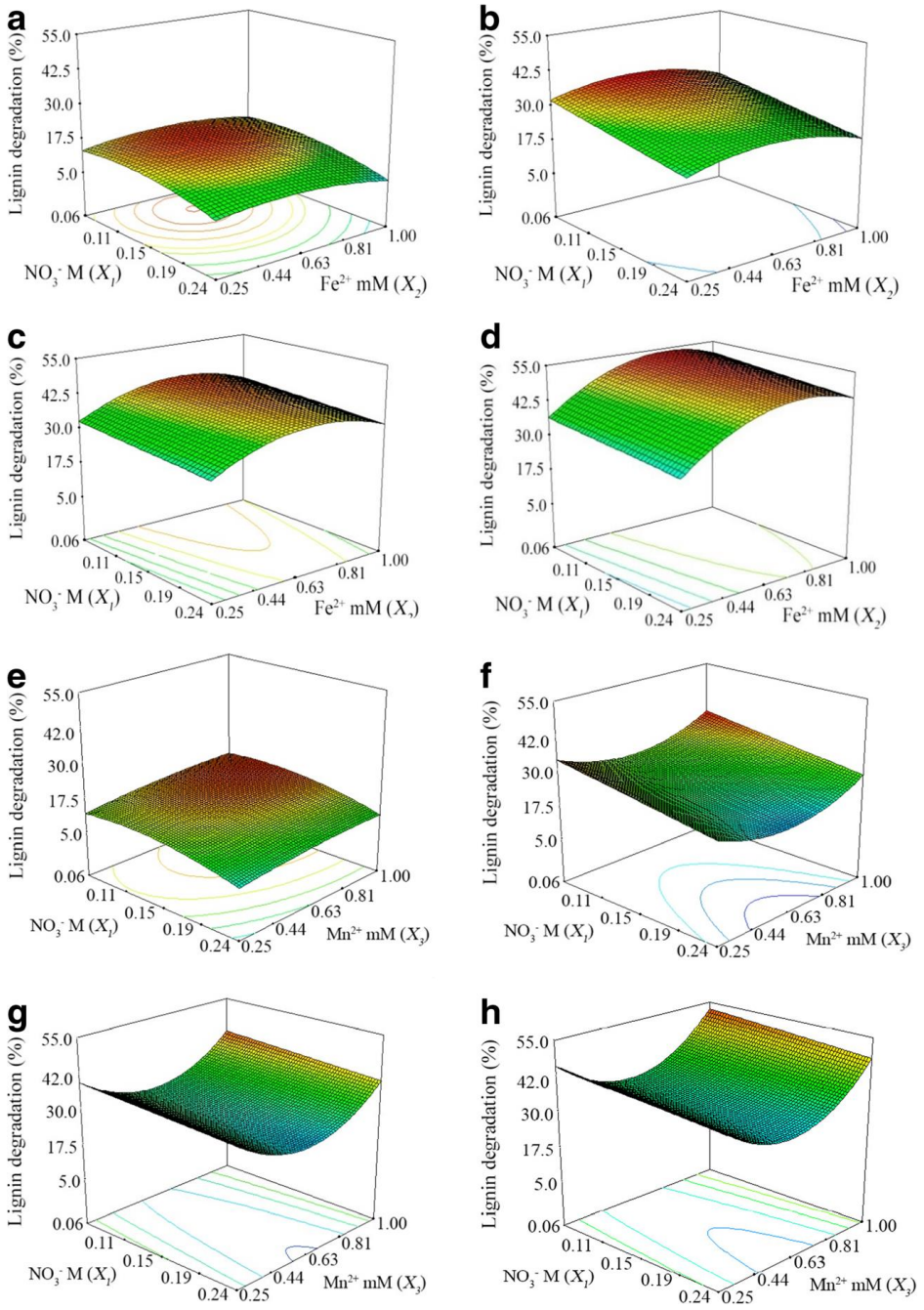

Figure 5 The 3D surface plots of modeled lignin degradation (%) in wheat straw by *G. lobatum* as functions of Fe^{2+} and NO_3^- after 10 (a), 20 (b), 30 (c) and 40 days of incubation (d) with 1.00 mM Mn^{2+}, and the modeled lignin degradation (%) as functions of NO_3^- (X_1) and Mn^{2+} (X_3) after 10 (e), 20 (f), 30 (g) and 40 days of incubation (h) with 0.25 mM Fe^{2+}.

Table 3 Maximization of lignin degradation and minimization of weight loss according to response-surface models.

Incubation times	Weight loss (%)		Lignin degradation (%)		Relative selectivity (Srel) of lignin degradation	
	Predicted value	Exp. value	Predicted value	Exp. value	Predicted value	Exp. Value
10 days	4.5	4.9	13.7	17.4	0.86	0.98
20 days	8.6	9.2	29.7	36.7	0.91	0.78
30 days	16.4	15.5	40.5	44.7	0.68	0.72
40 days	18.5	20.6	50.7	54.6	0.72	0.60

Optimal solution: 0.18 M NO_3^- (X_1). 0.73 mM Fe^{2+} (X_2) and 1 mM Mn^{2+} (X_3). *Desirability:* 0.78 for lignin degradation, 0.55 for weight loss and 0.68 for combined variables. *Exp. value*: maximum lignin degradation, the associated weight loss and the relative selective-lignin-degradation index measured experimentally at different incubation times.

3.3.4 FTIR analysis

To enhance our understanding of the chemical changes that occur during the biodegradation of wheat straw by *G. lobatum*, we analyzed untreated and fungal-treated samples by FTIR. In Figure 7, the FTIR spectra were divided into 1800-1400 and 1400-800 cm^{-1} ranges to identify the most characteristic bands of lignin and carbohydrates, respectively. The observed lignin bands included a characteristic triplet at 1600, 1505 and 1429 cm^{-1}attributed to aromatic-ring vibrations and a region at 1726 – 1710 cm^{-1} corresponding to the stretching of C=O bonds unconjugated to aromatic rings (Oliva-Taravilla et al., 2015; Sun et al., 2005; Xu et al., 2013). The carbohydrate bands observed at 900 cm^{-1} were attributed to β-glycosidic linkages and to amorphous cellulose types, with an additional region at 1160-1035 cm^{-1} (stretching vibration of carboxylic-acid side groups) and a band at 1373 cm^{-1} (symmetric bending of aliphatic C-H; Oliva-Taravilla et al., 2015; Xu et al., 2013).

A decrease in the absorbance at 1505 – 1510 cm^{-1} corresponding to the vibrations of aromatic rings of lignin can be observed in fungal-treated wheat-straw samples after 20 and 40 days, as well as in the 1726 - 1710 cm^{-1} range after 40 days in samples of fungal-treated wheat straw

with combination of 0.15 M NO_3^-, 0.63 mM Fe^{2+} and 1 mM Mn^{2+}. The largest decrease in the absorbance of lignin bands was observed in samples fungal-treated at 40 days corroborating the lignin degradation. On the other hand, the reduction of absorbance at 1726 – 1710 cm^{-1} suggest that the structure of lignin was modified by fungal pretreatment. An increase in the absorbance at 1373 cm^{-1} in fungal-pretreated samples was observed after 20 days. However, this band decreased again after 40 days, reaching a level similar to that seen in untreated wheat straw. Additionally, a marked decrease in carbohydrate bands at 1160 cm^{-1} and 900 cm^{-1} was related to amorphous cellulose, which is a readily degradable type of cellulose (Sawada et al., 1995). This change indicated that higher carbohydrates consumption by the fungus at 40 days than 20 days. These results confirm that the selective degradation of lignin by *G. lobatum* was higher until 20 days than 40 days, which are in concordance with the increase in weight loss. This in turn is consistent with an increase in CMCase and xylanase activities observed after 20 days, which implies an increase in the degradation of cellulose and hemicellulose.

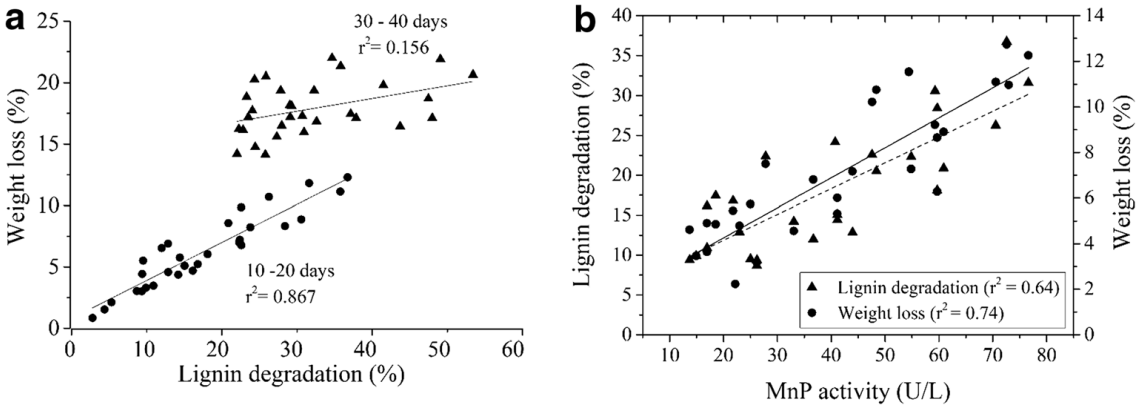

Figure 6 (a) Correlation analysis between lignin degradation (%) and weight loss (%) in wheat straw treated with *G. lobatum* under different culture conditions, *circles* represent data from 10 and 20 days of incubation, and *triangles* represent data from 20 and 40 days of incubation. (b) Correlation analysis between MnP activity and lignin degradation (%) and weight loss (%) in wheat straw treated with *G. lobatum* under the different culture conditions tested at 10 and 20 days of incubation (the *dotted trend line* represents lignin degradation and MnP activity, and the *solid trend line* represents the correlation between weight loss and MnP). *Circles* represent data of weight loss, and *triangles* represent data of lignin degradation.

3.3.5 Visualization of lignin degradation

The CLSM analysis was used to visualize the lignin degradation on wheat straw caused by fungi attack. Although some components of cell wall, such as lignin, have autofluorescence in the ultraviolet range (Bond et al., 2008), the use of specific dyes allows to visualize chemical changes component in biomass. In these sense, some dyes traditionally used for staining plant tissues in optical microscopy are also fluorescent. Samples of untreated and fungal-treated wheat straw after 40 days were stained with calcofluor (Monheit et al., 1984), safranin O (Bond et al., 2008) and Congo red (Verbelen and Kerstens, 2000) which allows to differentially and simultaneously observe the fluorescence of fungal mycelia, lignin and cellulose, respectively. Figure 8a shows untreated wheat straw, in which the green fluorescence is predominant because the intact lignin and Congo red could not penetrate the cell wall efficiently to stain cellulose. In contrast, Figure 8b shows a significant decrease in green fluorescence as result of lignin degradation by fungal treatment and an increase in red fluorescence, indicating the availability of cellulose. The three-dimensional reconstruction of the serial optical sections (Figure 8f-h) shows that the fungus (blue fluorescence) was capable of penetrating wheat straw and degrading lignin at different depths of wheat straw. Figure 8c shows another pattern in which lignin degradation was localized to the sites of fungal colonization. Figure 8g shows that the fungus colonized wheat straw only superficially. Figure 8d shows a third type of lignin degradation that generates pores that grant access to cellulose, as shown by the peaks of increased red fluorescence intensity. Congo red is a direct dye with high affinity for cellulose hydroxyl groups that has been used to quantify the area of the accessible cellulosic surface (Chandra et al., 2008). According to the CLSM images, treatment with *G. lobatum* generated areas of accessible cellulose, which were identified by Congo red staining. Treatment could thus significantly increase the digestibility of wheat straw for use in a downstream process, such as enzymatic hydrolysis or anaerobic digestion. Arantes and Saddler (2011) reported that cellulose

accessibility is directly correlated with the effectiveness of enzymatic hydrolysis in a pretreated lignocellulosic substrate, such as wheat straw. Furthermore, the accessible cellulose fraction may become more important than low lignin content to the efficiency of enzymatic hydrolysis (Wiman et al., 2012). Therefore, we demonstrated the ability of *G. lobatum* to degrade lignin selectively and increase the availability of cellulose, which can be improved by the addition of enzyme inducers (Fe^{2+} and Mn^{2+}) and nitrate, and applied as a biological pretreatment of lignocellulosic biomass oriented to the production of added-value products derived from the polysaccharide fraction.

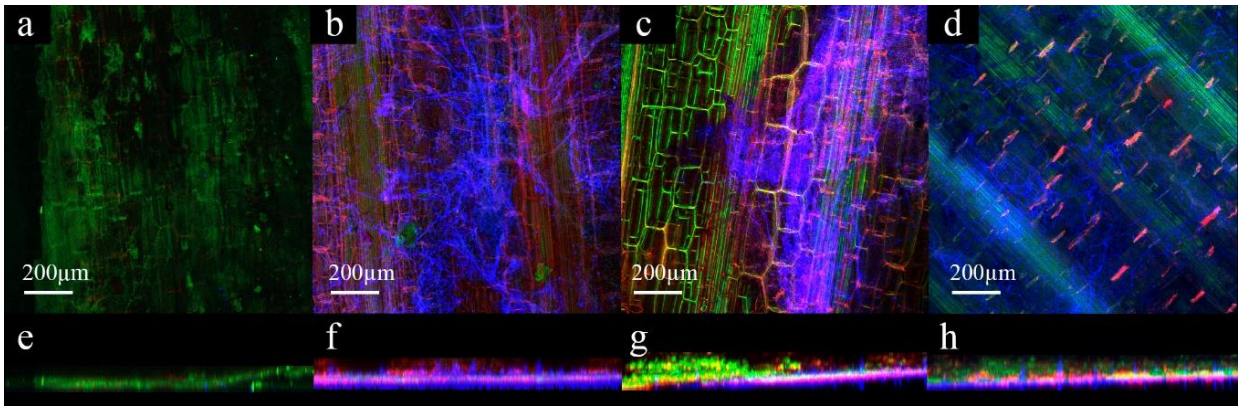

Figure 8 Confocal laser-scanning microscopy (CLSM) images of colonization and lignocellulosic-component degradation by *G. lobatum* on wheat straw after 40 days of incubation. (a) Untreated wheat straw, (b) Trial No 5: 0.06 M NO_3^-, 0.25 mM Fe^{2+} and 1 mM Mn^{2+}, (c) Trial No 6: 0.24 M NO_3^-, 0.25 mM Fe^{2+} and 1 mM Mn^{2+}, (d) Trial No 7: 0.06 M NO_3^-, 1 mM Fe^{2+} and 1 mM Mn^{2+}. The lateral views from three-dimensional reconstruction, (e), (f), (g) and (h) corresponding to samples (a), (b), (c) and (d), respectively. The fluorescence in images (merge) corresponds to lignin (*green channel*), cellulose (*red channel*) and fungal mycelium (*blue channel*).

3.4 Conclusions

The fungus *G. lobatum* showed selective lignin degradation of wheat straw independent of the tested factors until 20 days of incubation. The Mn^{2+} level had the strongest influence on lignin degradation and weight loss and, consequently, on the selectivity of lignin degradation. The addition of 0.72 mM Fe^{2+} had positive effects on lignin degradation by *G. lobatum*. The addition

of 0.18 M NO_3^- promotes selective lignin degradation by controlling weight loss. The condition that maximized selective lignin degradation resulted in 50% lignin degradation and 18.5% weight loss after 40 days of incubation.

3.5 Acknowledgements

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CHAPTER IV

Metal ions ($Mn^{2+}$and Fe^{2+}) and nitrate effects on the degradation of wheat straw by brown-rot fungus *Gloeophyllum trabeum*

Manuscript in preparation

Metal ions ($Mn^{2+}$and Fe^{2+}) and nitrate effects on the degradation of wheat straw by the brown-rot fungus *Gloeophyllum trabeum*

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Abstract

In this work, the effects of metal ions, Mn^{2+} and Fe^{2+} (as exogenous Fenton reactants) and NO_3^- (as nitrogen source) were evaluated on wheat straw weight loss and total crystallinity index, and on the brown-rot fungus *G. trabeum* extracellular activities (Fe^{3+}-reducing activity, catechol-type compounds, and hollocellulolytic enzymes). The Fe^{2+} promoted weight loss and Fe^{3+}-reducing activity, and at the same time, it dimished total crystallinity index, and cellulase and xylanase activity. Mn^{2+} had a negative effect on weight loss, cellulase, xylanase and Fe^{3+}-reducing activity, and a positive effect on catechol compounds. Whereas NO_3^{-1} had a negative effect on weight loss, xylanase activity and total crystallinity index, and a positive effect on cellulase activity, and total catechol compounds production. According to multiple optimization, the *G. trabeum* culture in wheat straw at 0.24 M NO_3^{-1}, 0.95 mM Fe^{2+} and 0.85 mM Mn^{2+}, resulted in 11.3% weight loss and 0.47 total crystallinity index after 40 days. The enzymatic hydrolysis assay revealed that the pretreatment of wheat straw by *G. trabeum* for 10 days, increased glucose recovery in 26.1% compared to untreated wheat straw. After 10 days of incubation, the changes caused in the wheat straw by the fungus decreased glucose recovery.

Keywords: Brown-rot fungi, Fenton reactants, nitrate, wheat straw, non-enzymatic mechanisms, biomass pretreatment.

4.1 Introduction

The route to obtain cellulose-derived products from lignocellulosic biomass require multiple steps: (1) pretreatment (to increase cellulose accessibility in the lignocellulose matrix), (2) saccharification or cellulose enzymatic hydrolysis (to obtain glucose), (3) glucose fermentation (for producing biofuels or chemical compounds), and (4) recovery and purification of the desired product (such as chemical compounds, biofuels, among others) (Sánchez 2009). From these, the pretreatment is the critical step for a feasible conversion of cellulose at industrial scale. Nowadays, the most established pretreatment technologies include physicochemical processes, such as steam explosion, ammonia fiber expansion (AFEX), diluted-acid and alkaline-catalyzed hydrolysis (Bals et al. 2010; O'Donovan et al. 2014; Silva et al. 2017; Taherzadeh and Karimi 2008). However, these pretreatments are expensive and have technical disadvantages such as machinery corrosion, formation of salts and by-products that inhibit enzymatic hydrolysis. The biological alternative to physicochemical pretreatments has been focused on the use of wood-rotting fungi (white-rot, brown-rot and soft-rot), which provide a cost-efficient, environmentally friendly and safe process (Mtui 2009; Saha et al. 2016; Salvachúa et al. 2013; Wan and Li 2012). Currently, lignocellulosic biomass biological pretreatment has been mainly carried out by using strains of white-rot fungi with the ability to selectively degrade lignin (Hermosilla et al. 2017; Saha et al. 2016; Wang et al. 2013). There are lesser number of studies about pretreatment by brown-rot fungi than about white-rot, which include the use of the whole-organism for lignocellulose modification (Monrroy et al. 2011) or the use of crude enzymatic extracts from brown-rot fungi for enzymatic hydrolysis step

(increased of 33% compared to untreated wood) (Lee et al. 2008; Schilling et al. 2009). Very few studies on pretreatment by brown-rot fungi have been carried out using non-wood biomass (Rasmussen et al. 2010; Schilling et al. 2012).

Brown-rot fungi degrade cellulose and hemicellulose in lignocellulosic biomass through non-enzymatic and enzymatic mechanisms, without significant lignin degradation. In early stages of the degradation by brown-rot fungi, an oxidative radical-based system (via Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + {}^{-}OH$) attacks the plant cell wall causing important modifications on the lignocellulose matrix, including cellulose and hemicellulose chains cleavage and conformational changes in the structure of lignin (Arantes et al. 2012; Goodell et al. 2017). Hydrogen peroxide, oxalic acid (to solubilize iron contained in biomass) and iron-reducing compounds (IRCs) that propagate the Fenton reactions are produced by the brown-rot fungus during the non-enzymatic degradation step. Cellulases and xylanases are also secreted by the fungus, but they are too large to penetrate intact cell wall. After the non-enzymatic attack, voids large enough for allowing the access of polysaccharide-degrading enzymes are created (Hammel et al. 2002), which could be favorable in its application as a pretreatment for enhancing enzymatic hydrolysis by commercial enzymes. Monrroy et al. (2011) showed that the enzymatic hydrolysis performance increase (14% yield) was directly related to cellulose polymerization degree and total crystallinity index decrease (calculated by FTIR analysis), and hemicellulose degradation produced by brown-rot fungus *G. trabeum* in *Pinus radiata* and *Eucalyptus globulus* wood.

The addition of iron ($FeSO_4$ salt) as a supplementary Fenton reactant has increased the amount of hydroxyl radical production and pollutant degradative abilities by brown-rot fungi, *Gloeophyllum trabeum* and *Daedalea dickinsii*, in liquid and soil cultures (Purnomo et al. 2010, 2011). In addition to iron, manganese and copper are also involved in the generation of hydroxyl radicals via Fenton reaction. The effect of the addition of non-ferrous Fenton reactants as

manganese or copper on lignocellulose degradative abilities of brown-rot fungi have not been evaluated yet. On the other hand, lignocellulosic biomass has very low nitrogen content, which is below optimal level for fungal growth; therefore, for accelerating the fungal growth in an initial stage of biomass colonization it is necessary to increase nitrogen content (Bisaria 1998; Jonathan and Adeoyo 2011; Mane et al. 2007). It is know the production of enzymes involved in lignin degradation by white-rot fungi is promoted under nitrogen starvation conditions (Kersten and Cullen 2007; Wu and Zhang 2010). However, a variable nitrogen concentration effect has been reported on enzymes and extracellular compounds involved in lignocellulose degradation by brown-rot fungi. Therefore, in this work, the effects of Mn^{2+} and Fe^{2+} (as exogenous Fenton reactants) and NO_3^- (as nitrogen source) were evaluated on wheat straw degradation by *G. trabeum* in terms of weight loss and total crystallinity index, and extracellular activities (Fe^{3+}-reducing activity, catechol-type compounds, and hollocellulolytic enzymes) in order to find culture conditions that stimulate an adequate lignocellulose biodegradation for biological pretreatment application.

4.2 Materials and methods

4.2.1 Fungal strains

The *Gloeophyllum trabeum* CCCT16.04 fungal strain was collected from a temperate forest in Antuco, located at the Bio-Bio Region, Chile (latitude, 38° 39'S; longitude, 72° 35'W). The fungal isolate was obtained by placing small fragments of the fungi fruiting bodies on glucose malt-extract agar plates (per liter: 15 g of agar, 3.5 g of malt extract, and 10 g of glucose), which were kept at 25°C. The pure fungus culture was kept in slant culture tubes with malt-extract agar medium at 4 °C and periodically sub-cultured. The strain was molecularly identified and deposited in Colección Chilena de Cultivos Tipo of Scientific and Technological Bioresource Nucleus at Universidad de La Frontera, Temuco, Chile.

4.2.2 Inoculum preparation

For inoculum preparation, an Erlenmeyer flask (500 mL) containing 100 mL of modified Kirk medium (per liter: 10 g of glucose, 2 g of peptone, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 500 µM $MnSO_4xH_2O$, 2 mg of thiamine, and 10 mL of mineral salts) was autoclaved at 121°C for 15 min. Thereafter, the flask was inoculated with five agar disks (6-mm diameter) of active mycelia from a five-day-old culture on malt-extract agar cultivated in Petri dishes, and incubated at 30°C for 10 days. Then, the fungal culture was homogenized in a sterilized blender for 1 min and used as an inoculum (blended fungal mycelia) (Rubilar et al. 2007).

4.2.3 Solid-state fermentation

The biodegradation of wheat straw by *G. trabeum* was carried out in 250 mL Erlenmeyer flasks containing 5 g wheat straw (~50 mm pieces) and 25 mL sterile water (Singh et al. 2011) supplemented with $NaNO_3$, $FeSO_4$, $MnSO_4$ at concentrations according to treatments established in the experimental design (Table 1). Flasks were stoppered and autoclaved at 121°C for 25 min. Afterwards, they were cooled and then inoculated with 0.5 mL blended fungal mycelia and finally, they were kept in the dark at 25 °C ±1 for 10, 20, 30 and 40 days. Uninoculated flasks were carried out as a negative control.

4.2.4 Experimental design

The effect of NO_3^{-1} (X_1), Fe^{2+} (X_2) and Mn^{2+} (X_3) on the biodegradation of wheat straw by *G. trabeum* was evaluated according to a response-surface methodology (RSM) of central composite design (CCD). All factors were set at three levels in 17 experiments (Table 1). All experiments were carried out in triplicate, and the means of each value were considered as the dependent variables. The evaluated responses were weight loss, catechol-type compounds, Fe^{3+}-reducing activity, total crystallinity index and cellulase and xylanase activities. The Design

Expert software, v.8.0 trial-version (https://www.legacy.statease.com/dx8trial.html) was used to build the experimental design, evaluate the statistical significance of effects and to model the above-mentioned responses as a function of factor levels based on numerical optimizations. Where required, the obtained quadratic, polynomial and cubic models were reduced by incorporating all individual effects as well as the most significant interactions to simplify the model and increase its statistical significance. Design Expert software allows the simultaneous optimization of multiple responses. Hence, a multiple optimization using the models of total crystallinity index and weight loss was carried out to find the optimal levels of NO_3^-, Fe^{2+} and Mn^{2+}, which should result in both minimum weight loss and total crystallinity index in wheat straw after 40 days of fungal-treatment in order to promote an adequate wheat straw degradation by *G. trabeum*.

4.2.5 Sample treatment

The extracts were obtained from untreated and fungal-treated wheat-straw samples after the addition of 25 mL of sterile water and subsequent sample incubation in a rotary shaker (150 rpm) at room temperature for 2 h. Then, the extracts were collected by filtration through Whatman N°1 filter paper (pore size 11 µm), and aliquots were used to quantify Fe^{3+}-reducing activity, catechol-type compounds, Fe^{3+}-reducing activity and enzyme activities. Wheat straw from each flask was washed with tap water to remove fungal mycelia and was dried at 105 °C until it reached a constant weight. This value was used to calculate sample weight loss.

4.2.6 Determination of enzyme activity

Endoglucanase (CMCase) activity was measured by the release of reducing sugars from carboxymethylcellulose according to Wood and Bhat (1988), with some modifications. The reaction mixture contained 125 µL of enzyme extract and 125 µL of 1% w/v CMC (carboxymethylcellulose) in 250 mM citrate buffer (pH: 4.5). The reaction was incubated in a

water bath at 37°C for 30 min and stopped by the addition of 750 µL of 3,5-dinitro salicylic acid (DNS) reagent and then incubated in a water bath at 100°C for 5 min. Finally, 4 mL of distilled water were added, and the reaction mixture absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of glucose per min. Xylanase activity was measured in a reaction mixture containing 125 µL of enzyme extract and 125 µL of 1% w/v xylan-birchwood in 250 mM citrate buffer (pH: 4.5). The reaction was stopped by adding 750 µL of 3,5-dinitro salicylic acid (DNS) reagent. The mixture was incubated in a water bath at 100°C for 5 min, and then 4 mL of distilled water was added. The absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that released 1 µmol of xylose per min. The amount of reducing sugars released during CMCase and xylanase activities was calculated using calibration curves for glucose and xylose, respectively. The reducing sugars were measured by DNS method (Miller 1959). The mixture reaction contained 250 µL sample and 750 µL DNS reagent. It was incubated in a water bath at 100°C for 5 minutes, and after incubation, 4000 µL distilled water were added. Finally, absorbance was measured at 540 nm, and reducing sugars were calculated from a glucose or xylose calibration curve (0.1 – 4 mg L^{-1}).

4.2.7 Analysis of aqueous extracts

Catechol-type compounds were measured by a spectrophotometric assay, according to Aguiar and Ferraz (2012). The mixture reaction contained 500 µL aqueous extract, 500 µL 0.5 mM HCl, 500 µL 10%(m/v) $NaMoO_4$, 500 µL 10% (m/v) $NaNO_2$, and 500 µL 1 M NaOH. Finally, absorbance was measured at 510 nm, and total catechol content was calculated from a catechol (1,2-dihydroxybenzene) calibration curve (0.1-1 mM). In the reference cell, aqueous extract was replaced by distilled water. Total catechol content was also corrected using a mixture reaction without $NaMoO_4$ and $NaNO_2$. The Fe^{3+}-reducing activity in extracts was estimated by the formation of ferrozine-Fe^{2+} complex, according to Aguiar et al. (2013). Briefly, the reaction

was conducted in a quartz cuvette containing 375 µl of extract, 330 µl of sodium acetate buffer (50 mM, pH4,5 and 25 µl freshly prepared $FeCl_3 \cdot 6H_2O$ (8 mM)). After 30 min, 70 µl of NaF (1% v/v) was added to stop the reaction and 200 µl of Ferrozine (1% v/v) was added to complex Fe^{2+} ions. The absorbance of the reaction mixture was measured at 562 nm (ε=27,900 mol^{-1} l cm^{-1}). As the extracts contained Fe^{2+} ions, another reaction mixture was carried out replacing $FeCl_3 \cdot 6H_2O$ with deionized H_2O_2. These values were subtracted from the amount of Fe^{3+} reduced by the extract.

4.2.8 Fourier-transform-infrared spectroscopy (FTIR) analysis

Solid samples were dried in an oven at 105°C for 24 h, milled into fine powder and homogenized before FTIR analysis. The infrared spectra were collected at room temperature in the 4000-600 cm^{-1} range at 4 cm^{-1} resolution, with an average of 32 scans per sample, using a Cary-630 spectrometer equipped with an ATR accessory. Spectra were obtained using Agilent Resolution Pro software (Agilent Technologies Inc., Santa Clara, CA). The total crystallinity index (TCI) was estimated by the ratio of absorbance areas A_{1375}/A_{2900} from FTIR spectra (Nelson and O'Connor 1964). Values of relative changes in bands of functional groups were calculated according to equation 1 (Kumar et al. 2009).

$$\% \text{ Relative change} = 100 * (\text{Intensity of UWS} - \text{intensity of FPWS}) / \text{intensity of UWS} \quad (1)$$

where UWS is untreated wheat straw and FPWS is fungal-pretreated wheat straw. Positive values of relative change indicate reduction.

4.2.9 Enzymatic hydrolysis

Enzymatic hydrolysis assays were carried out using samples of pretreated wheat straw and commercial enzymes Celluclast 1.5L (a mixture of cellulases produced by *Trichoderma reesei*) and β-glucosidase produced by *Aspergillus niger* . Tests were carried out in Erlenmeyer flasks

containing 2.5 g (5% w/w) of wheat straw and an enzyme solution prepared in 50 mM sodium citrate (pH 4.8) with enzyme loading of 15 FPU g^{-1} biomass and 45 IU β-glucosidase per g biomass. Then, they were incubated at 50 °C, 200 rpm in a rotary shaker for 72 h. In all trials, sodium azide (0.1% w/v) was added in order to prevent microbial contamination. Samples were withdrawn at 1, 2, 4, 6, 24, 48 and 72 h, and the amount of sugars released from the biomass was measured by HPLC. Untreated wheat straw was used as a negative control. All assays were carried out in triplicate.

4.2.10 Scanning electron microscopy (SEM)

A SEM (SU 3500, Hitachi, Japan) equipped with a module for EDX elemental microanalysis was used to take images of untreated and fungal-pretreated wheat straw samples (at different magnifications such as 100x, 200x, and 500x).

Table 1 Experimental values versus predicted values of weight loss, TCI and accumulated lignocellulolytic activity of wheat straw treated with *G. lobatum* after 10 days of incubation.

Trial no.	Coded levels			Real levels			Responses									
	X_1	X_2	X_3	X_1	X_2	X_3	Y_1 TCI (A_{1375}/A_{2900})		Y_2 Weight loss (%)		Y_3 Fe3+-reducing activity		Y_4 Catechol (UI L^{-1})		Y_6 Xylanase Activity (UI g^{-1})	
	$NaNO_3$ (M)	$FeSO_4$ (mM)	$MnSO_4$ (mM)	$NaNO_3$ (M)	$FeSO_4$ (mM)	$MnSO_4$ (mM)	Exp. Value	Pred. Value	Exp. Value	Pred. Value	Exp. Value	Exp. Value	Exp. Value	Pred. Value	Exp. Value	Pred. Value
1	-1	-1	-1	0.06	0.25	0.25	0.53	0.53	15.1	13.8	2.08	0.86	0.15	0.14	6.71	6.68
2	+1	-1	-1	0.24	0.25	0.25	0.49	0.50	4.3	5.2	2.37	1.93	0.20	0.22	5.94	6.11
3	-1	+1	-1	0.06	1.00	0.25	0.50	0.50	14.1	19.9	2.29	4.03	0.08	0.08	5.65	6.01
4	+1	+1	-1	0.24	1.00	0.25	0.41	0.41	8.8	11.3	3.98	5.11	0.15	0.18	5.42	5.44
5	-1	-1	+1	0.06	0.25	1.00	0.44	0.45	6.4	12.3	2.72	3.06	0.29	0.26	7.18	6.54
6	+1	-1	+1	0.24	0.25	1.00	0.43	0.43	4.4	3.7	3.87	4.14	0.35	0.36	5.97	5.97
7	-1	+1	+1	0.06	1.00	1.00	0.52	0.52	22.1	18.3	7.63	6.24	0.09	0.08	5.34	5.87
8	+1	+1	+1	0.24	1.00	1.00	0.44	0.44	8.5	9.7	9.35	7.31	0.20	0.21	5.12	5.31
9	-1	0	0	0.06	0.63	0.63	0.49	0.47	18.6	16.1	3.48	3.55	0.13	0.18	6.28	6.06
10	+1	0	0	0.24	0.63	0.63	0.43	0.42	7.2	7.5	4.01	4.62	0.36	0.28	5.85	5.49
11	0	-1	0	0.15	0.25	0.63	0.47	0.45	8.3	8.8	3.26	2.50	0.18	0.19	5.81	6.33
12	0	+1	0	0.15	1.00	0.63	0.44	0.44	15.1	14.8	6.92	5.67	0.13	0.08	6.76	5.66
13	0	0	-1	0.15	0.63	0.25	0.43	0.41	17.3	12.5	4.84	2.98	0.24	0.19	6.68	6.16
14	0	0	+1	0.15	0.63	1.00	0.40	0.39	10.7	11.0	3.01	5.19	0.25	0.27	6.10	6.03
15	0	0	0	0.15	0.63	0.63	0.38	0.41	12.2	11.8	3.33	4.08	0.20	0.20	5.85	5.94
16	0	0	0	0.15	0.63	0.63	0.40	0.41	10.7	11.8	3.21	4.08	0.18	0.20	5.37	5.94
17	0	0	0	0.15	0.63	0.63	0.38	0.41	16.6	11.8	3.08	4.08	0.17	0.20	5.42	5.94

Coded levels: $NaNO_3$ 0.06 (-1), 0.15 (0) and 0.24 (+1); $FeSO_4$ 0.25 (-1), 0.63 (0) and 1.00 (+1); $MnSO_4$ 0.25 (-1), 0.63 (0) and 1.00 (+1).

4.3 Results and discussion

This is the first report about the effect of Fe^{2+}, Mn^{2+} or NO_3^- on the degradation of lignocellulosic biomass and extracellular activities by a brown-rot fungus. These effects were assessed using an *ex*perimental CCD design, which provided important information about the influence of each factor and their interactions on responses.

4.3.1 Weight losses from wheat straw

Weight loss is an important variable due to the fact that it gives an estimation of the degradation extent of the wheat straw lignocellulosic components. The maximum weight loss obtained during the biodegradation of wheat straw by *G. trabeum* was 9.2%, 14.7%, 14.9% and 22.1% after 10, 20, 30 and 40 days, respectively. Weight loss values were fitted to linear and polynomial models (Table 2). All factors (NO_3^-, Fe^{2+} and Mn^{2+}) showed a significant effect ($p < 0.05$) on weight loss by *G. trabeum*. Considering co-efficient of each factor and their interactions in the equations, NO_3^- is the main factor that negatively affected weight loss during the wheat straw biodegradation by *G. trabeum*. The NO_3^--Fe^{2+} interaction (at 20 and 30 days) and Mn^{2+} also have a negative effect on weight loss. Whereas, Fe^{2+} and Fe^{2+}-Mn^{2+} interaction (at 20 and 30 days) had a positive effect. Figure 1 shows the surface responses of weight loss after 10, 20, 30 and 40 days of incubation according to the fitted models. In Figure 1a-d, it can be seen that the positive effect of Fe^{2+} on weight loss occurs only at low concentrations of NO_3^-, and the negative effect of Mn^{2+} occurs at a low concentration of Fe^{2+} (Fig 1 e-h). In our previous results without supplementary nitrogen source or metal ions, *G. trabeum* produced lower weight loss with a maximum of 10% weight loss after 40 days of incubation (Appendix B). The increase in weight losses could indicate that non-enzymatic mechanisms of degradation are promoted by the positive effect of Fe^{2+} and Fe^{2+}-Mn^{2+} interaction (at 20 and 30 days), which would be contributing to wheat straw biodegradation via Fenton reaction. Therefore, the addition of Fe^{2+} and Mn^{2+}

accelerated the biodegradation of wheat straw by *G. trabeum*. On the other hand, an increase in nitrogen source generates low weight loss.

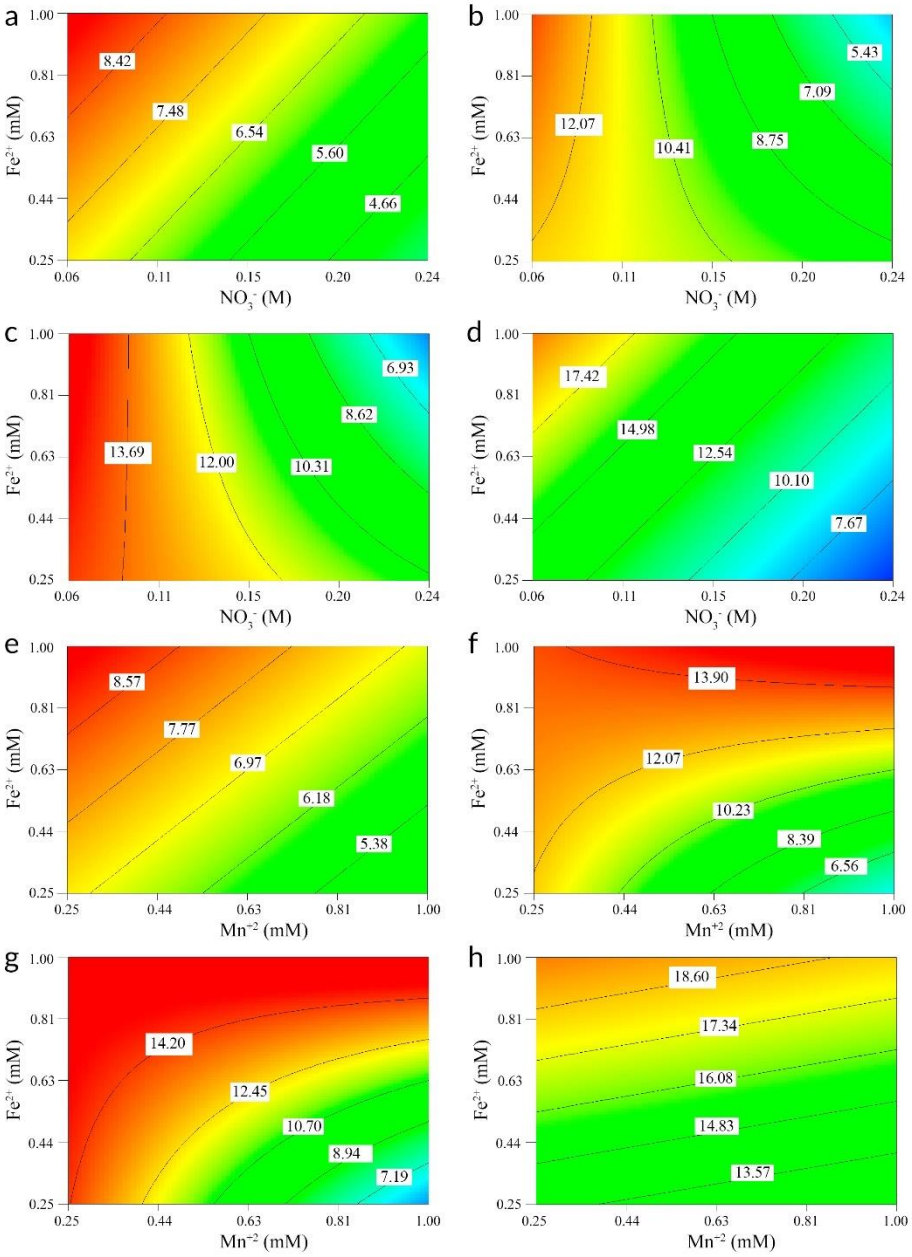

Figure 1 Response surface plots of weight loss (%) after fungal pretreatment of wheat straw. Plots (a - d) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 0.25 mM Mn^{2+} (X_3) for 10, 20, 30 and 40 days of incubation, respectively. Plots (e-h) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.06 M NO_3^- (X_1) for 10, 20, 30 and 40 days of incubation, respectively.

Table 2 Fitted model equations for each response

Response		Model equation in terms of coded factors	r^2	*P* value	A.P.
Weight loss	10 days	$+5.27 - 1.71X_1* + 1.11X_2 - 1.28X_3*$	0.49	0.028	7.8
	20 days	$+8.19 - 3.34X_1* + 1.40X_2* - 1.47X_3* - 1.81X_1X_2* - 0.17X_1X_3 + 2.30X_2X_3*$	0.95	<0.001	21.7
	30 days	$+9.80 - 2.96X_1* + 1.32X_2* - 1.54X_3* - 1.59X_1X_2* + 0.52X_1X_3 + 2.35X_2X_3*$	0.87	0.001	10.9
	40 days	$+11.78 - 4.30X_1* + 3.01X_2* - 0.76X_3$	0.66	0.002	9.9
Catechol	10 days	$+0.46 - 0.017X_1 - 0.12X_2* - 0.012X_3 - 0.046X_2X_3 - 0.17X_1^2*$	0.61	0.040	6.36
	20 days	$+0.15 + 0.021X_1 - 0.019X_2 - 0.002X_3 - 0.070X_1X_2* + 0.023X_1X_3 - 0.030X_2X_3*$	0.85	0.001	11.4
	30 days	$+0.23 + 0.037X_1 - 0.098X_2* + 0.050X_3*$	0.66	0.002	10.4
	40 days	$+0.20 + 0.051X_1* - 0.053X_2* + 0.036X_3 + 0.007\ X_1X_2 + 0.006X_1X_3 - 0.029X_2X_3 + 0.027X_1^2 - 0.064X_2^2 + 0.028X_3^2$	0.83	0.040	7.39
Fe^{3+}-reducing activity	10 days	$+117.01 + 3.45X_1 + 17.62X_2* - 1.53X_3 - 2.11X_1X_2 - 1.31X_1X_3 - 1.65X_2X_3 - 9X_1^2 - 18.61X_2^2* + 0.42X_3^2$	0.94	0.001	10.2
	20 days	$+93.16 + 4.65X_1 + 8.55X_2* - 3.61X_3 - 3.61X_1^2 - 14.10X_2^2*$	0.83	0.001	10.0
	30 days	$+13.46 + 0.75X_1 + 2.12X_2 + 4.06X_3* + 1.12X_1X_2 + 1.28X_1X_3 + 3.06X_2X_3 - 9.75X_1^2 - 1.69X_2^2 + 3.03X_3^2$	0.86	0.026	6.9
	40 days	$- 1.29 + 0.72X_1 + 4.23X_2* + 2.94X_3*$	0.61	0.005	9.54

A *P* value < 0.05 indicates that the fit of the model is statistically significant. A.P. adequate precision ratio: If A.P. is > 4, the model is adequate and can be used to navigate the design space. The model equation in terms of coded factors indicates how each factor changes the response Y. *Factors or interactions that have significant effects on the variable model according to ANOVA ($p < 0.05$)

Continued table 2…

Response		Model equation in terms of coded factors	r^2	*P* value	A.P.
CMCase	10 days	$+4.81 + 0.08X_1 - 0.87X_2* - 0.41X_3$	0.51	0.020	6.8
	20 days	$+2.82 + 0.07X_1 - 0.607X_2* - 0.58X_3*$	0.60	0.006	8.6
	30 days	$+2.96 + 0.20X_1 - 0.98X_2* - 0.17X_3 - 0.042X_1X_2 - 0.17X_1X_3 - 0.045X_2X_3 + 0.79X_1^2* - 0.053X_2^2 + 0.12X_3^2$	0.90	0.009	9.6
	40 days	$+2.81 + 0.065X_1 - 0.47X_2* - 0.1X_3 + 0.18X_1X_2 - 0.14X_1X_3 - 0.21X_2X_3 + 1.25X_1^2*$	0.78	0.020	6.8
Y_5 Xylanase	10 days	$+5.24 - 0.65X_1 - 1.70X_2* + 0.019X_3 - 0.63X_1^2 + 0.23X_2^2$	0.62	0.03	5.7
	20 days	$+4.38 - 0.031X_1 - 0.62X_2* - 0.65X_3* - 0.13X_1X_2 - 0.058X_1X_3 + 0.51X_2X_3* + 0.16X_1^2 + 0.043X_2^2 + 0.43X_3^2$	0.84	0.04	7.3
	30 days	$+4.26 - 0.061X_1 - 0.28X_2* - 0.32X_3* - 0.07X_1^2 - 0.22X_2^2 + 0.13X_3^2$	0.70	0.03	6.9
	40 days	No model adjustment			
Y_6 TCI	10 days	$+0.41 - 0.027X_1* - 5.50E^{-3}X_2 - 7.10E^{-3}X_3 - 0.021X_1X_2* + 0.014X_1X_3 + 0.017X_2X_3* + 0.033X_1^2* + 0.018X_2^2 + 0.013X_3^2$	0.93	0.003	9.8
	20 days	$+0.41 - 0.034X_1* + 1.00E^{-3}X_2 - 8.00E^{-3}X_3 - 0.021X_1X_2 + 8.75E^{-3}X_1X_3 + 0.024X_2X_3* + 0.056X_1^2* + 0.011X_2^2 - 3.80E^{-3}X_3^2$	0.92	0.004	9.1
	30 days	$+0.40 - 0.033X_1* + 6.00E^{-3}X_2 - 0.017X_3* - 0.018X_1X_2* + 5.00E^{-3}X_1X_3 + 0.017X_2X_3* + 0.049X_1^2* + 0.034X_2^2* - 1.34E^{-3}X_3^2$	0.94	0.002	11.1
	40 days	$+0.41 - 0.028X_1* - 5.00E^{-3}X_2 - 0.013X_3 - 0.015X_1X_2 + 5.00E^{-3}X_1X_3 + 0.025X_2X_3* + 0.039X_1^2* + 0.034X_2^2* - 5.84E^{-3}X_3^2$	0.91	0.006	9.1

A *P* value < 0.05 indicates that the fit of the model is statistically significant. A.P. adequate precision ratio: If A.P. is > 4, the model is adequate and can be used to navigate the design space. The model equation in terms of coded factors indicates how each factor changes the response Y. *Factors or interactions that have significant effects on the variable model according to ANOVA ($p < 0.05$)

4.3.2 Catechol production and Fe^{3+}-reducing activity in extracts

The most studied auxiliary compounds in Fenton reaction are dihydroxybenzenes (DHB), mainly catechol (1,2-dihydroxybenzene) and its derivatives have been described as Fe^{3+}-reducing compounds, produced by brown-rot fungi (Aguiar et al. 2007). The DHB is secreted outside the fungal mycelium to reduce Fe^{3+} to Fe^{2+}, which react with H_2O_2, generating hydroxyl radicals (Arantes et al. 2012; Contreras et al. 2006). Therefore, the presence of catechol compounds indirectly indicates that Fenton reactions occur during the degradation of wheat straw by *G. trabeum*. The production of catechol fluctuated during incubation times (Figure 3). The highest catechol concentration (0.61 mg g^{-1}) by *G. trabeum* was detected during the first 10 days of incubation (Figure 3). The catechol production was adjusted to quadratic, polynomial and linear models after 10, 20 and, 30 and 40 days, respectively (Table 2). All evaluated factors and their interactions showed negative effects on catechol production after 10 and 20 days, whereas NO_3^{-1} and Mn^{2+} presented significant positive effect after 30 and 40 days. Although it was expected that the addition of exogenous iron would promote the Fenton reaction as well the involved reactants, Fe^{2+} had a significant negative effect on catechol production during wheat straw degradation by *G. trabeum* (Figure 3). The maximum catechol production in all incubation times was obtained at 0.23 M NO_3^-, 0.25 Fe^{2+} and 1 mM Mn^{2+}. A commercial strain of *G. trabeum* ATCC 11539 showed concentrations of catechol below 0,06 mg g^{-1} with a production peak at 7 days of incubation in *Pinus taeda* wood chips (Aguiar et al. 2013).

The Fe^{3+}-reducing activity in extracts was evaluated due to phenols derived from extractives or lignin degradation, which can also reduce Fe^{3+}. The Fe^{2+} showed a significant positive effect on Fe^{3+}-reducing activity in extracts. The Mn^{2+} showed a negative effect on Fe^{3+}-reducing activity until day 20, and a positive effect at 30 and 40 days of incubation. The highest Fe^{3+}-reducing activity (120 µmoles Fe^{3+} reduced) was detected at 10 days of

incubation, at 0.15 M NO_3^-, 0.63 mM Fe^{2+} and 0.63 mM Mn^{2+}. After 20 days, the Fe^{3+}-reducing activity in extracts decreased markedly with means of 8.5 and 4.0 µmoles reduced Fe^{3+}, after 30 and 40 days of incubation, respectively. This decrease has been attributed to the consumption of phenol compounds by the fungus (Aguiar et al. 2013). Our results suggest that phenol compounds participate in Fe^{3+}-reduction until day 20, whereas catechol compounds would participate in the Fe^{3+}-reduction throughout incubation time.

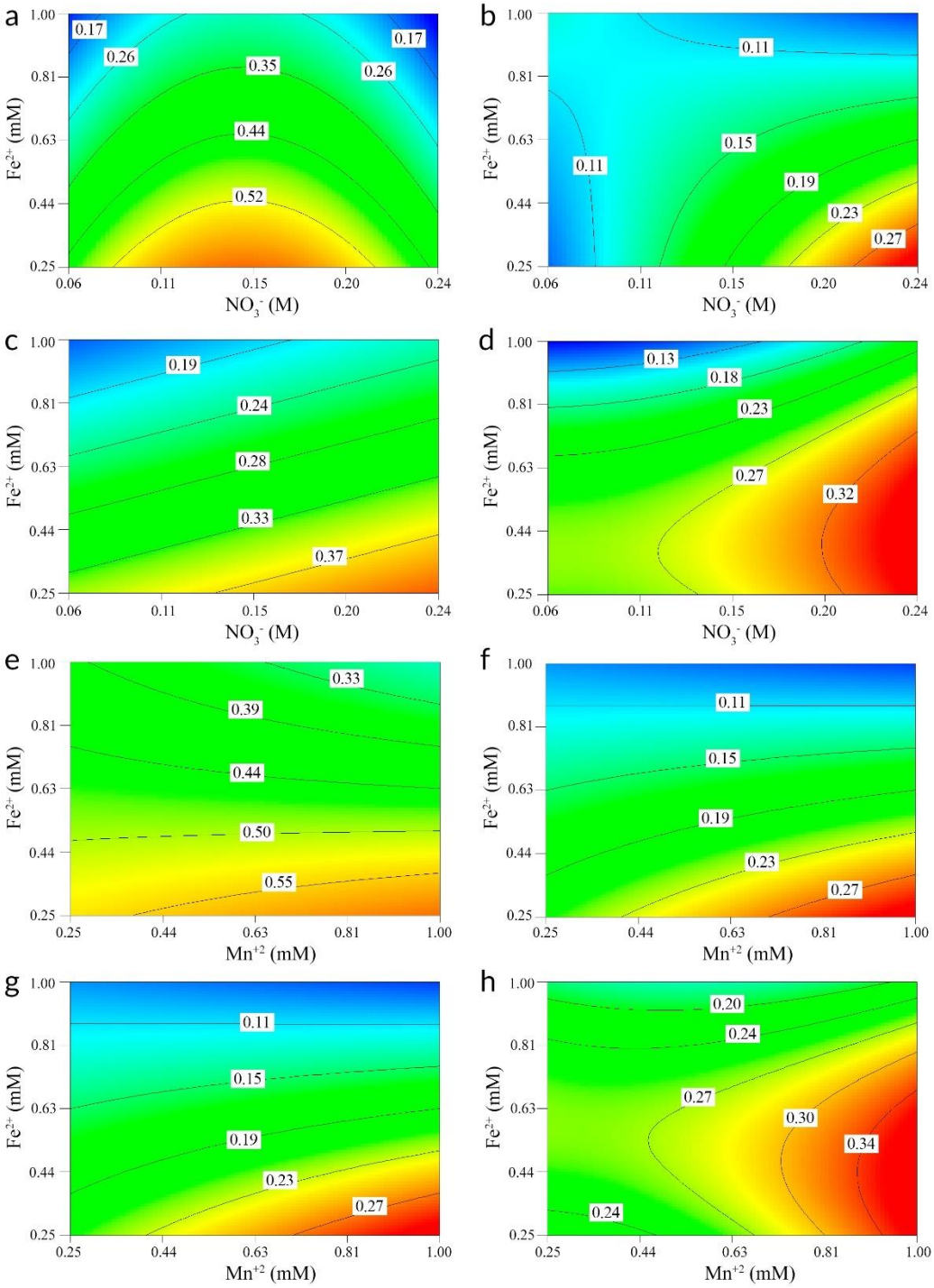

Figure 2 Response surface plots of catechol-type compounds (mg g^{-1}) in extracts from fungal-treated wheat straw. Plots (a-d) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 1.00 mM Mn^{2+} (X_3) for 10, 20, 30 and 40 days of incubation, respectively. Plots (e-h) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.24 M NO_3^- (X_1) for 10, 20, 30 and 40 days of incubation, respectively.

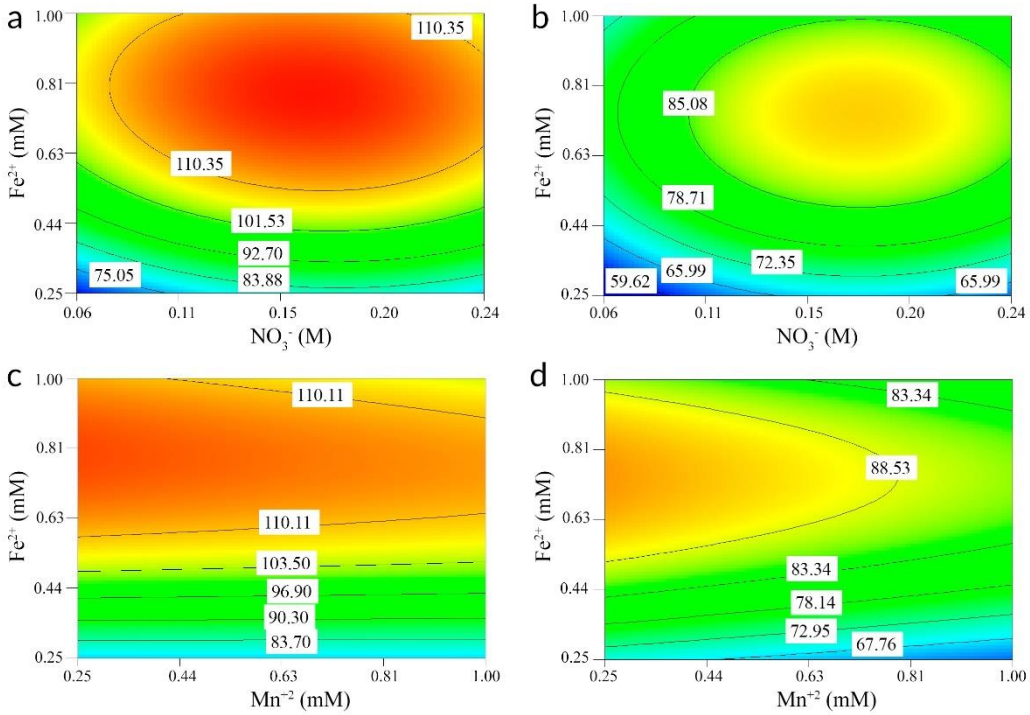

Figure 3 Response surface plots of Fe^{3+}-reducing activity (µmol l^{-1} Fe^{3+} reduced after 30 min reaction) in extracts from fungal-treated wheat straw. Plots (a-b) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 1.0 mM Mn^{2+} (X_3) for 10 and 20 days of incubation, respectively. Plots (c-d) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.06 M NO_3^- (X_1) for 10 and 20 days of incubation, respectively.

4.3.3 Cellulolytic and hemicellulolytic enzymes

Cellulase activity was adjusted to linear model after 10 and 20 days and to quadratic models after 30 and 40 days. Cellulase activity was affected negatively by Fe^{2+} and Mn^{2+} during all incubation times, while NO_3^{-1} had a positive effect (Table 2). However, only Fe^{2+} showed a significant effect throughout incubation time. The interaction between NO_3^{-1} and NO_3^{-1} negatively affected cellulase activity after 30 and 40 days, which indicates that at the lowest and the highest concentrations of NO_3^{-1} cellulase activity decreased (Figure 3). The maximum cellulase activity (6.3 IU g^{-1}) was detected after 10 days of incubation (at 0.15 M NO_3^{-1}, 0.25 mM Fe^{2+} and 0.63 mM Mn^{2+}). The optimum solution obtained for maximizing cellulase activity during 40 days of incubation was 0.24 NO_3^{-1}, 0.25 mM Fe^{2+} and 0.25 mM

Mn^{2+}). In relation to xylanase activity, it was possible to adjust a linear model after 10, 20 and 30 days. The Fe^{2+} showed a significant negative effect after 10, 20 and 30 days, and Mn^{2+} also showed a significant negative effect after 20 and 30 days (Table 2). The NO_3^{-1} showed a non-significant effect on xylanase activity.

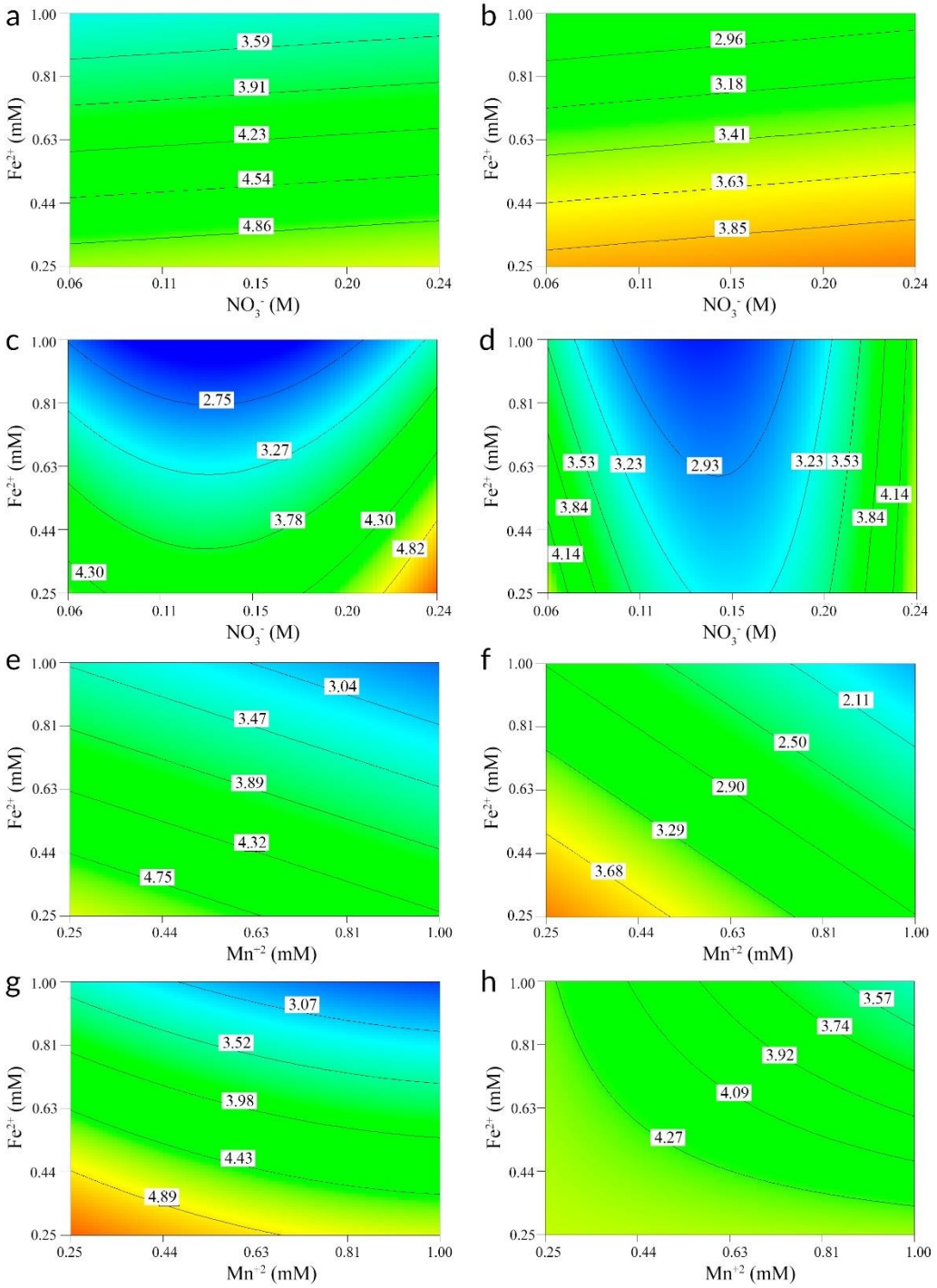

Figure 4 Response surface plots of CMCase activity (U g^{-1}) in extracts from fungal-treated wheat straw. Plots (a-d) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 0.25 mM Mn^{2+} (X_3) for 10, 20, 30 and 40 days of incubation, respectively. Plots (e-h) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.06 M NO_3^- (X_1) for 10, 20, 30 and 40 days of incubation, respectively.

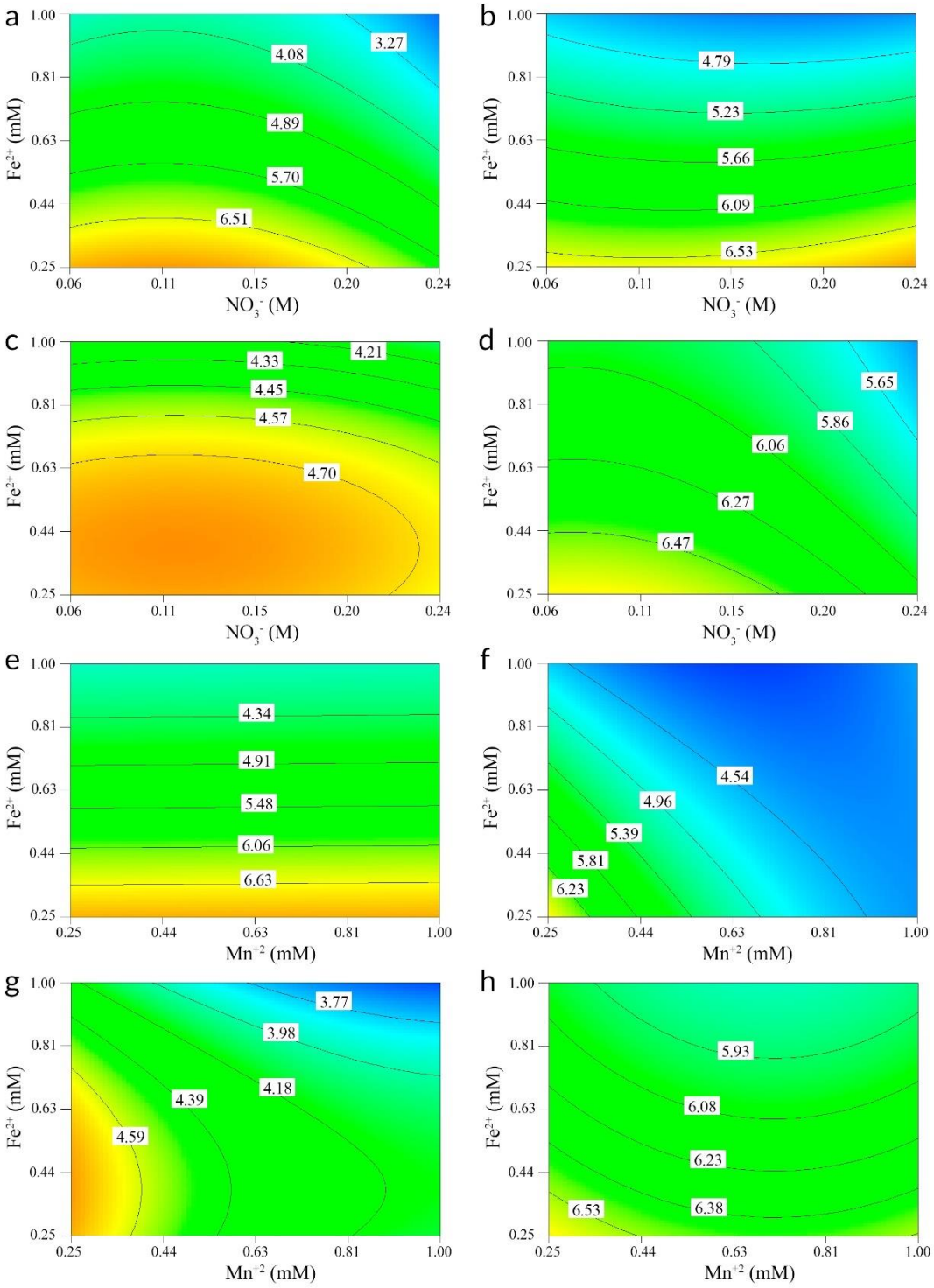

Figure 5 Response surface plots of xylanase activity (U g^{-1}) in extracts from fungal-treated wheat straw. Plots (a-d) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 0.25 mM Mn^{2+} (X_3) for 10, 20, 30 and 40 days of incubation, respectively. Plots (e-h) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.06 M NO_3^- (X_1) for 10, 20, 30 and 40 days of incubation, respectively.

4.3.4 FTIR analysis and TCI

Fourier transform mid-infrared spectroscopic analysis is a nondestructive and rapid technique for qualitative and quantitative evaluation changes in the composition of each plant cell wall polymer and in functional groups involved in the cross-links inter-polymers, after pretreatments of lignocellulosic materials (Kumar et al. 2009; Xu et al. 2013). It is important to emphasize that the biodegradation of wheat straw by fungi only generates changes in the intensity of FTIR spectrum bands associated to functional groups of the cell wall polymers, which does not generate changes in the peaks because it only partially degrades polymers. Figure 7 shows the FTIR spectra of untreated and fungal-treated wheat straw after 10, 20, 30 and 40 days of incubation of *G. trabeum*. The intensity of 3348 cm^{-1} band associated to O–H stretching, and 1159, 1078, 1032 cm^{-1} associated to C-O stretch of polysaccharides, decreased in fungal-treated samples. The intensity of 1159 cm-1 has been also associated with changes in cellulose crystallinity (Pozo et al. 2016). The intensity of 900 cm^{-1} band decreased progressively after 40 days. This band is associated to the amorphous cellulose readily degradable region of cellulose; consequently, fungi consume it before crystalline cellulose (Sawada et al. 1995). Therefore, it could have negative effects generating less digestible and recalcitrant wheat straw to the post-processing such as enzymatic hydrolysis. It was reported that hydroxyl radicals are probably responsible for the initial attack on these amorphous regions, because the porosity of wood during early brown-rot degradation is too low to admit enzymes (Cohen et al. 2005). The decrease in 1270 and 1230 cm^{-1} bands are associated with acetyl and carboxyl vibrations in xylan, which suggests a partial hemicellulose degradation. Likewise, the decrease in 1735 band indicates depolymerization of hemicellulose caused by the oxidative process of brown-rot fungi (Pandey and Pitman 2003). On the other hand, very slight changes were observed in 1600 cm^{-1} band, which indicates that the fundamental structure of lignin in wheat straw did not

change during the degradation by *G. trabeum*. The changes observed in the FTIR spectra of fungal-treated wheat straw in this study were consistent with those reported previously in wood degraded by brown-rot fungi (Pandey and Pitman 2003; Pozo et al. 2016).

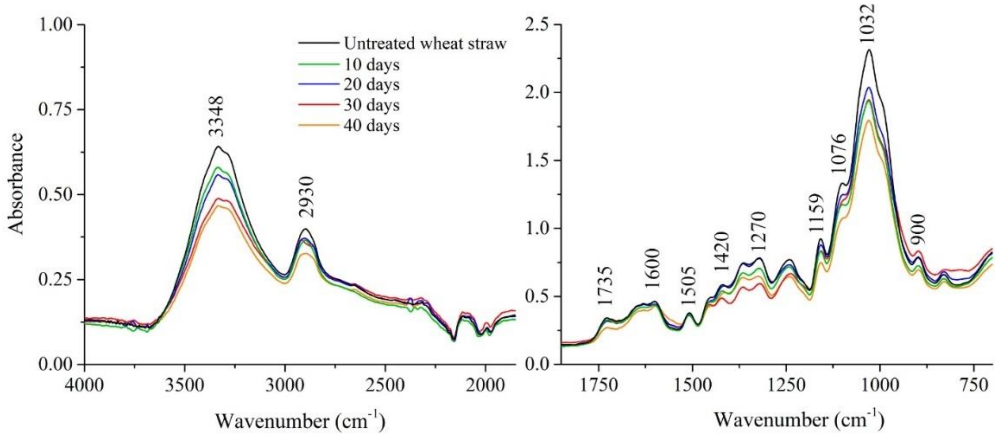

Figure 7 ATR-FTIR spectra of untreated and fungal pretreated wheat straw after 10, 20, 30 and 40 days. Data shown correspond to the experiment carried out under optimal conditions.

The reduction of cellulose crystallinity is another effect of the degradation process by brown rot fungi (Goodell 2003). In this work, FTIR spectroscopy was also used to assess changes in total crystallinity index (TCI). The untreated wheat straw showed TCI value of 0.52, while fungal-treated samples showed TCI values in the range of 0.53-0.38. A decrease in TCI value is a positive change in lignocellulose matrix, which could indicate the disaggregation of cellulose microfibrils in crystalline regions. By contrast, an increase in TCI value could indicate that amorphous regions of cellulose were degraded. The lowest TCI values were detected in the trial carried out at 0.15 M NO_3^-, 0.63 mM Fe^{2+} and 0.63 mM Mn^{2+}. Figure 5 shows the TCI surface response of fungal-treated wheat straw during incubation time. The NO_3^- showed a significant negative effect on TCI, while Fe^{2+} and Mn^{2+} showed a variable effect during incubation time. The Fe^{2+} showed a negative effect on TCI only at a high concentration of NO_3^- and low concentrations of Mn^{2+} until 20 days and at high

concentrations of Mn^{2+} after 30 days. According to the fitted models, the lowest TCI can be obtained at 0.16 NO_3^-, 0.49 mM Fe^{2+} and 1 mM Mn^{2+}. Cohen et al. (2005) showed that *G. trabeum* ATCC11539 can grow on microcrystalline cellulose (Avicel) as a unique carbon source. Howell *et al*., 2009 showed that *G. trabeum* ATCC11539 significantly decreases pine blocks cellulose crystallinity. Monrroy et al., (2011) showed that *G. trabeum* ATCC11539 showed a decrease 21% total crystallinity index in wood chips calculated by FTIR, increasing 14% enzymatic hydrolysis. In our results, *G. trabeum* decreased the TCI value to 27%. XRD diffractograms of wood degraded by the brown-rot fungi, *G. trabeum* and *R. placenta*, showed a slight decrease in the relative crystallinity (Goodell et al. 2017).

According to previous studies, *G. trabeum* increase enzymatic hydrolysis of lignocellulosic materials, decreasing the crystallinity of the lignocellulose matrix (Howell et al. 2009; Monrroy et al. 2011a; Schilling et al. 2012). It is important to highlight that since both lignin and hemicellulose are amorphous polymers, the total crystallinity of lignocellulose is mainly related to the crystallinity of cellulose. In cellulose, the hydroxyl groups of glucose form hydrogen bonds with oxygen molecules on the same or on a neighboring chain, holding the chains firmly together side by side, forming strong crystalline aggregates called microfibrils (Brett 2000) that are interrupted by amorphous regions (Silveira et al. 2015). Crystalline regions prevent the conversion of cellulose into glucose. In order to establish a suitable degradation of wheat straw by *G. trabeum*, a multiple optimization of TCI responses and weight loss was carried out in order to obtain the lowest value of total crystallinity index and minimize the associated weight loss to prevent the cellulose loss. The optimum solution was 0.24 M NO_3^{-1}, 0.95 mM Fe^{2+} and 0.85 mM Mn^{2+}. Under these culture conditions, it is expected to obtain a TCI value about 0.43 associated to 3.89% of weight loss after 10 days. In the experiment carried out at the optimum solution to corroborate the optimization, a TCI value of 0.41 was obtained after 10 days, associated to 3.15% of weight loss (Table 3). To

evaluate the efficacy of the pretreatment by *G. trabeum*, the enzymatic hydrolysis of the treated sample was carried out using commercial enzymes. Untreated wheat straw reached a maximum of glucose recovery 68.2 mg g^{-1} wheat straw after 72 h enzymatic hydrolysis. A maximum of glucose recovery of 86.0 mg g^{-1} wheat straw was archived in samples treated by *G. trabeum* after 10 days, 26.1% higher than untreated samples. Interestingly, longer incubation times showed lower glucose recovery. The pretreatment for 40 days of incubation showed glucose recovery 11% lower than untreated wheat straw.

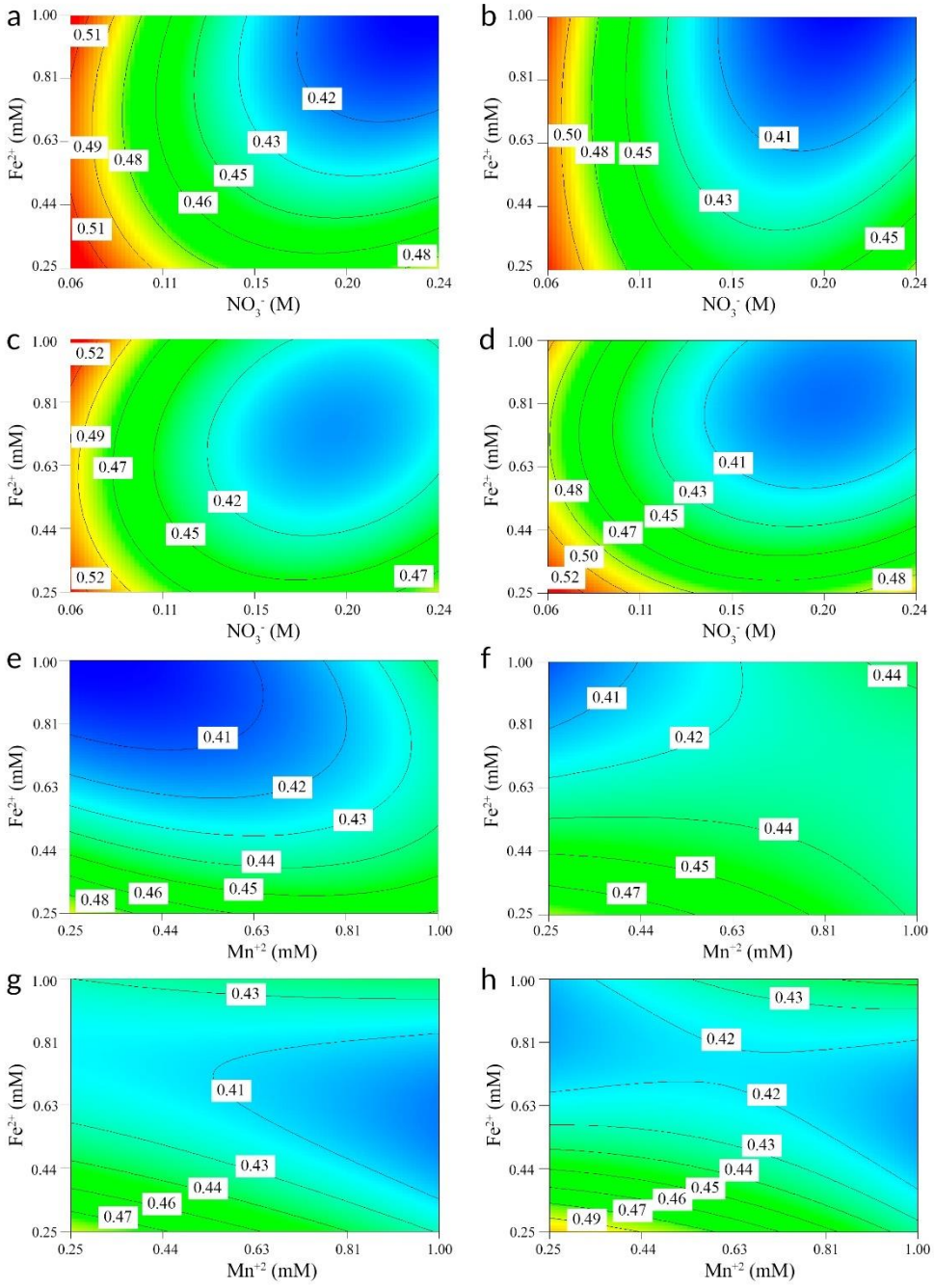

Figure 8 Response surface plots of TCI (A_{1375}/A_{2900}) in fungal-treated wheats straw. Plots (a-d) as a function of NO_3^- (X_1) and Fe^{2+} (X_2) at 0.25 mM Mn^{2+} (X_3) for 10, 20, 30 and 40 days of incubation, respectively. Plots (e-h) as a function of Fe^{2+} (X_2) and Mn^{2+} (X_3) at 0.06 M NO_3^- (X_1) for 10, 20, 30 and 40 days of incubation, respectively.

Table 3 Minimization of TCI (A_{1375}/A_{2900}) and weight loss (%) according to response surface models.

Incubation time	Weight loss (%)		TCI (A_{1375}/A_{2900})		Enzymatic Hydrolysis (mg glucose g^{-1} wheat straw)
	Exp. values	Pred. values	Exp. values	Pred. values	Exp. values
10 days	3.15 (0.98)	3.89	0.41 (0.01)	0.43	86.0 (26.1)
20 days	5.75 (1.32)	5.09	0.41 (0.05)	0.43	74.43 (9.1)
30 days	7.49 (1.56)	7.52	0.41 (0.06)	0.43	67.73 (-0.7)
40 days	11.3 (1.93)	9.96	0.47 (0.03)	0.43	60.77 (-10.9)

Solution 0.24 M NO_3^- (X_1), 0.95 mM Fe^{2+} (X_2) and 0.85 mM Mn^{2+} X_3). Desirability: 0.65 for weight loss, 0.71 for TCI and 0.69 for combined variables. Exp. values correspond to experiment carried out under optimum conditions. *Parenthesis values* in weight loss and TCI represent standard deviation, and parenthesis values in enzymatic hydrolysis column represent the increase (%) compared with untreated wheat straw.

4.3.5 Relationship between evaluated responses

To assess the relationship between the evaluated responses during incubation time, Pearson correlation coefficients were calculated for all pairs of responses from collected data for periods of 10 to 20 days and 30 to 40 days of incubation. Figure 9 shows the scatter plot matrix and calculated Pearson coefficients. Until day 20 (Figure 9A), weight loss showed a positive correlation (r = 0.44) with TCI values. Total catechol concentration in extracts showed a positive correlation (r = 0.48 and 0.49, respectively) with CMCase and Xylanase activities. A negative correlation (r = -0.38) was obtained between Fe^{3+}-reducing activity and TCI. In the period comprised between 30 to 40 days (Fig 6B), weight loss showed a negative correlation (r = -0.56 and -0.39, respectively) with catechol and CMCase, and a positive correlation (r = 0.37) with TCI. This result indicates that fungus decreased wheat straw crystallinity without generating a high weight loss percentage. Catechol showed a positive correlation (r = 0.53) with CMCase and a negative correlation (r = -0.34) with TCI. Fe^{3+}-activity in extracts showed a negative correlation (r = -0.47 and -0.46, respectively)

with CMCase and xylanase activities. The negative correlation between Fe^{3+}-reducing activity and catechol with TCI could indicate a relationship between Fenton reactions and the decrease of wheat straw crystallinity, where the Fe^{3+}-reduction is carried out mainly by phenols compounds until 20 days, and by total catechols in the period of 30 to 40 days. It has been reported that Fe^{3+}-reducing activity is not correlated with total catechols (Aguiar et al. 2013).

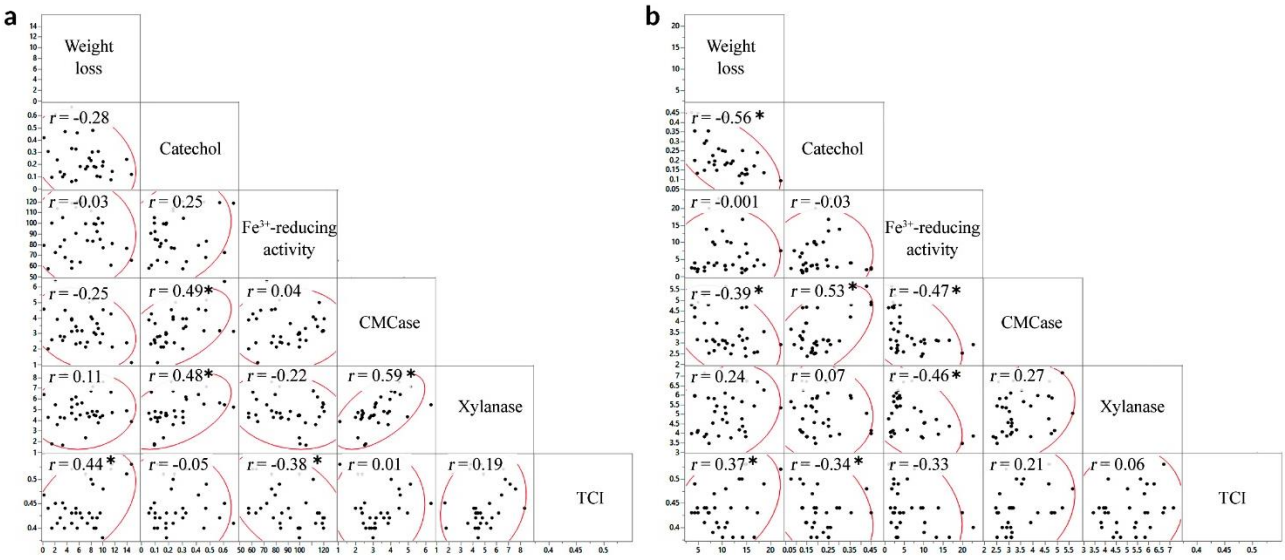

Figure 9 Scatter plot matrix and Pearson coefficients (*r*-value) derived from data correlation between all responses (weight loss, catechol, Fe^{3+}-reducing activity, CMCase, xylanase and TCI) after 10 - 20 days (a) and 30 - 40 days (b) of wheat straw fungal-treatment. * indicates significant correlation coefficient ($p < 0.05$). Showed values correspond to all data collected from all trials.

4.3.7 Scanning electron microscopy

Scanning electron microscopy was carried out to visualize the fungal colonization and potential structural changes in wheat straw after fungal treatment (Figure 7). Figure 7A and 7B show untreated wheat straw lateral and transversal views, which exhibit the intact and inaccessible wheat straw structure. Figure 7C and 7D show wheat straw samples after 30 days of *G. trabeum* treatment, where structural changes are observed in plant cells, which were enlarged and even perforated (Figure 7C). These perforations could allow the infiltration of cellulolytic enzymes, thus facilitating glucose recovery through enzymatic hydrolysis. In Figure 7D fungal mycelia can be observed in cellular lumen of a fungal-treated wheat straw cross-section, which indicates that the fungus colonized wheat straw superficially and intracellularly.

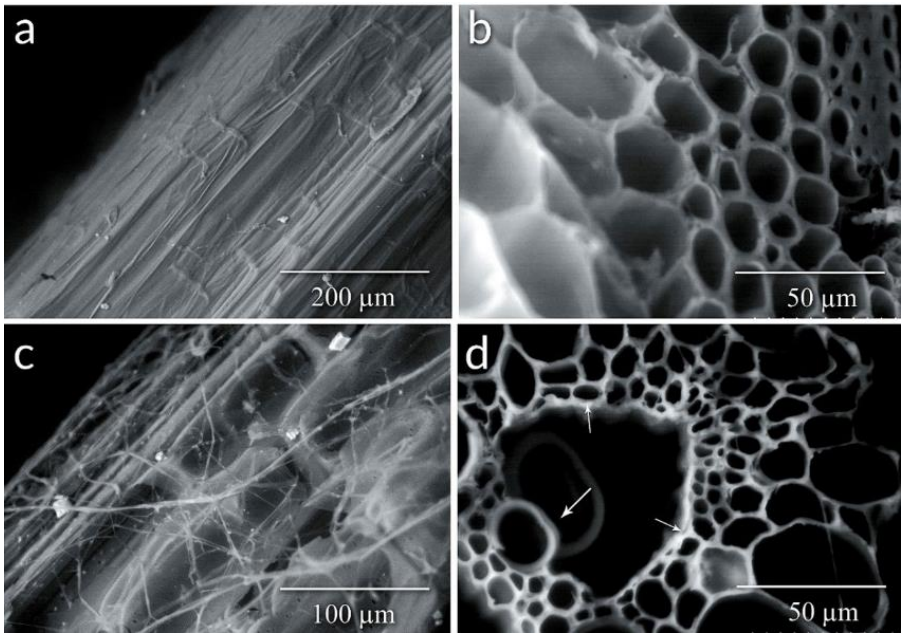

Figure 10 SEM images of colonization wheat straw by *G. trabeum* on wheat straw after 30 days of incubation. **(a)** lateral and **(b)** transversal views of untreated wheat straw, and **(c)** lateral and **(b)** transversal views of fungal-colonized wheat straw. Arrows indicate fungal-mycelia.

This report contributes to the understanding of the effects of metal ions as exogenous Fenton reactants and nitrate on degradative abilities and extracellular activities of the brown-rot

fungus *G. trabeum* for the pretreatment of wheat straw. Moreover, it allowed finding the best combination of these factors for improving the potential of *G. trabeum* for the pretreatment of wheat straw, enhancing its digestibility. The enzymatic hydrolysis assays showed that *G. trabeum* incubation for 10 days generates the highest increase of glucose recovery from wheat straw. These results were used to design the subsequent experiment to evaluate the *G. trabeum* (brown-rot fungus) and *G. lobatum* (white-rot fungus) co-culture effect on the pretreatment of wheat straw.

4.4 Conclusions

Based on the results, we conclude that the addition of exogenous Fe^{2+} and Mn^{2+} accelerated the degradation of wheat straw by *G. trabeum*, which was reflected in an increase of 2-fold weight loss compared with previous results. The above suggests that these ions participate as reactants in the non-enzymatic degradation process via Fenton reaction. In addition, Fe^{2+} promotes weight loss and Fe^{3+}-reducing activity, and decreases total crystallinity index, cellulase and xylanase activity. The Mn^{2+} had a negative effect on weight loss, cellulase, xylanase and Fe^{3+}-reducing activity, and a positive effect on catechol compounds. Whereas NO_3^{-1} had a negative effect on weight loss, xylanase activity and total crystallinity index, and a positive effect on cellulase activity and total catechol compound production. Finally, according to the multiple optimization, the culture of *G. trabeum* in the wheat straw, at 0.24 M NO_3^{-1}, 0.95 mM Fe^{2+} and 0.85 mM Mn^{2+}, promotes the adequate biodegradation of wheat straw resulting in 11.3% weight loss and 0.47 total crystallinity index after 40 days. The enzymatic hydrolysis assay revealed that the pretreatment of wheat straw by *G. trabeum* for 10 days, increased glucose recovery in a 26.1%, compared to untreated wheat straw. After 10 days of incubation, the changes caused by the fungus in wheat straw decreased glucose recovery.

4.5 References

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CHAPTER V

Sequential white-rot and brown-rot fungal pretreatment of wheat straw as a promising alternative for complementary mild treatments

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Sequential white-rot and brown-rot fungal pretreatment of wheat straw as a promising alternative for complementary mild treatments

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Abstract

Single, sequential and co-inoculation of the white-rot fungus *Ganoderma lobatum* and the brown-rot fungus *Gloeophyllum trabeum* were evaluated as an alternative biological pretreatment of wheat straw. The single cultures of *G. lobatum* and *G. trabeum* exhibited preferential degradation of lignin and hemicellulose, respectively. The total crystallinity index decreased in pretreated samples by *G. trabeum* but not by *G. lobatum*. The pretreatment with single cultures of *G. lobatum* or *G. trabeum* increased glucose yields by 43.6% and 26.1%, respectively, compared to untreated straw. Although co-inoculation resulted in higher yields of glucose when compared with single cultures, no synergistic effect between fungi was observed. Otherwise, the sequential inoculation of *G. lobatum* incubated for 10 days followed by the inoculation of *G. trabeum* incubated for 10 days more, showed

a synergic effect on enzymatic hydrolysis. This co-culture showed the highest glucose yield (191.5 mg g^{-1} wheat straw), being 2.8-fold higher than untreated wheat straw.

Keywords: wheat straw, biological pretreatment, white-rot fungus, brown-rot fungus, cellulose hydrolysis.

5.1 Introduction

Nowadays the lignocellulosic biomass derived from agricultural waste has been established as a renewable alternative and socially acceptable raw material to obtain biofuels. Due to the high cellulosic content (35 - 50% of dry weight) in these materials, enzymatic hydrolysis can be used to convert cellulose into glucose that can be subsequently fermented into ethanol (Silveira et al., 2015) or for the production of other chemicals in a biorefinery concept (ref). However, the native state of lignocellulosic biomass is highly recalcitrant to enzymatic hydrolysis, because the cellulosic fraction is sheathed by lignin and hemicellulose which implies a physical barrier that hinders the access of cellulase enzymes during hydrolysis. Besides, cellulose fibers present intrachain and interchain hydrogen bonds forming highly resistant crystalline regions. Therefore, a feasible process for obtaining cellulose-derived products (biofuels or chemicals) from lignocellulosic biomass require a pretreatment step to increase the biodegradability and access to cellulose (Wan and Li, 2010; Mosier et al., 2005). Some microorganisms, especially wood-rotting fungi including white, brown and soft-rot fungi, are capable of degrading plant cell wall polymers through enzymatic and non-enzymatic mechanisms in an environmentally friendly and energy-efficient manner (Schilling et al., 2012; Wan and Li, 2012). In biological pretreatment, white-rot fungi have been used for their ability to degrade selectively lignin (Hermosilla et al., 2017; Saha et al., 2016; Wan and Li, 2010), whereas brown-rot fungi have been used for their ability to cause

extensive and fast degradation and depolymerization of cellulose and hemicellulose, with a minimal assimilation of degradation products (Lee et al., 2008; Machado and Ferraz, 2017; Rasmussen et al., 2010). Currently, the biological pretreatment of lignocellulosic biomass has been focused using single-culture of white-rot fungi, while few reports show the use of brown-rot and soft-rot fungi. However, in nature, the degradation process of lignocellulosic biomass is carried out by the interaction of various wood-rotting fungi. Therefore, the use fungal co-culture that mimics this type of interaction could shorten the pretreatment time or improve its efficiency. So far, the studies about co-culture of wood-rotting fungi in lignocellulosic biomass have been focused on the production of ligninolytic enzyme (Iakovlev and Stenlid, 2000; Kannaiyan et al., 2015) or to facilitate the pulping process through partial degradation of lignin (Chi et al., 2007). On the other hand, most of co-culture studies have used combinations of strains of white-rot fungi in simultaneous inoculation (Iakovlev and Stenlid, 2000; Chi et al., 2007; Kannaiyan et al., 2015), while the effect of interaction of white-rot and brown-rot fungi in co-culture or sequential inoculation of fungi on lignocellulosic biomass pretreatment has been less studied.

In our previous studies, the white-rot fungus *Ganoderma lobatum* CCCT16.03 showed high potential to degrade lignin selectively in wheat straw (50%) associated to low weight loss (18.5%), which was improved by the addition of enzyme inducers (Mn^{2+} and Fe^{2+}) and nitrate as nitrogen source (Hermosilla et al., 2017). Additionally, the potential of the brown-rot fungus *Gloeophyllum trabeum* had been reported in the improvement of organosolv pretreatment and enzymatic hydrolysis yield in wood chips (Schilling et al., 2009; Monrroy et al., 2011).

Therefore, the aim of this study was to evaluate the effect of single cultures of *G. lobatum* (white-rot fungus) and *G. trabeum* (brown-rot fungus) and co-cultures using sequential and co-inoculation of these fungi for the pretreatment of wheat straw.

5.2 Materials and methods

5.2.1 Fungal strains

The fungal strains *Ganoderma lobatum* CCCT16.03 and *Gloeophyllum trabeum* CCCT16.04 were collected from a temperate forest of Antuco, located in the Bio-Bio Region, Chile (latitude, 38° 39'S; longitude, 72° 35'W). The fungal isolates were obtained by placing small fragments of the fungal fruiting bodies on glucose malt-extract agar plates (per liter: 15 g of agar, 3.5 g of malt extract, and 10 g of glucose) and kept at 25°C. The pure cultures were kept in slant culture tubes with malt-extract agar medium at 4 °C and periodically sub-cultured. The strains were molecularly identified and deposited in Colección Chilena de Cultivos Tipo of Scientific and Technological Bioresource Nucleus at Universidad de La Frontera, Temuco, Chile.

5.2.2 Intraspecific and interspecific mycelial interactions under co-culture

The intraspecific and interspecific interactions of *G. lobatum* and *G. trabeum* were evaluated in solid medium malt extract agar (per liter: 15 g of agar, 3.5 g of malt extract) with pH adjusted to 4.5. The plates were inoculated with agar-mycelia plugs (6 mm diameter) cut from the actively growing margin of fungal cultures on malt extract agar plates. To evaluate interspecific interactions, two agar plugs of *G. lobatum* and *G. trabeum,* respectively, were placed on opposite sides (40 mm apart) of on each assay plate. In addition, self-pairings of each species were included to evaluate intraspecific interactions. Three replicate plates were carried out for each fungal pairing studied to check reproducibility of the interaction. The assay plates were incubated in the dark at 25 °C ± 1°C for 3 weeks. The interactions between opposing fungal colonies were evaluated visually at least once in 3-4 days and classified according to Boddy (2000).

5.2.3 Fungal inoculum preparation

The inoculum preparation was carried out in Erlenmeyer flasks (500 mL) containing 100 mL of modified Kirk medium containing per liter 10 g of glucose, 2 g of peptone, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 84 mg of $MnSO_4•H_2O$, 2 mg of thiamine, and 10 mL of mineral salts (per liter: 0.1 g of $FeSO_4•7H_2O$, 0.1 g of $CoCl_2$, 0.1 g of $ZnSO_4•7H_2O$, 0.1 g of $CuSO_4$, 10 of mg $AlK(SO_4)_2•12H_2O$, 10 mg of H_3BO_3 and 10 mg of $Na_2MoO_4•2H_2O$). The flasks with medium were autoclaved at 121°C for 15 min. Each flask was inoculated with five agar disks (6-mm diameter) of active mycelia of *G. lobatum* or *G. trabeum* from a five-day-old culture on malt-extract agar cultivated in Petri dishes and incubated at 30°C for 10 days. Then, each fungal culture was homogenized in a sterilized blender for 1 min and used as a source for the inoculum (blended fungal mycelia) of biological pretreatments, as described below (Hermosilla et al., 2017).

5.2.4 Biological pretreatment assays

Wheat straw was provided by Maquehue experimental field of Universidad de La Frontera, Temuco, Chile. The pretreatments were carried out in 250-mL Erlenmeyer flasks containing 5 g (dry material) of chopped wheat straw (particle size of ~50 mm long) and moistened with 25 mL of water. The wheat straw was supplemented with NO^{3-}, Fe^{2+}, and Mn^{2+} at different concentrations established in previous studies from our laboratory, which depended on the fungal inoculum that was evaluated (Hermosilla et al., 2017 and unpublished data) (Table 1). The flasks were stoppered with hydrophobic cotton and gauze and autoclaved for 25 min at 121 °C. Sterilized wheat straw was inoculated with 0.5 mL of blended fungal mycelia (10 mg of dry weight fungal mycelia) and kept in the dark at 25 °C ± 1 °C without shaking for 10 to 40 days. In parallel, uninoculated flasks were incubated at the same conditions (untreated wheat straw), as negative controls. All pretreatment assays were carried out in triplicate.

Table 1 Conditions of fungal pretreatments of wheat straw.

Pretreatment	Conditions
G. lobatum	0.18 M $NaNO_3$; 0.73 mM $FeSO_4$; 1 mM $MnSO_4$*
G. trabeum	0.24 M $NaNO_3$; 0.95 mM $FeSO_4$; 0.85 mM $MnSO_4$ **
G. lobatum plus *G. trabeum*	0.24 M $NaNO_3$; 0.74 mM $FeSO_4$; 1 mM $MnSO_4$***

According to Hermosilla et al. (2017) (*), Chapter 3 (**) and Appendix C (***).

The effect of co-culture of fungal strains for the pretreatment of wheat straw was evaluated considering the results obtained from the assays of single cultures. Firstly, both *G. lobatum* (white-rot) and *G. trabeum* (brown-rot) were co-inoculated (CI) in a same flask and incubated for 20 days. Afterwards, sequential inoculation of the fungi was also evaluated. In a first strategy (S1), *G. lobatum* was inoculated in the wheat straw and, after 10 days, *G. trabeum* was added to the flasks that were incubated for another 10 days. The second strategy (S2) was similar to S1, except that *G. trabeum* was inoculated after 20 days. The third strategy (S3) consisted in inoculating *G. trabeum* firstly and, after 10 days, *G. lobatum* was added to the flasks that were incubated for another 20 days. After pretreatments, wheat straw was washed with tap water to remove fungal mycelia and used for characterization of lignocellulosic components and enzymatic hydrolysis assays.

Additional samples of each pretreatment (single- and co-cultures) were carried out in parallel and triplicate to determine the weight loss produced by fungi. These samples were also washed with tap water to remove the fungal mycelia and dried at 105°C until constant weight. This value was used to determine the difference in percentage between the initial weight and the final weight of wheat straw after each fungal pretreatment.

5.2.5 Chemical characterization

The composition of cellulose, hemicellulose and lignin in wheat straw was determined according to described procedures (Sluiter et al., 2011). Briefly, the pretreated and untreated wheat straw samples were extracted with water and ethanol to remove wax and resins (extractives content) by Soxhlet-extraction. Extractive-free samples (300 mg) were hydrolyzed for 1 h in 72% of H_2SO_4 at 30°C followed by dilution of H_2SO_4 to 4% by distilled water addition and autoclaved at 121°C for 1 hour. Thereafter, the acid-insoluble lignin (solid fraction) was filtered in a Gooch crucible and dried at 105°C until constant weight and expressed as percentage of the original sample. Liquid fraction from acid hydrolysis was neutralized with $CaCO_3$, filtered through a 0.20 μm filter and stored at -20°C until sugar analysis by high-performance liquid chromatography (HPLC). The percentage of cellulose, hemicellulose and lignin degradation mediated by fungus was calculated from the difference between its initial content in untreated wheat straw and the content in wheat straw after fungal pretreatment adjusted by weight loss according to the equation (1):

$$\% \text{ Component degradation} = [(1 - W(C_f))/(W_o(C_o))] \times 100 \quad (1)$$

where C_0 is the component content (%) in untreated wheat straw; C_f is the component content (%) in wheat straw after fungal-treatment, W is dry weight of wheat straw (g) after fungal-treatment and Wo is the dry weight of wheat straw (g) before fungal-treatment.

5.2.6 Enzymatic hydrolysis

Enzymatic hydrolysis assays were carried out using samples of pretreated wheat straw and commercial enzymes Celluclast 1.5L (a mixture of cellulases produced by *Trichoderma reesei*) and β-glucosidase produced by *Aspergilus niger* (Sigma Aldrich, St. Louis, MO).

The tests were carried out in Erlenmeyer flask containing 2.5 g (5% w/w) of wheat straw and an enzyme solution prepared in 50 mM sodium citrate (pH 4.8) with enzyme loading of 15 FPU g^{-1} biomass and 45 IU β-glucosidase per g biomass, and incubated at 50 °C, 200 rpm in a rotary shaker for 72 h. In all trials, sodium azide (0.1% w/v) was also added to prevent microbial contamination. Samples were withdrawn at 1, 2, 4, 6, 24, 48 and 72 h and the amount sugars released from biomass were measured by HPLC. Untreated wheat straw was used as control. All assays were carried out in triplicate. The cellulose to glucose conversion yield (%) was calculated according to the following equation (Silva et al., 2016):

$$\text{Glucose yield} = \frac{(C_{glucose} - C_{glucose_0})}{1.111 \left(\frac{W_t}{V_{h0}}\right) F_{ins0}\, F_{glucan}} \times 100 \qquad (2)$$

Where $C_{glucose}$ is the glucose concentration in the hydrolysates (g/L); $C_{glucose_0}$ is the initial glucose concentration in the hydrolysis assay; W_t is the total weight of the hydrolysis assay (g); V_{h0} is the initial volume of liquid (L); F_{ins0} is the initial mass fraction of insoluble solids in the total hydrolysis assay; F_{glucan} is the initial mass fraction of glucan in insoluble solids. V_{h0} is the volume that corresponded to the initial mass (g) of liquid added to the hydrolysis assay ($W_t - W_{ins0}$).

5.2.7 HPLC analysis

Sugar samples from chemical characterization and enzymatic hydrolysis were derivatizated with 1-phenyl-3-methyl-5-pyrazolone (PMP) before HPLC analysis according to Li et al. (2013). Briefly, a reaction mixture containing 100 µL of sugar sample, 100 µL of 0.3 M NaOH and 100 µL of 0.5 M PMP (in methanol) were added in a 2-mL centrifuge tube. The mixture was incubated at 70°C for 30 min, then cooled to room temperature and neutralized with 100 µL of 0.3 M HCl. The solution was mixed with 200 µL of chloroform, vortexed

and centrifuged, the organic phase was carefully discarded to remove the reagents excess. This extraction process was repeated three times. Finally, the aqueous phase was filtered through a 0.22 µm membrane before HPLC analysis. The analysis of PMP derivatives of saccharides was carried out in a HPLC Merk-Hitachi (Elite LaChrom) using a Purospher® STAR RP-18 Endcapped (250 mm x 5 µm, Darmstadt, Germany) at 30°C. The mobile phase was 40 mM phosphate buffer (pH 8.0)/acetonitrile in proportion 79:21 (v/v) at 0.5 mL min^{-1}. The derivatized compounds were detected at 248 nm.

5.2.8 Fourier-transform-infrared spectroscopy (FTIR) analysis

The solid samples dried in an oven at 105°C for 24 h were milled into a fine powder and homogenized before FTIR analysis. The infrared spectra were collected at room temperature in the 4000-600 cm^{-1} range with an average of 32 scans per sample using a Cary-630 spectrometer equipped with an accessory of attenuated total reflection (ATR). Spectra were obtained using Agilent Resolution Pro software (Agilent Technologies Inc., Santa Clara, CA). The total crystallinity index (TCI) was estimated by the ratio of absorbance areas A_{1375}/A_{2900} from FTIR spectra (Nelson and O'Connor, 1964).

5.2.9 Statistical analysis

Multiple linear regression analysis was carried out in order to determine interactions between glucose recovery from enzymatic hydrolysis with total crystallinity index and lignin degradation using JMP 11.0 software with a statistical significance the $p < 0.05$.

5.3 Results and Discussion

5.3.1 Mycelial interaction between fungi under co-culture

The intraspecific and interspecific interactions between the white-rot and brown-rot fungi were evaluated on petri dishes to relate it with the behavior of the fungi under co-culture conditions. In the assay plates inoculated with the same fungus (*G. lobatum* vs *G. lobatum* and *G. trabeum* vs *G. trabeum*), the mycelium of both opposing fungal colonies merged in the central zone of plate at 7 days and 21 days of incubation for *G. lobatum* and *G. trabeum*, respectively (Figure 1 a-f). No hyphae interference or non-inhibition of growth was observed between both colonies of the same fungus indicates a lack intraspecific competition. However, in the co-culture of the two-fungal species (*G. lobatum* vs *G. trabeum*), a clear interaction of competition was observed. In general, the fungal competition in a virgin or sterile substrate have two steps: a primary resource capture (where the fungi spread onto the uncolonized substrate) and a secondary resource capture (where fungi combat to extend the colonization to areas already colonized by other fungus) (Boddy, 2000). The fungus *G. lobatum* colonized the malt extract medium quicker than *G. trabeum*, impeding its growth by hyphal interference at 7 days of incubation. A barrage with high mycelial density was also observed when both fungal mycelia make contact at 7 days incubation, which was accompanied by production of a brown pigment in the interaction zone (Figure 1g). The brown pigmentation of barrages has been attributed to production of melanin compounds that could protect hyphal structures from hydrolytic enzymes and antagonistic organisms (Savoie et al., 2001), which can be produced inter and intracellularly (Boddy, 2000). At 14 days of incubation, zones of density reduction and lysis of mycelium in the colony of *G. lobatum*, and an increase in the production of the brown pigment were observed close to mycelial barrage (Figure 1h). At 21 days, the zone of mycelium lysis increased significantly and development of low density mycelia of *G. trabeum* was observed in the lysis zone,

suggesting a partial replacement of already colonized zones by *G. lobatum.* Owens et al. (1994) showed that *G. trabeum* and other strains of brown-rot fungi were highly combative when paired against several strains of white-rot fungi (*Bjerkandera adusta, lrpex lacteus, Phanerochaete chrysosporium, Phlebia brevispora, Schizophyllum commune* and *Trametes versicolor*) in co-culture on malt extract agar. Contrary, Giles et al. (2011) reported no combative interactions between the brown-rot fungus *P. placenta* and the white-rot fungus *C. subvermispora.* Our results suggest that *G. trabeum* have the potential to grow in wheat straw already colonized by a white-rot fungus, making feasible a sequential inoculation of *G. lobatum* followed by *G. trabeum*. By contrast, *G. lobatum* not showed combative potential against *G. trabeum*, therefore it would expect a poor growth of *G. lobatum* in wheat straw already colonized by *G. trabeum*. In case of co-inoculation we can expect that both fungi will have a lower growth when compared to its corresponding single-culture, which could be reflected in their degradation potentials.

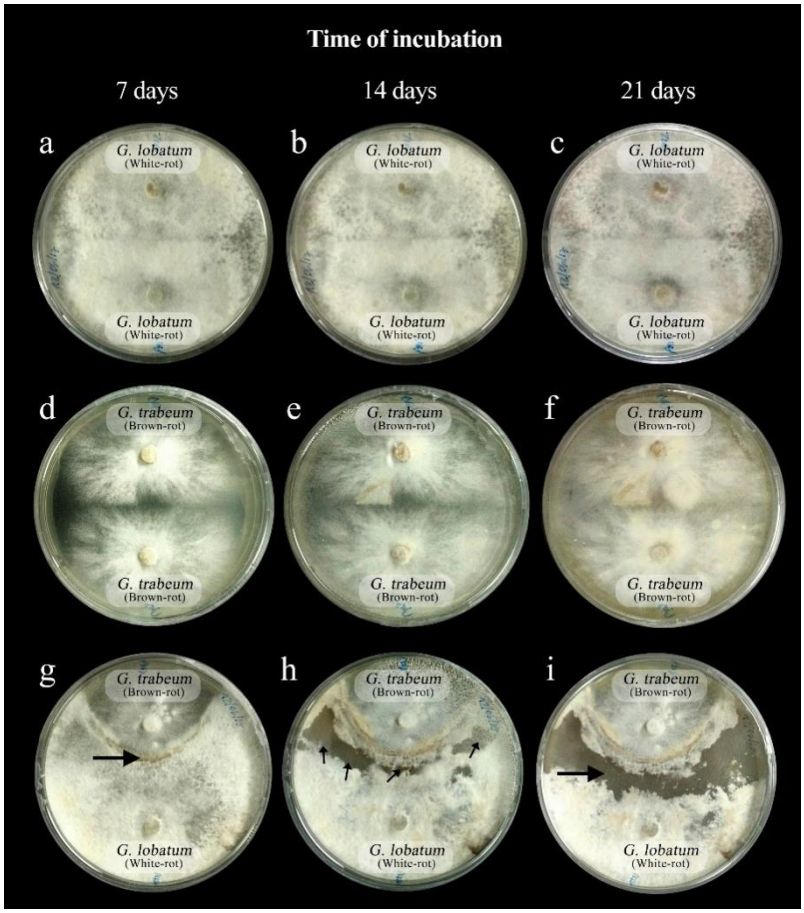

Figure 1 Intraspecific **(a-f)** and interspecific **(g-i)** interactions of *Ganoderma lobatum* and *Gloeophyllum trabeum* on malt extract agar medium incubated for 7, 14 and 21 days at 30°C. *Arrows* indicate the hypha interference between fungi (at 7 days) and mycelium lysis zone (at 14 days) preceding the partial replacement of *G. lobatum* by *G. trabeum* (at 21 days).

5.3.2 Effect of single inoculation of fungi on weight loss and wheat straw composition

The weight loss of wheat straw was monitored to determine the amount of lignocellulosic components of wheat straw that were degraded by the microorganisms. The weight loss of wheat straw was higher after the incubation with the white-rot fungus *G. lobatum* than with *G. trabeum*, at all incubation times (Table 2). Weight losses of wheat straw were 6.3% and 3.2% after 10 days of pretreatment with *G. lobatum* and *G. trabeum*, respectively. A clear increase of weight loss was observed after 20 days of wheat straw pretreatment with *G. lobatum*, doubling the percentage of weight loss between 20 days (8.4%) and 30 days

(15.7%), reaching 21% after 40 days, while *G. trabeum* had lower influence on weight loss, with a maximum of 11.3% after 40 days.

Wheat straw samples after pretreatments were characterized to determine the composition changes on biomass and estimate the degradation or loss of the lignocellulosic components. The untreated wheat straw was composed by 44.3% cellulose, 21.2% hemicellulose (15.8% xylan, 2.6% arabinan, 1.5% mannan and 1.3% galactan), 21.1% lignin, 6.6% extractives and 1.1% ash. The content and degradation of cellulose, hemicellulose and lignin in wheat straw after pretreatments by single culture are shown in Table 2. The single culture of the white-rot fungus *G. lobatum* promoted a selective and faster degradation of lignin than cellulose and hemicellulose, which resulted in 50.3% lignin degradation after 40 days. After 10 days, low degradation of cellulose and hemicellulose was detected, corresponding to 3.8% and 2.8%, respectively. There was no significant increase in cellulose degradation between 10 and 20 days (4.4%), suggesting that until 20 days, the fungus only consumed the more accessible parts of cellulose (Table 2). Additionally, the degradation of hemicellulose (9.22%) observed at 20 days indicates that *G. lobatum* preferentially consumes xylose as sugar source in this period, which is a feature of selective lignin-degrading fungi (Messner et al., 2003). After 20 days, the degradation of cellulose increased considerably, reaching 14.7% and 21.4%, after 30 and 40 days, respectively, which could be the result of lignin and hemicellulose degradation that increases the cellulose access to cellulolytic enzymes secreted by the fungus.

Table 2 Chemical composition and degradation of wheat straw before and after pretreatment by single fungal cultures.

Pretreatment	Incubation time (days)	Content (%)			Degradation (%)			
		Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin	Weight loss
Untreated wheat straw		44.26 ± 0.76	21.23 ± 0.64	21.09 ± 0.05	nd	nd	nd	nd
White-rot fungus								
G. lobatum	10	45.15 ± 2.89	22.02 ± 0.21	19.00 ± 0.09	3.80 ± 0.09	2.83 ± 0.03	15.60 ± 0.07	6.32 ± 1.03
G. lobatum	20	46.50 ± 1.16	21.05 ± 1.36	15.73 ± 0.07	4.44 ± 0.28	9.22 ± 0.60	31.70 ± 0.14	8.44 ± 1.20
G. lobatum	30	44.97 ± 2.97	21.98 ± 0.85	14.70 ± 0.09	14.32 ± 0.95	12.69 ± 0.49	41.22 ± 0.25	15.67 ± 1.95
G. lobatum	40	44.04 ± 0.17	22.03 ± 0.38	13.27 ± 0.82	21.43 ± 0.08	18.07 ± 0.31	50.32 ± 3.11	21.04 ± 1.23
Brown-rot fungus								
G. trabeum	10	43.87 ± 1.89	16.13 ± 1.02	22.39 ± 0.54	4.01 ± 0.17	26.42 ± 1.67	*	3.15 ± 0.98
G. trabeum	20	43.27 ± 0.10	16.21 ± 0.94	23.50 ± 0.94	7.86 ± 0.02	28.03 ± 1.63	*	5.75 ± 1.32
G. trabeum	30	42.87 ± 0.95	15.92 ± 0.33	24.30 ± 0.31	10.40 ± 0.23	30.64 ± 0.64	*	7.50 ± 1.56
G. trabeum	40	43.27 ± 0.96	14.93 ± 0.42	25.43 ± 0.24	13.29 ± 0.29	37.63 ± 1.06	*	11.31 ± 1.93

All values are expressed in terms of percentages of dry weights, nd = not determined. Data shown are expressed as means ± standard deviation (n=3). *lignin recoveries were between 102-106% of the original content. Considering the sensitivity of methods used, it can be considered that no lignin degradation occurred.

The brown-rot fungus *G. trabeum* showed a fast degradation of hemicellulose, observed in the first 10 days (26.4%), which increased slowly until 40 days. The single culture of *G. trabeum* showed a preferential degradation of hemicellulose than cellulose, reaching 37.6% and 13.2% after 40 days, respectively, with no quantifiable lignin degradation. Since the enzymes involved in cellulose and hemicellulose degradation are too large to penetrate into the lignocellulosic matrix of the intact biomass, a non-enzymatic oxidative mechanism of Fenton reactions based on hydroxyl radical production in brown-rot decay have been proposed by several authors (Aguiar and Ferraz, 2012; Arantes et al., 2012; Schilling et al., 2009; Shimokawa et al., 2004), which could explain the higher hemicellulose degradation in the first 10 days compared to *G. lobatum*. These results are consistent with a typical temporal pattern of degradation of lignocellulosic components in lignocellulosic biomass by brown-rot fungi showed by Arantes et al. (2012), where hemicellulose is degraded before cellulose, and lignin is not significantly degraded.

5.3.3 Effect of fungal co-cultures on weight loss and wheat straw composition

The effect of co-culture of fungal strains for the pretreatment of wheat straw was evaluated using co-inoculation (CI) and three strategies of sequential inoculation of fungi (S1, S2 and S3). All pretreatments by fungal co-culture resulted in lower weight losses than pretreatments with the single cultures (Table 3), indicating a decrease in growth and degradation potential of the fungi as expected from the results of mycelial interactions in petri dishes. Both CI and S2 pretreatment resulted in the highest weight loss of 15.5 and 15.5%, respectively, while S1 resulted in the lowest weight loss (7.6%). Arora (1995) also reported that the co-inoculation of the white-rot and brown-rot fungi, *Daedalea flavida* and *Polyporus palustris*, decreased weight loss in wheat straw compared to the fungal single cultivations. In the pretreatments by fungal co-cultures, S2 resulted in the highest degradation of lignin (33.8%). Although, in S2, *G. lobatum* was incubated by a

period of 30 days and only the last 10 days in co-culture with *G. trabeum*, the lignin degradation decreased 18.0% compared to the single inoculation of *G. lobatum* for 30 days (Tables 2 and 3) suggesting that the combative behavior of *G. trabeum* observed in petri dishes also takes place in when both fungi are co-cultivated in wheat straw. This behavior was also observed in S1 and CI pretreatment. By contrast, in S3 pretreatment, which corresponded to 10 days of *G. trabeum* growth, followed by the sequential inoculation of *G. lobatum* and incubated for 20 days more, resulted in a similar degradation of lignin when compared to the single *G. lobatum* culture for 20 days. Therefore, the subsequent or simultaneous inoculation with *G. trabeum* produced a decrease in lignin degradation by *G. lobatum*. According to the assay of mycelial interactions between fungi observed in petri dishes, *G. trabeum* could produce lysis and partial replacement of *G. lobatum* also in wheat straw, consequently leading to a decrease in the lignin degradation by *G. lobatum*. Arora (1995) showed that some strains of brown-rot fungi decreased the lignin degradation produced by white-rot fungi during simultaneous co-culture. This behavior was also reported by Parani and Eyini (2012) in the evaluation of the biodegradation of coffee pulp by co-inoculation of white-rot and brown-rot fungi. In relation to cellulose degradation in pretreated wheat straw by fungal co-cultures; CI, S1, S2 and S3 pretreatments showed degradation levels of 16.8, 9.2, 18.2 and 31.4%, respectively. In general, a synergistic effect between fungi was observed in the level of cellulose degradation when correlated to pretreatments by single fungal cultures, except in S1 pretreatment which showed an additive effect on cellulose degradation between fungi. This synergistic effect can be attributed to an increase in the availability of cellulose caused by each fungus. The hemicellulose degradation in wheat straw pretreated by fungal co-culture was lower than pretreatment by the single culture of *G. trabeum*, except in S3 pretreatment. In S1 and S2 pretreatment, *G. trabeum* was

inoculated after 10 and 20 days of growth of *G. lobatum*, respectively, therefore the efficiency of colonization of *G. trabeum* was lower than its single culture. As *G. trabeum* was more effective than *G. lobatum* to degrade hemicellulose in single culture, the lesser growth of *G. trabeum* in S1 and S2 pretreatment reflected lower hemicellulose degradation.

Table 3 Chemical composition and degradation of wheat straw before and after pretreatment by fungal co-cultures.

Pretreatment	Incubation time (days)	Content (%)			Degradation (%)			
		Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin	Weight loss
Wheat Straw		44.26 ± 0.76	21.23 ± 0.64	21.09 ± 0.05	Nd	nd	nd	nd
Co-inoculation								
G. lobatum plus G. *trabeum* (CI)	20	43.60 ± 0.92	18.94 ± 0.83	18.67 ± 1.83	16.78 ± 0.35	24.61 ± 1.08	25.21 2.47	15.52 ± 1.86
Sequential inoculation								
G. lobatum followed by *G. trabeum* (S1)	20	43.48 ± 0.82	16.85 ± 0.21	17.03 ± 0.05	9.24 ± 0.17	26.67 ± 0.33	25.39 ± 0.07	7.61 ± 1.05
G. lobatum followed by *G. trabeum* (S2)	30	42.88 ± 0.94	20.57 ± 0.22	16.53 ± 0.04	18.17 ± 0.40	18.17 ± 0.19	33.79 ± 0.08	15.53 ± 1.56
G. trabeum followed by *G. lobatum* (S3)	30	33.83 ± 1.72	16.73 ± 0.53	21.22 ± 0.52	31.36 ± 1.59	29.25 ± 0.93	9.62 ± 0.24	10.19 ± 1.32

Inoculation days: both at 0-day (CI); 0 and 10 days (S1); 0 and 20 days (S2); 0 and 10 days (S3), respectively.

All values are expressed in terms of percentages of dry weights, nd = not determined. Data shown are expressed as means ± standard deviation (n=3).

5.3.4 Fungal pretreatments effects on enzymatic hydrolysis

The enzymatic hydrolysis using a preparation of the commercial cellulolytic enzymes (Celluclast 1.5L and β-glucosidase) is a method widely accepted to evaluate increase in biodegradability of pretreated lignocellulosic biomass for biofuel production (Chandra et al., 2008; Machado and Ferraz, 2017; Saha et al., 2016). These enzymes release monomeric sugars from cellulose (glucose) mainly and in a lesser extent from hemicellulose contained in the pretreated and untreated lignocellulosic biomass. The enzymatic hydrolysis of untreated wheat straw reached a maximum of glucose recovery of 68.2 mg g^{-1} wheat straw with a 13.7% conversion yield of cellulose to glucose. All fungal pretreatments increased significantly the amount of glucose released after enzymatic hydrolysis from wheat straw, except when *G. trabeum* was incubated for 40 days, which decreased sugar recovery by 10.9%. The pretreatment by single culture of *G. lobatum* was more effective than *G. trabeum*. The highest concentration of glucose after enzymatic hydrolysis was observed in pretreated wheat straw by single cultures of *G. lobatum* (97.4 mg g^{-1} of wheat straw) and *G. trabeum* (86.0 mg g^{-1} of wheat straw) were obtained after 20 and 10 days of incubation, increasing by 42.9% and 26.0% compared to untreated wheat straw, respectively (Figure 2). The concentration of released glucose decreased significantly for wheat straw samples pretreated for incubation times superior to that. Based on these results, four methods of co-culture were carried out to evaluate potential additive and synergistic effects between the fungi, including co-inoculation (CI) and sequential (S1, S2 and S3) inoculation of fungi. Samples pretreated with CI co-culture for 20 days reached a maximum glucose concentration of 113.1 mg g^{-1} of wheat straw after enzymatic hydrolysis and 23.3% conversion yield. A synergistic effect between fungi on pretreatment was observed when *G. lobatum* (white-rot) was inoculated first followed by *G. trabeum* (brown-rot), in S1 and S2 co-cultures, resulting in higher glucose

concentration during enzymatic hydrolysis of samples pretreated at those conditions. Glucose concentration obtained after enzymatic hydrolysis of samples obtained by S1 pretreatment (191.9 mg g^{-1} of wheat straw) was slightly higher than by S2 pretreatment (181.0 mg g^{-1} of wheat straw) and, moreover, S1 pretreatment takes 20 days compared with 30 days of S2. This compares well with the pretreatments by single fungal cultures, indicating that a longer period of fungal exposure not necessarily involves an increase in glucose recovery during enzymatic hydrolysis of pretreated samples. The S1 pretreatment increased the glucose recovery in enzymatic hydrolysis by 2.8-fold compared to untreated wheat straw and achieved 39.7% conversion of cellulose to glucose (Figure 2). For S3 pretreated samples, where *G. trabeum* was inoculated first followed by *G. lobatum*, a maximum of glucose concentration of 96.6 mg g^{-1} wheat straw was obtained, with 25.7% of conversion yield. Overall, the pretreatment of lignocellulosic biomass by single fungal culture were less effective than the co-cultivation conditions evaluated, resulting in samples that were less prone for enzymatic hydrolysis. The conversion yield obtained after S1 pretreatment was higher than the yields presented in other studies of biological pretreatment of wheat straw with similar or longer fungal exposure time. In this sense, the pretreatment using *Trametes versicolor* C6915 for 112 days resulted in a 28.6% of cellulose to glucose conversion after enzymatic hydrolysis of pretreated samples (Zhang et al., 2016). Another study, using the fungus *Ceriporiopsis subvermispora* for 18 days of pretreatment reached 30% glucose yield (Wan and Li, 2010). On the other hand, the hydrolysis of wood samples pretreated by the application of white-rot and brown-rot fungi in two stages for 60 days of fungal exposure resulted in a maximum of sugar concentration of 119 mg g^{-1} *L. tulipifera* wood chips (Giles et al., 2011).

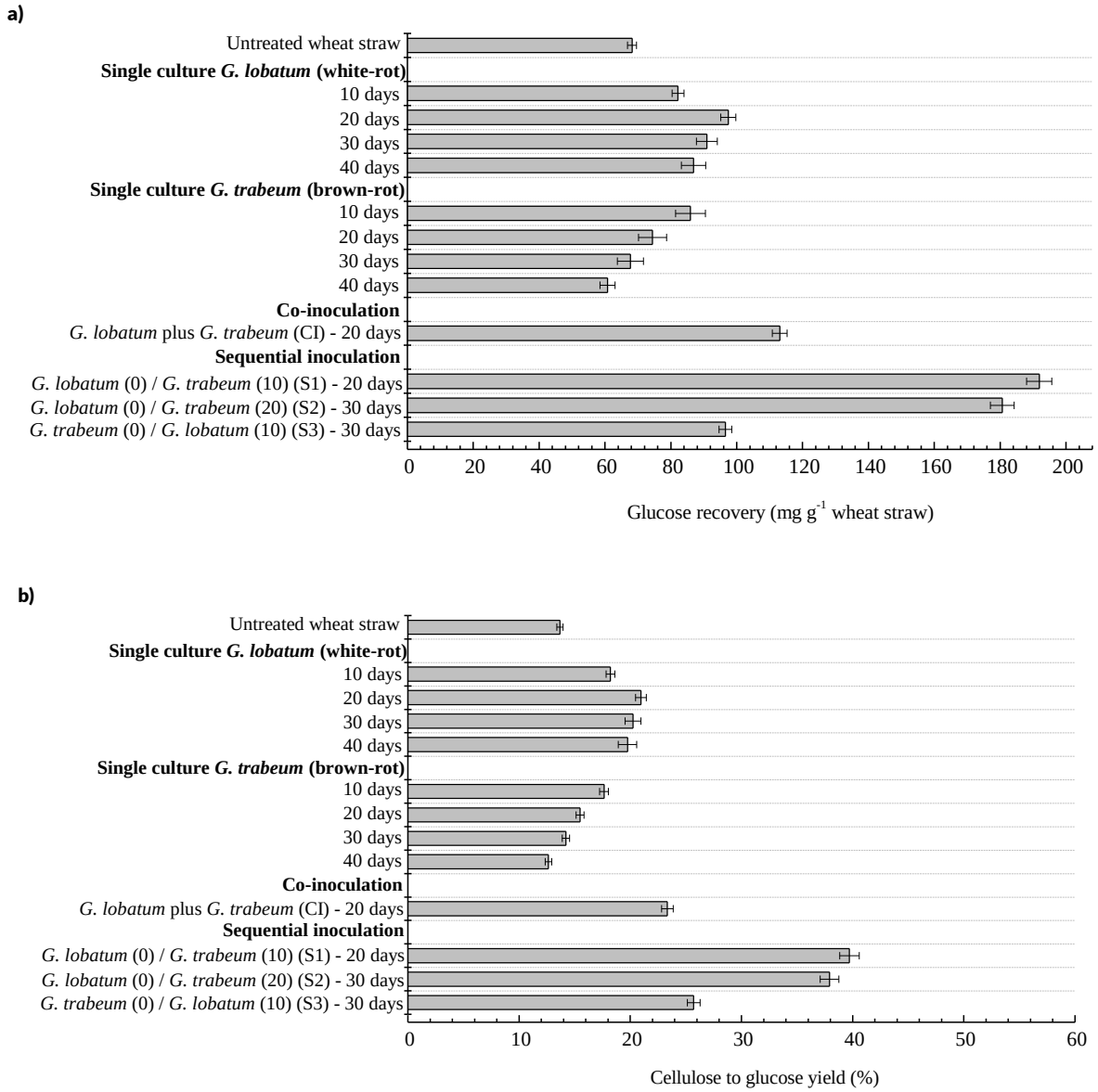

Figure 2 Glucose recovery (mg g^{-1} wheat straw) (a) and cellulose to glucose yield (%) (b) after 72 h of enzymatic hydrolysis of untreated and fungal-pretreated wheat straw. In sequential inoculation, number in parenthesis next to fungus name indicate inoculation day of each fungus. Data shown are expressed as means ± standard deviation (n=3).

Recent studies have revealed that the combination of physicochemical with biological pretreatments are more effective than the pretreatment methods performed alone. It allows the decrease the fungal exposure times or severity conditions of biological and physicochemical pretreatments, respectively. Wheat straw pretreated by the white-rot

fungus *I. lacteus* (21 days of incubation) combined with mild alkali pretreatment (NaOH (5% w/v), at 50°C and 165 rpm for 1 h) resulted in a 62% glucose yield (Salvachúa et al., 2013). The mild acid pretreatment (0.25% H_2SO_4) combined with biological pretreatment by a white rot fungus *Echinodontium taxodii* (10 days of fungal exposure) increased 2.11-fold times the enzymatic hydrolysis of *Eichhornia crassipes* (Ma et al., 2010). Similar to what occurs in the combination of biological with physicochemical pretreatments, in this study, the co-culture of white-rot and brown-rot fungi increased significantly enzymatic hydrolysis of wheat straw compared to single culture at moderately shorter period of fungal exposure. On the other hand, since the S1 pretreatment alone reached 40% of conversion, it represents a good alternative as a pre-pretreatment step, as its combination with any chemical or thermal pretreatment would significantly improve the effectiveness or allow a decrease in the severity conditions of a physicochemical pretreatment.

5.3.5 ATR-FTIR analysis and Total Crystallinity Index

The ATR-FTIR spectroscopy was used to identify chemical differences in samples of wheat straw before and after pretreatment. It is important to emphasize that the pretreatment of wheat straw by fungi only generates changes in intensity of spectrum bands associated to functional groups of the cell wall polymers, does not generate shift of bands, because fungus degrades partially the cell wall polymers. Therefore, it is difficult to discern differences between these spectra. However, the subtraction of normalized ATR-FTIR spectra allows to identify the differences between spectra of untreated and pretreated wheat straw. The Figure 3 shows the spectral subtractions of single and co-culture pretreatments that resulted in the best enzymatic hydrolysis yields, where positive bands indicate a higher relative content of functional group in the pretreated sample, while negative bands indicate a high content functional group in the untreated wheat straw. A clear reduction of band at 3348 cm^{-1} (associated to OH stretching) was appreciated in all

pretreated samples, especially in pretreatment by *G. trabeum*. Some authors suggest that the reduction in intensity of bands at 3348 cm^{-1} indicates that intramolecular hydrogen bonds of cellulose were disrupted, which could enhance accessibility of cellulose to hydrolytic enzymes (Kumar et al., 2009; Poletto et al., 2014). The band at 2900 cm^{-1} associated to CH stretching decreased in fungal pretreated wheat straw, with the strongest change in the pretreatment with *G. trabeum* at 10 days, indicating rupture of methyl and methylene portions of cellulose were ruptured (Abraham et al., 2015). The band position at 1720 cm^{-1} attributed to carbonyl C=O stretching unconjugated ketones showed higher intensity in all pretreatments. Pretreatment by *G. lobatum* and S1 showed similar level of change in intensity of band at 1720 cm^{-1}, while *G. trabeum* showed a lower change. Carbonyls are important functional groups in side chains of lignin, therefore the increase of intensity of such band suggest that the lignin structure was modified by fungal pretreatment. Reduction of intensity of lignin bands at 1598 and 1505 cm^{-1} attributed to aromatic-ring vibrations was observed in samples of wheat straw pretreated by *G. lobatum* and co-culture pretreatments. The intensity of lignin bands was significantly higher in samples pretreated by *G. trabeum* than samples of untreated wheat straw. Gelbrich et al. (2012) reported that the absorbance values of these lignin bands (1598 and 1505 cm^{-1}) are linearly correlated with the lignin content in lignocellulosic biomass. Additionally, Liu et al. (2014) reported a progressive reduction in absorbance of bands at 1598 and 1505 cm^{-1} during the biodegradation of purified lignin by a strain of *Penicillium simplicissimum*. The carbohydrate band position at 900 cm^{-1} was attributed to β-glycosidic linkages and indicates changes in amorphous cellulose types. The greatest reduction in this band was found in samples pretreated by *G. trabeum* and S1, while *G. lobatum* showed slight changes in this band until 20 days, which is in agreement with low level of cellulose degradation until 20 days.

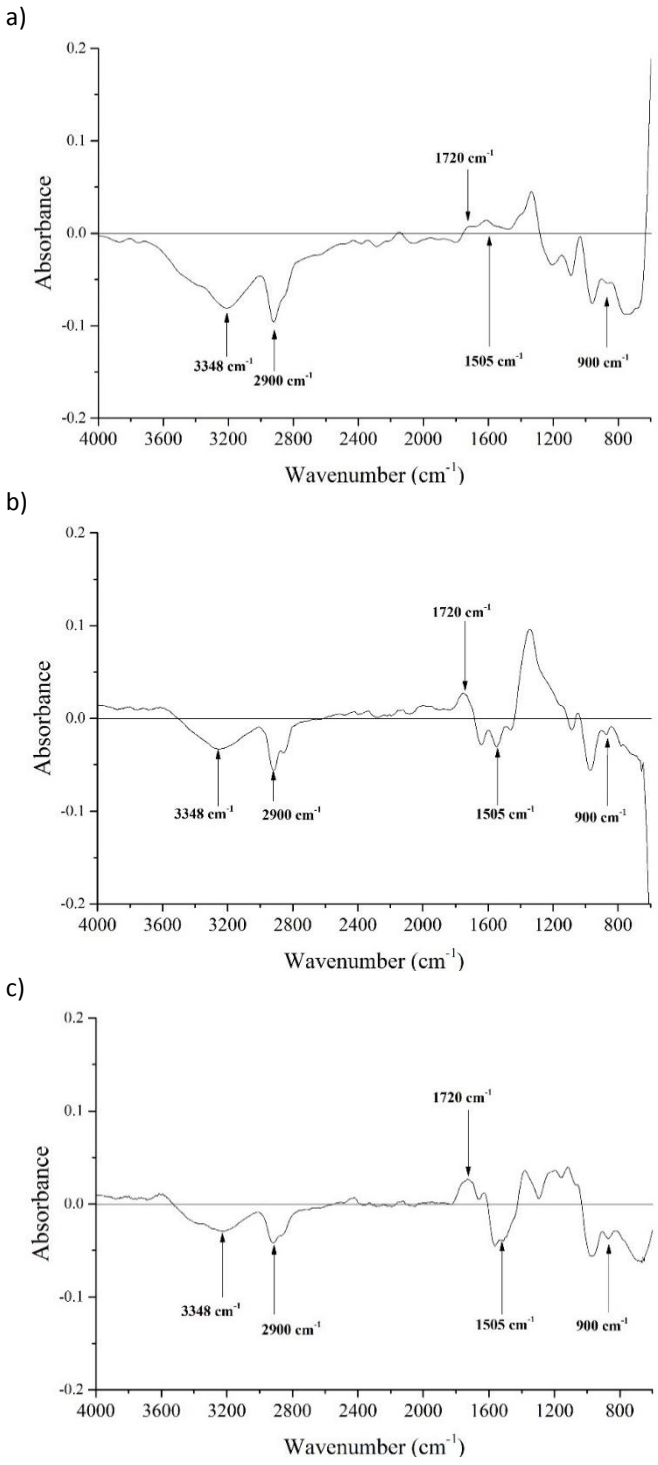

Figure 3 Subtraction of FTIR spectra of pretreatments by spectrum of untreated wheat straw for *G. trabeum* at 10 days (a), *G. lobatum* at 20 days (b) and *G. lobatum* followed by *G. trabeum* (S1 pretreatment) (c).

Cellulose is an unbranched polysaccharide exclusively composed of glucose. The hydroxyl groups of glucose in cellulose form hydrogen bonds with oxygen molecules on the same or on a neighboring chain, holding the chains firmly together side by side, forming strong crystalline aggregates called microfibrils that are interrupted by amorphous regions (Silveira et al., 2015). The crystalline regions prevent the releasing of glucose from cellulose. Kumar et al. (2009) suggest that FTIR analysis is a good tool for determining changes in crystallinity of biomass after pretreatments. The total crystallinity index (TCI) was estimated by the ratio of absorbance areas A_{1375}/A_{2900} from FTIR spectra (Nelson and O'Connor, 1964). Although, the TCI indicates crystallinity associated to all components interactions in biomass, the cellulose crystallinity had the strongest influence on the total crystallinity of a lignocellulosic biomass, since both lignin and hemicellulose are amorphous polymers. The TCI of samples of pretreated and untreated wheat straw are shown in Figure 4. Untreated wheat straw showed a TCI of 0.52, while the fungal pretreatments had lower TCI than untreated wheat straw, except the pretreatment by *G. lobatum* on 30 (0.52) and 40 days (0.56), and fungal combination S3 (0.61). The lowest TCI in single culture were found in wheat straw pretreated by *G. trabeum* and *G. lobatum* were after 10 days (0.41) and 20 (0.46), respectively. The combined pretreatment S1 showed the lowest TCI (0.29). Howell et al. (2009) showed that *G. trabeum* ATCC11539 significantly decreases cellulose crystallinity of pine blocks. In another study, Monrroy et al. (2011) indicated that the same strain of *G. trabeum* ATCC11539 showed 21% decrease of crystallinity index estimated by FTIR, increasing in 14% the enzymatic hydrolysis yields. The reduction in the crystallinity of cellulose by the pretreatment could enhance cellulose digestion during enzymatic hydrolysis due to a greater accessibility of the enzyme to the cellulose fibers (Kumar et al., 2009; Silva et al., 2011).

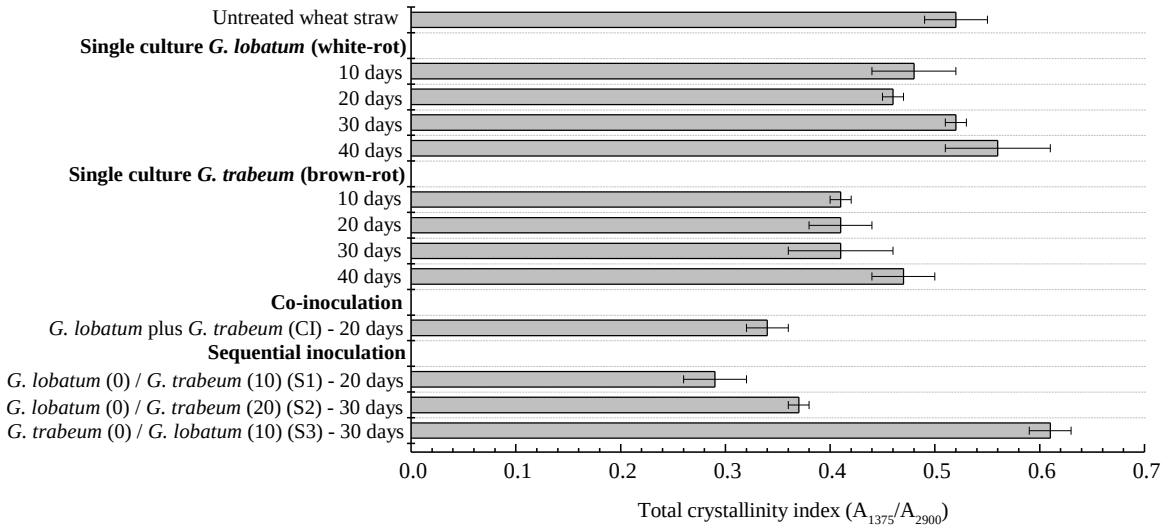

Figure 4 Total crystallinity index (A_{1375}/A_{2900}) calculated by FTIR of wheat straw samples before and after pretreatment by single and co-cultures of *G. lobatum* and *G. trabeum*. In sequential inoculation, number in parenthesis next to fungus name indicate inoculation day of each fungus. Data shown are expressed as means ± standard deviation (n=3).

5.3.6 Relationship between lignin degradation, TCI and glucose recovery

For a better understanding of the pretreatment effect on enzymatic hydrolysis, a correlation analysis was carried out between glucose recovery after enzymatic hydrolysis with weight loss, cellulose degradation, hemicellulose degradation, lignin degradation and TCI including data collected from the different pretreatments (Figure 5a-e). The variables weight loss (r = 0.25), cellulose degradation (r = 0.20) and hemicellulose degradation (r = 0.04) did not show correlation with glucose recovery. The variables lignin degradation (r = 0.42) and TCI (r = -0.57) showed positive and negative weak correlation with glucose recovery, respectively. In accordance with these results, Salvachúa et al. (2013) showed a positive correlation (r = 0.73) between lignin degradation and cellulose digestibility in pretreated wheat straw by the white-rot fungus *Irpex lacteus*. Monrroy et al. (2010) showed a slight correlation between TCI with the increase in the enzymatic hydrolysis of pretreated wood chips by *G. trabeum*.

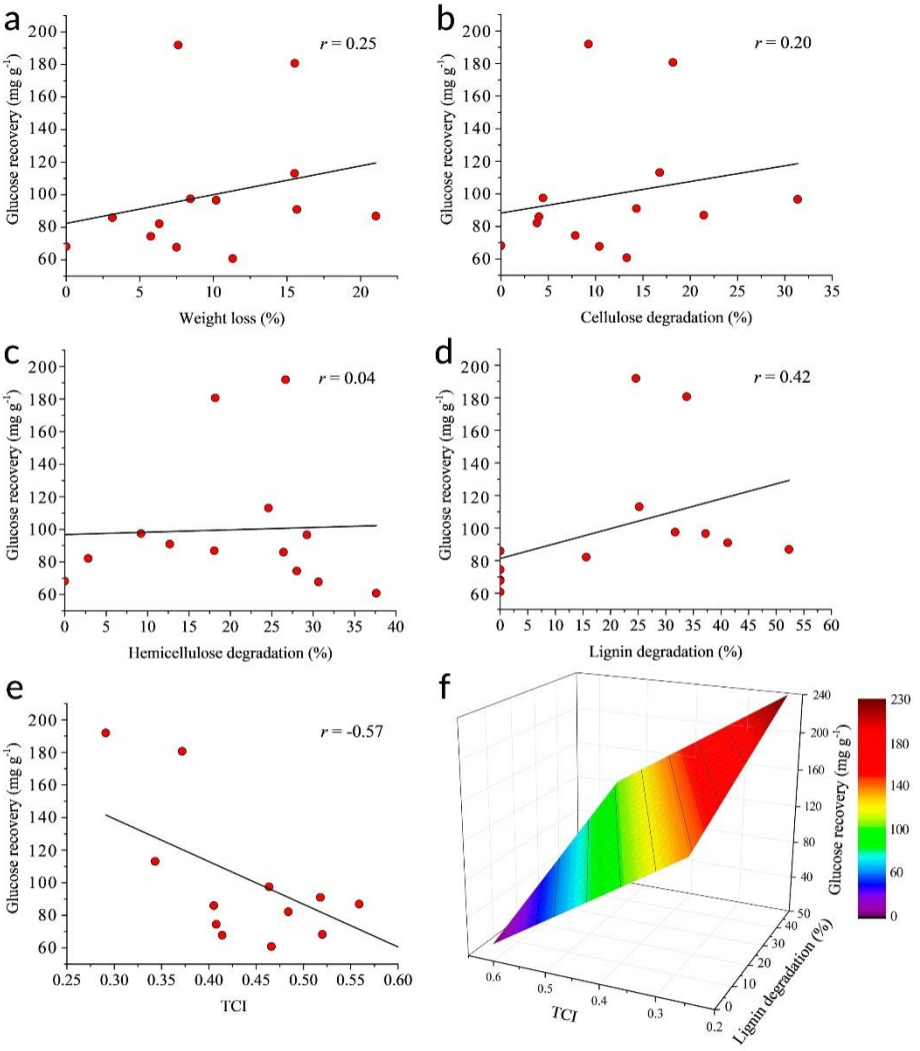

Figure 5 Correlation of glucose recovery after 72 h of enzymatic hydrolysis with weight loss (a), cellulose degradation (b), hemicellulose degradation (c), lignin degradation (d) and TCI (e), and multiple lineal regression between glucose recovery, TCI and lignin degradation (f) (r^2= 0.72, p value = 0.001). Values correspond to data from the different pretreatments.

Another analysis of multiple linear regression between glucose recovery, lignin degradation and TCI was carried out to determine the effect of these variables and its interaction on the enzymatic hydrolysis. This analysis showed a significant multiple linear regression (r^2 = 0.72) between the analyzed variables (Supplementary Table S1). According to F test, the TCI had a more significant effect (p value = 0.0013) than lignin degradation (p value = 0.004) on the amount of glucose released from straw in enzymatic hydrolysis (Supplementary Table S1).

Besides, it revealed that the influence of lignin degradation on enzymatic hydrolysis of wheat straw increased when TCI decreased (Figure 5f). It would indicate that the positive effect on the enzymatic hydrolysis of wheat straw pretreated by *G. lobatum* and *G. trabeum* is mainly related by their abilities to synergistically degrade lignin and modify the crystalline regions in the lignocellulosic matrix, respectively. Accordingly, although in the S1 pretreatment the lignin degradation was not the highest, TCI was the lowest detected, producing a high increase in the yield of enzymatic hydrolysis. S1 pretreatment represents the best alternative for pretreatment by fungal co-culture amongst the conditions studied and these results are in concordance with those obtained for pretreatments by single fungal culture in relation to the best incubation time for each fungus.

5.4 Conclusions

The pretreatments of wheat straw by co-culture of white-rot and brown-rot fungi provided higher yields of glucose after enzymatic hydrolysis when compared with pretreatments by their single cultures. It is also important to emphasize the shorter incubation time required in comparison to previous reported studies. The sequential inoculation of *Ganoderma lobatum* (white-rot) followed by *Gloeophyllum trabeum* (brown-rot) showed a synergistic effect on pretreatment and promoted higher yields in enzymatic hydrolysis of wheat straw, which was not observed in pretreatment by co-inoculation neither in sequential inoculation of brown-rot followed by followed by white-rot fungus. A multiple correlation between lignin degradation, total crystallinity index and enzymatic hydrolysis revealed that the influence of lignin degradation by the white-rot fungus on enzymatic hydrolysis of wheat straw increases when total crystallinity index decreases by the action of the brown-rot fungus. The S1 pretreatment represent a promising alternative that could be applied as a complementary step to any subsequent physicochemical pretreatment in order to increase its efficiency and decrease the severity conditions.

5.5 Acknowledgements

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CHAPTER VI
Global discussion, conclusions and future perspectives

6.1 Global discussion

Bioethanol can be produced from sugar-rich or starch-rich raw materials. The first-generation bioethanol is produced directly from food crops products such as sugarcane, sugar beet, sweet sorghum, barley, wheat, corn, among others. Whereas, the second-generation bioethanol is produced from non-food raw materials such as, wood, bagasse, straw and grass, which are called lignocellulosic biomass. Lignocellulosic biomass is a highly abundant and socially accepted feedstock for biofuel production. Currently, it is one of the most attractive alternatives to reduce the consumption of fossil-derived fuels in the automotive industry. Cellulose, the major component of lignocellulosic biomass, is a glucan polysaccharide that provides the real potential for its conversion into bioethanol. However, the complex structure of the lignocellulose is the main obstacle for the efficient utilization of the cellulose contained in the lignocellulosic biomass. (Silveira et al. 2015; Wan and Li 2010; Xu et al. 2017). Therefore, it is essential to submit lignocellulosic biomass to pretreatment processes, for increasing cellulose availability, and to enzymatic hydrolysis, for releasing glucose from cellulose, before fermentation into ethanol can be carried out. These two processes represent the highest cost of the ethanol production process, so all research aiming to improving it leads to an advance in the generation of biofuels. Biological pretreatment is an alternative to the commonly used physico-chemical methods that, although effective, are not environmentally friendly, and usually, generate inhibitors that harm the fermentation step.

This thesis investigated the potential of white-rot and brown-rot fungi on the biological pretreatment for improving the biodegradability of wheat straw, particularly focusing on the effect of fungi culture conditions (**Chapter III and IV**), incubation time, single-culture and co-culture, using simultaneous and sequential inoculation of the fungi (**Chapter V**). The pretreatment effectiveness was evaluated through enzymatic hydrolysis of pretreated wheat straw, using commercial enzymes. Preliminary screening assays were carried out to

qualitatively evaluate the lignocellulolytic potential of 14 Chilean native strains of wood-rotting fungi. This was performed in Petri dishes containing agar-solid medium with substrate indicators of ligninolytic (ABTS and RBBR), hemicellulolytic (Xylan from Birchwood), cellulolytic (CMC) and Fe^{3+}-reducing (Ferrozine) activity (**Chapter II**). Two white-rot fungal strains, *A. discolor* and *G. lobatum*, and two brown-rot fungal strains, *Daedalea* sp and *G. trabeum*, were selected due to the fact that they showed the highest lignin and holocellullose degrading activity, respectively. These strains were inoculated on wheat straw under solid-state fermentation conditions and incubated for 10, 20, 30 and 40 days. Wheat straw degradation by fungi was evaluated in terms of lignin content, lignin degradation selectivity, weight loss, released reducing-sugars, and lignocellulolytic enzymes (**Chapter II**). The white-rot fungus, *G. lobatum*, was selected because it generated similar levels of lignin degradation (43%) compared with *A. discolor* (42%) but associated to lower weight loss (24%) after 40 days of incubation, resulting in a higher lignin degradation selectivity. Fungal species belonging to the *Ganoderma* genre have been reported as lignin-degrading selective white-rot fungi (Blanchette 1984; Martínez et al. 2005). This selective lignin degradation pattern favors the conversion of lignocellulosic biomass because the cellulosic fraction is largely unaffected and becomes more available (Shrivastava et al. 2011). On the other hand, the main ligninolytic enzymes detected during wheat straw degradation by *G. lobatum* were manganese-dependent (MnP) and manganese independent peroxidase (MiP). The brown-rot fungus, *G. trabeum*, was selected because it produces high cellulase and xylanase activity and weight loss lower than 10% after 40 days of incubation. Moreover, *Daedalea* sp. showed poor growth in wheat straw. The potential of *G. trabeum* had been reported in the improvement of organosolv pretreatment and enzymatic hydrolysis yield in wood chips .

As lignocellulosic biomass is often rich in carbon, but low in nutrients (nitrogen, phosphorus and trace elements) and water content; the addition of nutrients is an important factor on the

biological pretreatment because it influences the effectiveness of wood-rotting fungi for degrading lignocellulosic biomass (Baldrian et al. 2005; Salvachúa et al. 2013; Shrivastava et al. 2012). To increase the ligninolytic potential of white-rot fungi, Mn^{2+}, Cu^{2+}, Fe^{2+}, unsaturated fatty acids, and aromatic compounds have been used as enzymes inducers or mediators on submerged and solid-state fermentation cultures (Acevedo et al. 2011; van Kuijk et al. 2016). In previous studies, metal ions Mn^{2+} and Fe^{2+} had shown a positive effect on MnP production by white-rot fungi (Baldrian et al. 2005; Salvachúa et al. 2013). On the other hand, the brown-rot fungi degrade cellulose and hemicellulose in lignocellulosic biomass through non-enzymatic and enzymatic mechanisms, without significant lignin degradation. In early stages of the degradation by brown-rot fungi, an oxidative radical-based system (via Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + {}^{-}OH$) attacks the plant cell wall causing important modifications in the lignocellulose matrix, including cellulose and hemicellulose chain cleavage and conformational changes in the structure of lignin (Arantes et al. 2012; Goodell et al. 2017). These early modifications in lignocellulose via Fenton reaction is more favorable than enzymatic degradation for the pretreatment of lignocellulose biomass by brown-rot fungi, generally due to the fact that early changes generate cellulose availability increase with minimal polysaccharide loss (Aguiar et al. 2013; Schilling et al. 2009).

The addition of Fe^{2+} as supplementary Fenton reactant has been shown to increasing the amount of hydroxyl radical production and pollutant degradative abilities of brown-rot fungi (Purnomo et al. 2010, 2011). In addition to Fe^{2+}, Mn^{2+} and Cu^{2+} are also involved in the generation of hydroxyl radicals via Fenton reaction (Pozo et al. 2016). The low nitrogen content (usually lower than 1% dry weight) is below optimal level for fungal growth; therefore, it is necessary to increase the nitrogen content for accelerating the fungal growth, in an initial stage of biomass colonization (Bisaria 1998; Jonathan and Adeoyo 2011; Mane et al. 2007). Based on this information and in order to optimize an adequate and faster wheat straw degradation by the

fungi, the selected fungi were incubated on wheat straw containing different NO_3^-, Fe^{2+} and Mn^{2+} concentrations for 10, 20, 30 and 40 days (**Chapter III and IV**). These assays were carried out using a central composite experimental design of response surface in order to obtain mathematical models of the main variables affecting the availability and biodegradability of cellulose in wheat straw after fungal pretreatment. Hence, a multiple optimization using the models of lignin degradation and weight loss was carried out so as to find the optimal levels of NO_3^-, Fe^{2+} and Mn^{2+}, to promote selective lignin degradation by *G. lobatum*, which resulted in both minimum weight loss and maximum lignin degradation in wheat straw after 40 days of incubation (**Chapter III,** Hermosilla et al. 2017). The addition of Mn^{2+} and Fe^{2+} promoted selective lignin degradation in wheat straw, increasing lignin degradation and decreasing weight loss. Mn^{2+} also showed a positive effect on MnP activity and a negative effect on MiP, cellulolytic and hemicellulolytic activities. Therefore, Mn^{2+} promoted selective lignin degradation decreasing weight loss derived from polysaccharides degradation by cellulolytic and hemicellulolytic enzymes. The addition of NO_3^- showed a negative effect on weight loss and lignin degradation. This negative effect of NO_3^- was also observed on activities of ligninolytic, cellulolytic and hemicellulolytic enzymes, suggesting that the addition of exogenous nitrogen source decreases *G. lobatum* degradation potential. However, the culture condition that promotes lignin degradation selectivity after 40 days was 0.18 M NO_3^-, 0.73 mM Fe^{2+}, and 1 mM Mn^{2+}, which resulted in 50.0% lignin degradation and 18.5% weight loss. Nitrogen concentration plays an important role on the regulation of the growth and the lignocellulolytic enzyme production by wood-rotting fungi (Kachlishvili et al. 2006; Kanwal and Reddy 2010). Besides, Labosky et al. (1991) reported that inorganic nitrogen sources (ammonium nitrate) at 0.95% nitrogen increased lignin degradation and weight loss caused by *P. chrysosporium* on red oak chips, but at 2.87% nitrogen, lignin degradation and weight loss significantly decreased. Boyle (1998) showed that 1% ammonium chloride significantly

decreased lignin degradation and weight loss caused by *Lentinula edodes* on wood. In this thesis, the lowest (0.06 M) and the highest NO_3^- (0.24 M) concentrations correspond to 0.41% and 1.68% nitrogen, respectively. Therefore, the combination of $Mn^{2+}$and Fe^{2+} effects that increased lignin degradation and the NO_3^- effect that decreased weight loss, stimulated a selective lignin degradation in wheat straw caused by the white-rot fungus. Compared with the previous *G. lobatum* incubation assay on wheat straw without additional nutrients, lignin degradation increased 7% and weight loss decreased 5.5% under optimal culture conditions. Studies with other selective lignin-degrading fungi showed similar levels of lignin degradation compared with *G. lobatum*; however, their results showed high weight loss. In this way, *P. ostreatus* HP-1 showed 40% lignin degradation with 40% weight loss after 32 days of incubation (Thakur et al. 2012), *I. lacteus* showed 45.9% lignin degradation with 38% weight loss after 21 days (Salvachúa et al. 2011), and *C. subvermispora* showed 39.2% lignin degradation with 18.8% weight loss after 42 days (Wan and Li 2010). In this study, a new method of confocal microscopy was developed to visualize wheat straw zones with high cellulose availability generated after lignin degradation in lignocellulosic biomass caused by white-rot fungi (Hermosilla et al. 2017). This method allows simultaneously and differentially visualizing lignin, cellulose and fungal mycelia fluorescence in fungal treated samples. It was observed that cellulose fluorescence increased in zones where the lignin fluorescence decreased, indicating that cellulose availability increased in fungal treated wheat straw. It was also observed that wheat straw lignin degradation by *G. lobatum* occurs in a heterogeneous manner; lignin degradation zones were observed in: areas surrounding fungal mycelia, only in areas with fungal mycelia and generation of degradation pores.

According to previous studies, the increase of glucose yield, during the enzymatic hydrolysis of *G. trabeum* pretreated lignocellulosic biomass, was related to a decreasing in lignocellulose crystallinity produced by the fungus (Howell et al., 2009; Monrroy et al., 2011; Schilling et al.,

2012). It is important to highlight that since both lignin and hemicellulose are amorphous polymers; therefore, total lignocellulose crystallinity is mainly related to cellulose crystallinity. A study on rice straw and sugarcane bagasse pretreatment, mimicking natural lignocellulose degradation by brown-rot fungi via Fenton reaction, showed that a decrease in the crystallinity index after pretreatment increased hydrolytic enzyme accessibility in treated samples (Sakdaronnarong and Jonglertjunya 2012). Therefore, in order to establish a suitable degradation of wheat straw by *G. trabeum*, a multiple optimization of the total crystallinity index (TCI) and weight loss was carried out so as to determine the optimal levels of NO_3^-, Fe^{2+} and Mn^{2+} that allow obtaining the lowest TCI value and minimizing the associated weight loss after 40 days of incubation (**Chapter IV**). The addition of NO_3^- and Mn^{2+} decreased weight loss and TCI value, whereas the addition of Fe^{2+} increased weight loss and also decreased the TCI value. The culture condition that promotes TCI decrease with a low weight loss was 0.24 M NO_3^-, 0.95 mM Fe^{2+} and 0.85 mM Mn^{2+}, which resulted in 0.43 TCI value (a 17.3% lower than TCI value of untreated wheat straw) and 11.3% weight loss after 40 days of fungal treatment. The molecules directly involved in the degradation of lignocellulose via Fenton reactions such as, hydroxyl radicals or hydrogen peroxide, are difficult to measure adequately in a complex sample as wheat straw colonized by fungus, due it contains a wide variety of chemical compounds either derived from lignocellulose degradation or secreted by the fungus that interfere with the detection. Besides, these compounds are unstable and have an extremely short half-life (10^{-9} s) (Arantes et al. 2012) as compared with the times in which samples were collected. Catechol and Fe^{3+}-reducing activity were evaluated since they are more stable compounds, which participate in metal ions redox cycling for the regeneration of Fenton-active metal ions oxidation state (Fe^{3+} to Fe^{2+} and Mn^{3+} to Mn^{2+}). Our results showed that the TCI value decrease was correlated with an increase of Fe^{3+}-reducing activity in extracts from the pretreated wheat straw up to 20 days. After 30 and 40 days, the TCI value decrease was

correlated to an increase of catechol compounds. Furthermore, the addition of Fe^{2+} showed a positive effect on Fe^{3+}-reducing activity in extracts, but a negative effect on the production of catechol compounds and cellulolytic and hemicellulolytic enzymes activities. This coupled with the positive effect of Fe^{2+} in weight loss could suggest that the Fe^{2+} promotes the degradation of wheat straw by *G. trabeum* via Fenton reaction with no-catechol compounds as Fe^{3+}-reducing reactants. Similar to the effect of NO_3^- observed on degradation by *G. lobatum*, NO_3^- under optimal conditions also controls weight loss produced by *G. trabeum*.

The effect of pretreatment of wheat straw by *G. lobatum* and *G. trabeum* under optimal culture conditions, on the availability of cellulose was evaluated through enzymatic hydrolysis (**Chapter V**). The effect of incubation time (10, 20, 30 and 40 days) on the pretreatment by the fungi was also evaluated. All fungal pretreatments significantly increased the amount of glucose released after enzymatic hydrolysis from wheat straw, except when *G. trabeum* was incubated for 40 days, which decreased sugar recovery by 10.9%. The pretreatment by *G. lobatum* single culture resulted more effective than *G. trabeum*. The highest concentration of glucose after enzymatic hydrolysis was observed in the pretreated wheat straw by *G. lobatum* and *G. trabeum* single cultures were obtained after 20 and 10 days of incubation, increasing by 42.9% and 26.0% glucose yield compared to untreated wheat straw, respectively. The concentration of released glucose significantly decreased for wheat straw samples pretreated for incubation times superior to that. One explanation for these phenomena may relate to the fact that extensive degradation of lignocellulose facilitates availability and consumption of cellulose for fungus growth, which would decrease the final glucose recovery (Capelari and Zadrazil 1997).

The main goal of this thesis was to evaluate the combined action of white-rot and brown-rot fungi for improving the wheat straw biodegradability. Until now, all our experiments had been directed towards the promotion of an adequate degradation of wheat straw by fungi. Therefore, based on the obtained results from single fungal cultures, four co-culture methods were carried

out to evaluate potential additive and synergistic effects between the fungi, including co-inoculation (CI) and sequential (S1, S2 and S3) inoculation of fungi (**Chapter V**). In CI pretreatment, both *G. lobatum* (white-rot) and *G. trabeum* (brown-rot) were co-inoculated in the same flask and incubated for 20 days. The first strategy of sequential inoculation, in S1 pretreatment, *G. lobatum* was inoculated in the wheat straw and, after 10 days, *G. trabeum* was added to the flasks that were incubated for another 10 days. The S2 pretreatment was similar to S1, except that *G. trabeum* was inoculated after 20 days. The S3 pretreatment consisted of inoculating *G. trabeum* firstly and, after 10 days, *G. lobatum* was added to the flasks that were incubated for another 20 days. The fungal co-cultures were carried out at a common culture condition of the concentration of NO_3^-, Fe^{2+} and Mn^{2+}, which was obtained using the mathematical models from the previous assays of single cultures in order to keep their degradative abilities (**Appendix C**). Overall, the pretreatments of lignocellulosic biomass by single fungal cultures were less effective than the co-cultivation conditions evaluated, resulting in samples that were less prone to enzymatic hydrolysis. A synergistic effect between fungi on pretreatment was observed when *G. lobatum* (white-rot) was inoculated first followed by *G. trabeum* (brown-rot), in S1 and S2 co-cultures, resulting in higher glucose concentration during enzymatic hydrolysis of samples pretreated at those conditions. The synergistic effect was not observed in pretreatment by co-inoculation (CI) neither in sequential inoculation of the brown-rot followed by followed by the white-rot fungus (S3). Glucose concentration obtained after enzymatic hydrolysis of samples obtained by S1 pretreatment (191.9 mg g^{-1} of wheat straw) was slightly higher than by S2 pretreatment (181.0 mg g^{-1} of wheat straw) and, moreover, S1 pretreatment takes 20 days compared with 30 days of S2. The S1 pretreatment increased the glucose recovery in enzymatic hydrolysis by 2.8-fold compared to untreated wheat straw and achieved 39.7% conversion of cellulose to glucose. The conversion yield obtained after S1 pretreatment was higher than the yields presented in other studies of biological pretreatment of

wheat straw with similar or longer fungal exposure time. In this sense, the pretreatment using *Trametes versicolor* C6915 for 112 days resulted in a 28.6% of cellulose to glucose conversion after enzymatic hydrolysis of pretreated samples (Zhang et al. 2016). Another study, using the fungus *Ceriporiopsis subvermispora* for 18 days of pretreatment reached 30% glucose yield (Wan and Li 2010). On the other hand, the hydrolysis of wood samples pretreated by the application of white-rot and brown-rot fungi in two stages for 60 days of fungal exposure resulted in a maximum of sugar concentration of 119 mg g^{-1} *L. tulipifera* wood chips.

For a better understanding of the pretreatment effect on enzymatic hydrolysis, a correlation analysis was carried out between glucose recovery after enzymatic hydrolysis with weight loss, cellulose degradation, hemicellulose degradation, lignin degradation and TCI including data collected from the different pretreatments. Only the lignin degradation (r = 0.42) and TCI (r = -0.57) showed positive and negative weak correlation with glucose recovery, respectively. These results corroborated that the lignin degradation and the decrease of the crystallinity in the pretreated wheat straw by the white-rot and brown-rot fungi, respectively, have a positive effect on an increment in the enzymatic hydrolysis. Additionally, a multiple correlation between lignin degradation, total crystallinity index and enzymatic hydrolysis revealed that the influence of lignin degradation by the white-rot fungus on enzymatic hydrolysis of wheat straw increases when total crystallinity index decreases by the action of the brown-rot fungus. This multiple correlation could explain the synergistic effect between fungi observed in S1 and S2 pretreatments. Considering that wheat straw has shown a higher resistant to biological pretreatment than other lignocellulosic biomass, like corn stover, switchgrass, hardwood, sugarcane bagasse (Wan and Li 2011), this method of fungal pretreatment could show higher yields in others lignocellulosic biomass.

6.2 Conclusions

- The screening of fungal strains allowed selecting a white-rot fungus, *G. lobatum*, which showed high selectivity for lignin degradation in wheat straw and a brown-rot fungus, *G. trabeum*, which showed fast hemicellulose degradation and cellulose crystallinity decrease in wheat straw.
- The combined effect of metal ions, Fe^{2+} and Mn^{2+} as enzyme inducers (for the white-rot fungus) or Fenton reactants (for the brown-rot fungus), and nitrate as additional nitrogen source promotes wheat straw degradation by the wood-rotting fungi for pretreatment. Overall, the Mn^{2+} had the strongest positive effect on lignin degradation selectivity by *G. lobatum*, while the Fe^{2+} has the strongest positive effect on the degradation of wheat straw and crystallinity decrease by *G. trabeum*. The addition of nitrate showed a negative effect on weight loss, without significant effect on lignin degradation or decrease in cellulose crystallinity caused by *G. lobatum* and *G. trabeum*, respectively.
- The wheat straw pretreatment in single-cultures of *G. trabeum* and *G. lobatum* after 10 and 20 days of incubation increased glucose yields by 26% and 43% after enzymatic hydrolysis, respectively. However, higher lignin degradation produced by *G. lobatum* after 30 and 40 days of incubation did not produce higher glucose yields. On the other hand, the changes produced in crystallinity and hemicellulose content of wheat straw by *G. trabeum* after 20 days of incubation, generated lower glucose yields as compared to untreated wheat straw.
- The pretreatment, with the combined action of white-rot and brown-rot fungi in co-culture conditions, provided higher yields of glucose after enzymatic hydrolysis compared with single-culture pretreatments. The sequential inoculation (S1) of white-rot fungus followed by brown-rot fungus after 10 days (for 20 days of total incubation time) showed a synergic effect after enzymatic hydrolysis, reaching a 39.7% of glucose

yield, 2.8-fold compared to untreated wheat straw. The sequential inoculation allowed to overlap the incubation times required for an effective wheat straw degradation by each fungus in single culture.

6.3 Future perspectives

The sequential inoculation is a promising method for applying biological pretreatment by fungal co-culture, which showed higher glucose yields compared with single fungal cultures, co-cultures using the co-inoculation method and even other biological pretreatments previously reported. Although the glucose yield of S1 pretreatment is still not comparable with the currently physicochemical methods, new studies have been focusing on the use of biological pretreatments as a complementary step to physicochemical pretreatments. Biological pretreatment allows to decrease the severity conditions and increase the effectiveness of physicochemical pretreatments. In this sense, a biological pretreatment with a high glucose yield results in a good alternative to being applied as a complementary method. Therefore, these results contribute to the new technologies development for lignocellulosic biomass pretreatment to enhance its conversion into biofuel; however, fungal pretreatment must be scaled-up to promote sustainable industrial development. Therefore, future pilot-scale projects must be carried out to assess and validate the fungal pretreatment performance for biofuel production at high-scale and even to pretreat different lignocellulosic biomass. On the other hand, it is important to highlight that several other by-products can be also obtained from the lignocellulosic biomass pretreatment by fungi, including fungal enzymes and chemical compounds (organic acids, xylitol, furfural, cresols, catechol, among others), which can be recovered and purified. So, another challenge ahead is to integrate a biorefinery model to obtain commercially viable biofuels and other bioproducts to take full advantage of lignocellulosic biomass in order reduce costs, improve performance, and achieve competitiveness with fossil fuels.

6.4 References

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Appendix
Optimization of co-culture conditions

Table 1 Predicted values of degradative parameters of *G. trabeum* and *G. lobatum* at optimal co-culture conditions (0.24 M $NaNO_3$, 0.74 mM $FeSO_4$ and 1 mM $MnSO_4$) in wheat straw.

Fungus		Predicted values		
		Weight loss (%)	TCI (A_{2900}/A_{1375})	Lignin degradation (%)
Brown-rot fungus *G. trabeum*	10 days	2.87	0.42	nd
	20 days	4.36	0.4	nd
	30 days	6.61	0.38	nd
White-rot fungus *G. lobatum*	10 days	4.37	nd	12.9
	20 days	8.36	nd	29.2
	30 days	16.6	nd	39.7

Nd= No determined response.

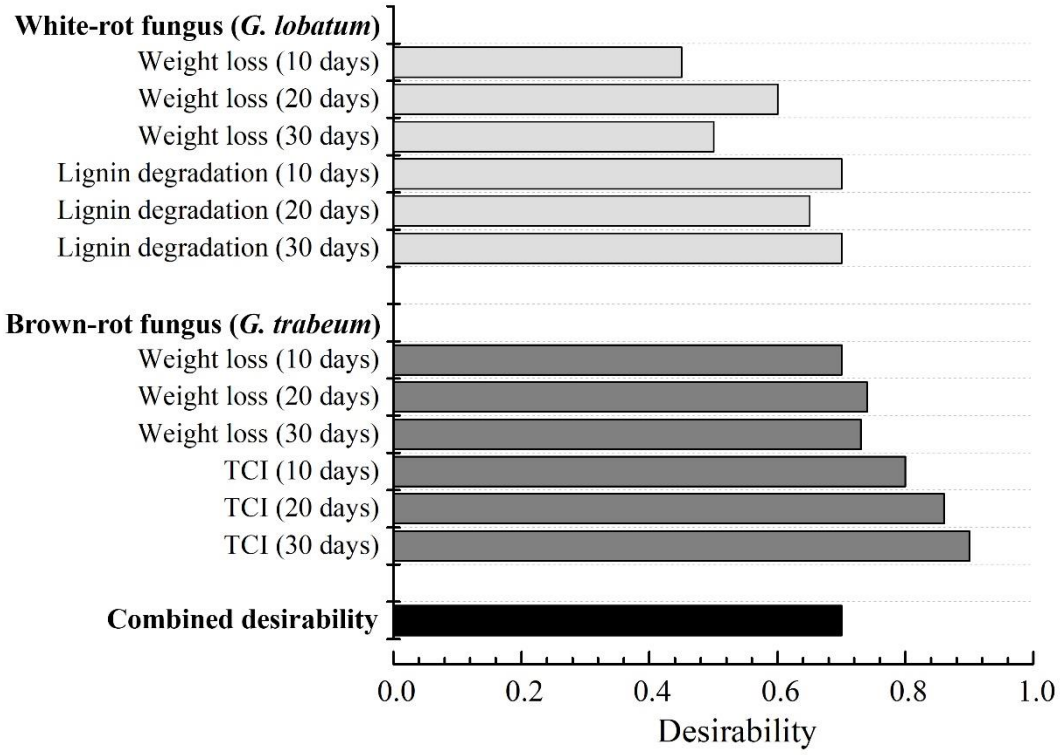

Figure 1 Desirability of each response at optimized culture conditions for the fungal co-culture that kept the degradative abilities of *G. lobatum* and *G. lobatum* in wheat straw.