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“DEVELOPMENT OF AN OLIVE MILL SOLID WASTE TREATMENT USING WHITE-ROT FUNGI WITH CU AND MN AS INDUCERS/COFACTORS FOR IMPROVING ITS BIODEGRADABILITY”

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"DEVELOPMENT OF AN OLIVE MILL SOLID WASTE TREATMENT USING WHITE-ROT FUNGI WITH CU AND MN AS INDUCERS/COFACTORS FOR IMPROVING ITS BIODEGRADABILITY"

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Summary and thesis outline

Lignocellulosic biomass, the most abundant type of biomass, is vastly underutilized despite its enormous production, with significant portions being relegated to landfilling, open dumping, or incineration. Exploiting this biomass in biorefineries could yield valuable products, but the molecular structure of lignin can hinder the process due to its strong association with cellulose and hemicellulose. Biological treatments offer a promising avenue to overcome this challenge with lower energy demands than physical, chemical, or thermal treatments. White-rot fungi (WRF) and their enzymatic machinery have emerged as effective tools for delignifying lignocellulosic residues. However, enhancing bioprocesses using fungal treatments faces additional challenges, including improving enzyme activity and stability and ensuring that residual fractions are environmentally benign. Strategies to optimize enzyme activity include the addition of organic and inorganic inducers to the culture medium and using co-substrates like lignocellulosic biomass. Notably, copper (Cu) and manganese (Mn) play crucial roles in enhancing the activities of ligninolytic enzymes in WRF. Cu acts as a cofactor in laccase catalytic centers, while Mn activates peroxidase enzymes and directly contributes to lignin degradation.

Olive mill solid waste (OMSW), a byproduct of olive oil extraction, is rich in lignocellulosic fibers and polyphenols. Polyphenols in OMSW confer antimicrobial activity, complicating its valorization through microbial processes and contributing to phytotoxicity. Therefore, a feasible process for obtaining derived products from OMSW and achieving its detoxification requires a treatment step to increase both its biodegradability and remove phenolic compounds.

Therefore, the general aim of this doctoral thesis was to develop an olive mill solid waste treatment using the native fungi *Anthracophyllum discolor* and *Stereum hirsutum* supplemented with Cu and Mn as enzyme inducers for improving biodegradability. These fungi were selected based on preliminary studies by researchers of the Environmental Nanobiotechnology Laboratory at the University of La Frontera.

Chapters I and II contextualize the thesis and establish the situation of the olive sector that makes the proposed scientific development necessary, as well as the framework at a scientific level that justify the novelty of the research work. Concretely, in **chapter I**, a general introduction of this doctoral thesis is presented, showing the hypothesis and aims of this study. **Chapter II** consists of a literature review highlighting the biotechnological potential of basidiomycete fungi in enzyme production under Cu and Mn supplementation, given their major relevance in the literature, which could significantly improve biodegradation and biomass-derived products. The review provides information on the effect of Cu and Mn on enzyme activity and describes the metal-fungus interaction mechanisms underlying the effects on enzyme activity. In **chapter III**, the evaluation of direct application of *A. discolor* and *S. hirsutum* as a biological pretreatment for OMSW is described. The assays determined the need to add supplementary nutrients and an exogenous carbon source or use agitation systems and evaluated WRF growth, enzyme activity, phenolic compound removal, and lignin degradation in order to determine the appropriate conditions to improve the OMSW biodegradability. The results demonstrate for the first time that *A. discolor* and *S. hirsutum* were able to degrade lignin (94.1% and 94.4% after 24 days of treatment, respectively) and remove phenolic compounds (more than 80% with both strains) from OMSW using this as the sole substrate without adding other nutrients or using agitation systems.

Afterward, in **chapter IV**, the effect of Cu and Mn added in different doses on OMSW inoculated with *A. discolor*, and *S. hirsutum* is explained. Ligninolytic enzyme activity, lignin degradation and phenolic compound removal were the main variables reported. This chapter conducted to enhance the conditions of OMSW biodegradability previously describe in the Chapter III. Optimal dosages of metals increased the enzyme activity depending on the strain. A positive effect associated with Mn addition could be improving the MnP activity in both strains. The best results in terms of lignin degradation (41.5±3.2%) were achieved using S. hirsutum supplemented with 2.5 mg Kg^{-1} of Cu and 7.3 mg Kg^{-1} of Mn, enhancing the degradation in a 18% respect to the treatment without supplementation. This strain also achieved a stable phenol removal efficiency of 65-75% in OMSW at 25 days, while in its leachate, the values were 80-95% at 30 days, where no representative differences were observed with the absence or presence of added Cu and Mn.

Chapter V evaluates the valorization of OMSW through the recovery of high added value compounds such as enzymatic extracts, volatile fatty acids (VFA) and/or phenolic compounds through the application of a fungal treatment with *A. discolor* and *S. hirsutum* with addition of metals, simultaneously determining the possible reduction of the phytotoxicity of OMSW. The laccase enzyme activity of both fungi was enhanced by the addition of Cu-Mn at about half the time tested (88.7% at day 15 for *A. discolor* and 32.3% at day 20 for *S. hirsutum*). The addition also increased the MnP activity of *S. hirsutum* by 97.2%. Regarding the release of phenols from the OMSW to a liquid fraction, *S. hirsutum* showed a higher release capacity (6001 ± 236 mg eq L^{-1} of gallic acid) compared to *A. discolor*. This phenol release to the liquid phases allowed the partial dephenolization of the OMSW solid phases. Concretely, in these solid phases, *A. discolor*

allowed 40.8% reduction of phenols, while *S. hirsutum* only reached 20%. *S. hirsutum* was able to generate a higher concentration of FV (1626.97±325.39 mg L^{-1}), mainly isobutyric acid and acetic acid, than *A. discolor*. With the treated OMSW, the solid fraction showed slightly lower phytotoxicity than the raw OMSW for both strains, although still showed severe phytotoxicity effects at OMSW doses higher than 10%.

Finally, in Chapter VI, all these results are discussed. Thus, this doctoral thesis concludes that (1) *A. discolor* and *S. hirsutum* are two potential white rot fungi that can be used for the future design of an in-situ treatment for the valorisation of OMSW. (2) The addition of Cu and Mn allows the enhancement of specific enzymes, which opens an opportunity to design specific supplements according to the application of the fungus. Moreover, Cu-Mn addition is strongly associated with the strain, showing a degradation selectivity associated with the physiology of phenol degradation by the studied strains. (3) *S. hirsutum* is a promising fungus for recovery of added-value compounds such as VFAs and phenolic compounds from the OMSW.

Resumen

La biomasa lignocelulósica, es de las más abundantes y está ampliamente subutilizada a pesar de su enorme producción, con importantes cantidades relegadas a vertederos, depósitos a cielo abierto o incineración. Explotar esta biomasa en biorrefinerías podría generar productos de alto interés, si bien la estructura molecular de la lignina plantea un desafío debido a su fuerte asociación con la celulosa y la hemicelulosa. Los tratamientos biológicos ofrecen una vía promisoria para superar esta limitación con menores demandas energéticas que los tratamientos físicos, químicos o térmicos ya existentes. Los hongos de pudrición blanca y su maquinaria enzimática han surgido como herramientas efectivas para la deslignificación de residuos lignocelulósicos. Sin embargo, llevar a cabo bioprocesos mediante tratamientos fúngicos enfrenta desafíos adicionales, como mejorar la actividad y estabilidad de las enzimas y garantizar que las fracciones residuales sean amigables con el ambiente. Las estrategias para optimizar la actividad enzimática incluyen la adición de inductores orgánicos e inorgánicos al medio de cultivo y el uso de co-sustratos como la biomasa lignocelulósica. Por ejemplo, el cobre (Cu) y el manganeso (Mn) juegan roles cruciales en mejorar las actividades de las enzimas ligninolíticas en los hongos de pudrición blanca. El Cu actúa como cofactor en los centros catalíticos de la lacasa, mientras que el Mn activa las enzimas peroxidasas y contribuye directamente a la degradación de la lignina.

Los residuos semi-sólidos de almazara, genéricamente denominados alperujo, un subproducto de la extracción de aceite de oliva, son ricos en fibras lignocelulósicas y polifenoles. Los polifenoles en el alperujo le confieren actividad antimicrobiana, complicando su valorización a través de procesos microbianos y contribuyen a su fitotoxicidad. Por lo tanto, un proceso factible para obtener productos derivados de alperujo y lograr su desintoxicación requiere una

etapa de tratamiento para aumentar tanto su biodegradabilidad como para eliminar compuestos fenólicos.

El objetivo general de esta tesis doctoral fue desarrollar un tratamiento para el alperujo, utilizando hongos nativos *Anthracophyllum discolor* y *Stereum hirsutum* suplementados con Cu y Mn como inductores de enzimas para mejorar la biodegradabilidad del residuo. Estos hongos fueron seleccionados con base a estudios preliminares realizados por investigadores del Laboratorio de Nanobiotecnología Ambiental de la Universidad de La Frontera, Chile.

Los **capítulos I y II** contextualizan la tesis y establecen el marco tanto a nivel científico como de la situación del sector del olivar que hacen necesario el desarrollo científico propuesto. Concretamente, en el **capítulo I** se presenta una introducción general de esta tesis doctoral, exponiendo la hipótesis y los objetivos de este estudio. El **capítulo II**, consiste en una revisión de literatura que destaca el potencial biotecnológico de los hongos basidiomicetos en la producción de enzimas bajo suplementación con Cu y Mn, dada su mayor relevancia en la bibliografía, lo que podría mejorar significativamente la biodegradación y la obtención de productos derivados de la biomasa. La revisión aporta información sobre el efecto de Cu y Mn en la actividad enzimática y describe los mecanismos de interacción metal-hongo que justifican los efectos sobre la actividad enzimática.

En el **capítulo III**, se describe la evaluación de la aplicación directa de *A. discolor* y *S. hirsutum* como un pretatamiento biológico para el alperujo. Los resultados determinaron que no hay necesidad de agregar nutrientes suplementarios o una fuente de carbono exógena, como tampoco utilizar sistemas de agitación. Las variables evaluadas fueron el crecimiento de los

microorganismos, la actividad enzimática, la remoción de compuestos fenólicos y la degradación de lignina, con el fin de determinar las condiciones apropiadas para mejorar la biodegradabilidad del alperujo. Los resultados demostraron por primera vez que *A. discolor* y *S. hirsutum* fueron capaces de degradar lignina (94,1% y 94,4% después de 24 días de tratamiento, respectivamente) y eliminar compuestos fenólicos del alperujo (más del 80% con ambas cepas) utilizándolo como único sustrato.

A continuación, en el **capítulo IV**, se explica el efecto de diferentes dosis de Cu y Mn agregadas en el alperujo inoculado con *A. discolor* y *S. hirsutum*. La actividad enzimática ligninolítica, la degradación de lignina y la remoción de compuestos fenólicos fueron las principales variables reportadas. Este capítulo se llevó a cabo para mejorar las condiciones de biodegradabilidad del alperujo previamente descritas en el Capítulo III. Las dosis óptimas de metales aumentaron la actividad enzimática dependiendo de la cepa. Un efecto positivo asociado con la adición de Mn podría mejorar la actividad de MnP en ambas cepas. Los mejores resultados en términos de degradación de lignina (41.5±3.2%) se lograron utilizando *S. hirsutum* con la adición de 2.5 mg Kg^{-1} de Cu y 7.3 mg Kg^{-1} de Mn, mejorando la degradación en un 18% en comparación con el tratamiento sin adición. Esta cepa también logró una eficiencia estable de remoción de fenoles del 65-75% en el alperujo a los 25 días, mientras que, en su lixiviado, los valores fueron del 80-95% a los 30 días, donde no se observaron diferencias representativas con la ausencia o presencia de Cu y Mn.

En el **capítulo V** se evalúa la valorización del alperujo mediante la recuperación de compuestos de alto valor añadido como extractos enzimáticos, ácidos grasos volátiles (AGV) y/o compuestos fenólicos mediante la aplicación de un tratamiento fúngico con *A. discolor* y *S.*

hirsutum con adición de metales, determinando simultáneamente la posible reducción de la fitotoxicidad del alperujo. La actividad de la enzima lacasa de ambos hongos fue mejorada con la adición de Cu-Mn en aproximadamente la mitad del tiempo evaluado (88.7% en el día 15 por *A. discolor* y 32.3% en el día 20 por *S. hirsutum*). La adición también incrementó la actividad MnP de *S. hirsutum* en un 97.2%. En cuanto a la liberación de fenoles desde el alperujo a una fracción líquida, *S. hirsutum* mostró una mayor capacidad de liberación (6001 ± 236 mg eq L^{-1} de ácido gálico) en comparación con *A. discolor*. Esta liberación de fenoles a una fase líquida permitió la defenolización parcial de la fase sólida del alperujo. Concretamente, en dichas fases sólidas, *A. discolor* permitió un 40.8% de reducción de los fenoles, mientras que *S. hirsutum* alcanzó solo el 20%. *S. hirsutum* fue capaz de generar una mayor concentración de AGVs (1627±325 mg L^{-1}), principalmente ácidos isobutírico y acético, que *A. discolor*. Con el alperujo tratado, la fracción sólida mostró una fitotoxicidad ligeramente menor que el alperujo crudo para ambas cepas, aunque a dosis de alperujo superiores al 10% aun presentaron una fitotoxicidad muy severa.

Finalmente, en el **capítulo VI** se discuten todos los resultados obtenidos. Por lo tanto, esta tesis doctoral concluye que (1) *A. discolor y S. hirsutum* son dos posibles hongos de pudrición blanca que se pueden usar para el futuro diseño de un tratamiento in situ para la valorización del alperujo. (2) La adición de Cu y Mn permite el aumento de enzimas específicas, lo que abre una oportunidad para diseñar suplementos específicos según la aplicación del hongo. Además, la adición de Cu-Mn está fuertemente asociada con la cepa, mostrando una selectividad de degradación asociada con la fisiología de la degradación de fenoles por las cepas estudiadas. (3) *S. hirsutum* es un hongo prometedor para recuperar compuestos de valor agregado como ácidos grasos volátiles y compuestos fenólicos desde el alperujo.

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CHAPTER I

General Introduction

1.1 Introduction

The escalating global population's heightened energy and food demands have led to a significant surge in waste production, with food and green waste accounting for 44% of this waste stream (Chen *et al*., 2021). These waste components, when released into the environment, degrade slowly and often without detoxification, resulting in pollution and endangerment of ecosystems and wildlife (Seah *et al*., 2023). Annually, an estimated 140 billion tonnes of biomass waste are generated globally from the agricultural sector, with only 40% being utilized for fuel, feed, and power generation. At the same time, the majority is incinerated, presenting a grave environmental and climate hazard (Tripathi *et al*., 2019).

Lignocellulosic biomass, comprising forestry and agricultural residues, represents the most abundant biomass type, with an annual production of 181.5 billion tonnes (Dahmen *et al*., 2019). However, a mere 8.2 billion tonnes are effectively utilized, leaving a substantial portion underutilized and subject to landfilling, open dumping, or incineration (Rouches *et al*., 2016). This vast biomass resource allows biorefineries to derive added-value products, including carbohydrate-enriched ruminant feed, chemical compounds, fertilizers, and biofuels (van Kuijk *et al*., 2016). Leveraging this biomass in biorefineries holds significant potential for sustainable resource utilization and producing valuable commodities (Wan & Li, 2012). The challenge posed by the molecular structure of lignin, coupled with its robust association with cellulose and hemicellulose, significantly hinders the acquisition of added-value products from lignocellulosic biomass (Dhiman *et al*., 2024). In response, biological treatments have emerged as a promising form for enhancing the utilization of lignocellulosic biomass in bioprocesses, offering a more energy-efficient alternative to physical, chemical, or thermal treatments (Bilal & Iqbal, 2020).

Specifically, WRF and their extracellular enzymatic machinery, including laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP), have been proposed as effective agents for delignifying lignocellulosic residues (Ntougias *et al*., 2015; Shirkavand *et al*., 2017; Suryadi *et al*., 2022). These biological approaches hold significant promise for enhancing the valorization of lignocellulosic biomass and advancing sustainable bioprocessing techniques.

Despite the potential of fungal treatments to biodegrade lignocellulosic waste and generate value-added by-products, some challenges persist in enhancing bioprocesses. These challenges included understanding in detail how to produce these compounds in a continuous way from wastes and improve the quality of enzymes or crude extracts in stages before obtaining the final product to maximize the content, stability, yields, productivity, and recovery for its final purpose, and define which variables must be controlled to develop effective and optimal bioprocesses. Also, it is imperative to ensure that residual fractions post-treatment yield valuable materials in organic amendments, with minimal risk for final disposal and containing low or no toxic compounds, such as polyphenols. Hence, there is a concerted effort to promote the management of biomass resources and advocate for zero-waste practices (Dahmen *et al*., 2019).

Some strategies to optimize processes in terms of enzyme activity enhancement include the addition of organic and inorganic inducers in the culture medium (Ellegaard-Jensen *et al*., 2014; Gutierrez-Correa *et al*., 1999), and/or the use of co-substrates like lignocellulosic biomass (Atilano-Camino *et al*., 2020; Majul *et al*., 2020; Mishra *et al*., 2017b). Particularly, inducers such as Cu, Mn, and Fe, including others play an essential role in improving the activities of ligninolytic enzymes in WRF (Acevedo *et al*., 2011; Baldrian, 2003; Hermosilla *et al*., 2017;

Reddy & Kanwal, 2022). Concretely, Cu plays a wide variety of functions mainly involving electron transfer, O^2 binding, activation, and reduction, NO^{2-} and N_2O reduction, and substrate activation (Solomon *et al*., 2014). In WRF, Cu plays an essencial role in the lignin degradation process, serving as a cofactor in the laccase catalytic center (Akpinar & Ozturk Urek, 2020; Baldrian, 2003). On its side, Mn has been shown to play a critical role in the transcriptional phase in WRF as well as in the activation of the peroxidase enzyme and directly participates in the process of lignin degradation through the reaction cycle of Mn-dependent peroxidase (MnP) (Baldrian, 2003). Culture supplementation with lignocellulosic biomasses can also produce many inducers during fermentation processes (Rocha *et al*., 2024). This transformation by-products, combined with laccases, can significantly improve the potential of synergic inducers-enzymes to produce enzyme extracts and degradation of recalcitrant organic pollutants (Atilano-Camino *et al*., 2020). In addition, the use of lignocellulosic biomasses supporting growth to various fungal strains has been tested to enhance the growth, colonization, and increase of the enzymatic activity and, with this, to enhance the efficiency of removal of recalcitrant lignin (Mishra *et al*., 2017b).

During olive oil extraction in two-phase processes, a semi-solid byproduct known as olive mill solid waste (OMSW) is produced. This byproduct is a complex matrix comprising olive husk, olive pulp, and olive vegetation water (Caroca *et al*., 2021; Serrano *et al*., 2017). OMSW exhibits a high lignocellulosic content, with approximately 35.2 ± 3% cellulose, 33.4 ± 4% lignin, and 45.5 ± 8% hemicellulose, along with a variable water content ranging from 60% to 95% (Serrano *et al*., 2021). These lignocellulosic fibers and polyphenols make OMSW a valuable substrate (Caroca *et al*., 2021; Serrano *et al*., 2017). However, polyphenols in OMSW confer strong antimicrobial properties, posing challenges for its valorization through microbe-mediated processes (Fernández-Prior *et al*., 2020). Additionally, these polyphenols are primarily

responsible for the phytotoxic effects associated with OMSW (Aranda *et al*., 2007; Reina *et al*., 2013). In addition, the incorrect management of OMSW has several deleterious environmental effects, such as coloration of natural waters, toxicity to aquatic life, degradation of soil quality by inhibiting plant germination and growth, phytotoxicity, and the generation of nuisance odors (Genethliou *et al*., 2020; Sounni *et al*., 2021). Alternatively, OMSW can also be used as a substrate or added on culture media to function as a support growth and promoter of extracellular ligninolytic enzymes from fungi as WRF mainly, and at the same time achieving higher levels of OMSW biodegradability (Benavides *et al*., 2022).

The olive oil industry in Chile, with an annual production of 16,500 tons in small areas, is projected to expand (ChileOliva, 2022). However, this growth brings environmental issues generated by residues that need to be solved, mainly in the current management of olive mill solid waste (OMSW) and the associated limitations of smaller farms. Therefore, the pretreatment application to enhance two-phase OMSW biodegradability and remove phenolic compounds is needed to reduce environmental issues generated by this industry. Reduce the amount of waste generated can be possible through biotechnological processes with ligninolytic enzymes from white-rot fungi, especially when they are promoted by mediators as metals because the ligninolytic capacity of these enzyme complexes can be a practical approach from the point of view of bioremediation to develop sustainable procedures in the recalcitrant waste treatment.

This doctoral thesis focused on advancing a novel technology based on fungal mechanisms for the biodegradation of OMSW, aiming to enhance its treatment and valorization. Introducing a fungal treatment incorporating Cu and Mn as inducers/cofactors marked a pioneering evaluation of OMSW. This study presented a novel approach by exploring enzymatic

extract production from indigenous WRF sourced from the forests of southern Chile, specifically targeting OMSW biodegradability and the conversion of waste biomass. Notably, the research underscored the potential for leveraging OMSW for agronomic applications post-biological treatment while outlining initial guidelines for a biorefinery concept and the valorization of derived products from OMSW utilizing *A. discolor* and *S. hirsutum*.

1.2 Hypothesis

Considering that:

OMSW management remains a significant environmental challenge within the olive oil industry.

The biodegradability of lignocellulosic biomasses correlates with the degree of lignocellulosic component degradation and reduction in polyphenol compounds.

Effective treatment of OMSW is essential to enhance its biodegradability and facilitate the production of bio-based products like biofertilizers.

Supplementation with inducers/cofactors such as Cu and Mn enhances enzymatic activities and supports lignin degradation in enzyme production.

Combining various enzymes into enzymatic extracts enhances the efficacy of enzymatic treatments.

Lignin degradation and phenol removal play crucial roles in enhancing organic matter and nutrient availability while reducing the phytotoxicity of lignocellulosic waste.

Therefore, this study's hypothesis posits that a biological treatment utilizing the synergistic action of white-rot fungi and cofactors such as Cu and Mn will enhance the OMSW biodegradability and, in turn, provide an organic matter and nutrient source for soil enrichment.

1.3 General objective

To develop an olive mill solid waste treatment using the action of native fungi such as *Anthracophyllum discolor* and *Stereum hirsutum* supplemented with Cu and Mn as enzyme inducers for improving biodegradability.

1.4 Specific objectives

1.4.1 To evaluate the combined effect of the white-rot fungi *Anthracophyllum discolor* and *Stereum hirsutum* and the use of Cu and Mn cofactors in enzyme production with ligninolytic potential.

1.4.2 To determine the operational conditions to improve the biodegradability of olive mill solid waste (OMSW) by using the white-rot fungi *Anthracophyllum discolor* and *Stereum hirsutum* and cofactors in a combined treatment.

1.4.3 To evaluate the feasibility of using OMSW pretreated as an organic matter and nutrient source to the soil.

1.5 Theoretical framework

1.5.1 Lignocellulosic biomass

Lignocellulosic biomass is an important renewable carbon source in recent decades, it has received special attention in the field of research for its use and valorization within the framework of global policies for waste treatment and sustainability (Chaudhary *et al*., 2023). The cell wall represents over 80% of the dry weight of lignocellulosic biomass, where cellulose, hemicellulose, and lignin are its primary constituents that can be fundamental structural components in various industrial and environmental processes (Dahiya *et al*., 2024). Cellulose represents the predominant component of lignocellulosic biomass, characterized as a linear homopolymer consisting of (1–4)-linked β-D-glucose units, whose β 1-4 bonds can only be cleaved by the exclusive action of microbial enzymes (Dhamaniya *et al*., 2018). Hemicellulose, constituting approximately 25% of the dry weight of lignocellulosic biomass, consists of a linear backbone with branched chains comprising 5 and 6 carbon sugar compounds (L-arabinose, D-glucose, D-mannose, D-xylose, L-galactose) (Fehér, 2018). Its structure and composition vary across taxonomic orders and even within specific tissues (Kubicek, 2012). As a bonding agent between lignin and cellulose, hemicellulose exhibits faster hydrolysis than cellulose due to its amorphous polymer nature. However, hemicellulose degradation is often limited due to its susceptibility to lignification (Abramson *et al*., 2010). Lignin is a complex aromatic macromolecule formed through the oxidative coupling of three primary hydroxy cinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohol. These alcohols give rise to corresponding phenylpropanoid units: p-hydrophenyl, guaiacyl, and syringyl, depending on the methoxy substitution on aromatic rings (Kai *et al*., 2018). Arranged randomly in an irregular three-dimensional network, these phenylpropanoid units provide lignin with its structural strength (Kai *et al*., 2018). Lignin interacts with cellulose by forming covalent cross-links and with hemicelluloses through diferulic acid bridges. Despite constituting a minor component of

lignocellulosic biomass (18-24% dry weight), its aromatic structure and strong association with cellulose and hemicellulose significantly hinder its effective degradation (Kubicek, 2012).

1.5.2 Two-phased olive mill solid waste (OMSW)

The growth of the olive oil industry is inherent in Chile, with a planted area of 21141 hectares and an annual production of 16500 tons (ChileOliva, 2022), it is projected as a growing industry. However, this production brings environmental issues generated by solid and liquid wastes. Two-phased olive mill solid waste (OMSW) is the main by-product generated during the extraction of olive oil by two-phase centrifugation system (Fernández-Rodríguez *et al*., 2019). Their high toxicity and low biodegradability characterize this waste (Ouazzane *et al*., 2017). In addition, OMSW, also has a high organic content and C/N ratio (Fernández-Rodríguez *et al*., 2019). Approximately, 800 kg of the OMSW are still produced for each tonne of milled olive (Fernández-Rodríguez *et al*., 2019). A current management of this waste is the direct application to soil like amendment, causing several toxicity problems when the doses increase, which can affect the soil properties, the normal growth crops and to change water quality (Alfano *et al*., 2011). Much of this waste, sometimes is accumulates in different oil presses without a clear destination, making the problem more serious. Several experimental treatments for OMSW have been evaluated, to make it re-usable as fertilizer and irrigation water or to obtain add-value compounds such as, proteins, minerals, and polysaccharides (Pantziaros *et al*., 2018; Sousa *et al*., 2018). Although OMSW is highly phytotoxic and contain phenolic compounds, it also contains high percentages of organic matter (OM) and a vast range of nutrients that could be reused as fertilizers for sustainable agricultural practices (Chowdhury *et al*., 2013). However, to produce suitable OM and an ideal final C/N ratio (10–20%), OMSW should be pretreated using the above-mentioned strategies. Moreover, is very important that OMSW have low concentrations of

organic compounds such as polyphenols, lignin, cellulose, hemicellulose, and pectin, mainly and a small surface area and particle size, together with a high Total Nitrogen Content concentration (Chowdhury *et al*., 2013; Tüzel *et al*., 2020). The treatments application to enhance its biodegradability and to remove phenolic compounds are indispensable (Pavel *et al*., 2013). Some research has highlighted vital factors such as temperature, pH, electrical conductivity (EC), moisture, bulk density, porosity, particle size, organic carbon (C), nitrogen (N) content, carbon to nitrogen ratio (C/N), and oxygen supply, to improve the composting process, because they regulate conditions for microbial growth and development, and organic matter (OM) decomposition (Chowdhury *et al*., 2013). In addition, phytotoxicity is an essential parameter, because is necessary to detect any substance capable of generating temporary or long-term stress on the germination capacity of seeds, roots growth and dry matter evolution (Pinho *et al*., 2017).

1.5.3 Biological treatments to degrade lignocellulosic waste and removal pollutant compounds

Several treatments to degrade lignocellulosic waste have been proposed as a solution to environmental issues generated by waste and to promote the use and valorization of the different types of lignocellulosic biomass. Physical pre-treatments such as the use of thermal gradients (Almansa *et al*., 2015; Rincón *et al*., 2013) and ultrasound application (Rincón *et al*., 2014) had been evaluated; chemical pre-treatments using hydrogen peroxide (Siciliano *et al*., 2016), the combined coagulation / flocculation with oxidation process of Fenton (Pekin *et al*., 2010) and alkaline pre-treatment (Pellera *et al*., 2016) also had been tested. Biological treatments for lignin degradation and phenolic compounds removal also had been studied widely (Duarte *et al*., 2014; Pointing, 1999; Rubilar *et al*., 2011; Singh, 2021). In recent years concerning deploying enzyme bio-catalysis, the ligninolytic fungi and their enzymes have appeared as alternative for

pretreatment processes, in particular interest to valorize the lignocellulosic biomass (Bilal & Iqbal, 2020). These biotechnological technologies have been useful in two processes delignification, and detoxification. In delignification, ligninolytic fungi or their enzymes have been used to reduce the lignin content in different types of feedstocks (Salvachúa *et al*., 2011; Vivekanand *et al*., 2008; Wan & Li, 2011), whereas, in detoxification, have been used in the removal of chemical compounds such as phenolic compounds and xenobiotics (Plácido & Capareda, 2015; Tortella *et al*., 2005, 2008).

The main groups of ligninolytic enzymes include lignin peroxidases (LiP; EC 1.11.1.14), manganese peroxidases (MnP; EC 1.11.1.13), versatile peroxidases (VP; EC 1.11.1.16), and laccases (Lac; EC 1.10.3.2) which are responsible for facilitating the degradation of phenolic and non-phenolic compounds (Wong, 2009). Lignin peroxidase (LiP) is responsible for catalyzing the H_2O_2-dependent oxidative depolymerization of lignin, is relatively nonspecific to its substrates and is used in the treatment of as well as a range of organic compounds (Wong, 2009); MnP catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}, being Mn^{3+} a small size compound with high redox potential, which diffuses readily in the lignified cell wall facilitating the penetration and action of the other enzymes (Plácido & Capareda, 2015); VP is a hybrids of lignin peroxidase and manganese peroxidase with a bifunctional characteristic (Wong, 2009), this enzyme group exhibit activities on aromatic substrates similar to LiP including veratryl alcohol, methoxybenzenes, and lignin model compounds and it is not only specific for Mn(II) as is the case of MnP (Rodakiewicz-Nowak *et al*., 2006). Laccase, has been found to oxidize non-phenolic model compounds and β-1 lignin dimers in the presence of a mediator, which plays a significant role in depolymerization of lignin and delignification (Bourbonnais & Paice, 1990), mainly, the fungal laccase is important in detoxification of phenol compounds produced during lignin

degradation, which through the synergistic action with peroxidases and other enzymes perform in the breakdown of lignin (Eggert *et al*., 1996).

Enzymes such as laccase and different peroxidases from fungi, have been studied widely for the treatment of lignocellulosic wastes (Dhiman *et al*., 2024). In this sense, there is a robust database of information about the characteristics and performance of enzymes. Some researchers have shown particular interest in the study of enzymatic extracts from microorganisms to metabolize lignocellulosic materials applied to different residues such as wheat (Rodrigues *et al*., 2008), cellulose-pulp (Soares *et al*., 2018), palm oil (Hafid *et al*., 2021), corn stover (Ding *et al*., 2019), and sweet sorghum bagasse (Mishra *et al*., 2017a), among many others. However, it is known that the enzyme systems are much more complicated in comparison with individual enzymes, so the full spectrum of effectors involved in the degradation of material lignocellulosic remains unknown yet (Vrsanska & Langer, 2016).

1.6 References

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CHAPTER II

Addition of copper and manganese for improving white rot fungi enzymatic activity -*Literature Review*-

Manuscript in preparation.

Addition of copper and manganese for improving white rot fungi enzymatic activity -

Literature Review-

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2.1 Introduction: Ligninolytic enzyme inducers/cofactors

White-rot fungi (WRF) are microorganisms with a high biotechnology potential thanks to their capability of producing extracellular oxidative enzymes for the degradation of lignocellulosic fibers (Kissi *et al.*, 2001; Ntougias *et al.*, 2015). These extracellular

lignocellulolytic enzymes are influenced by heavy metals both at the transcriptional level and during their function. For instance, during the breakdown of lignocellulosic compounds and xenobiotics by WRF and/or their enzymatic extracts, heavy metals can disrupt the activity of extracellular enzymes and fungal colonization (Baldrian, 2003). Although certain metals like manganese and copper are essential for the life of all organisms, they may not always be present in sufficient bioavailable levels for fungi due to their limited concentrations in aerobic and aqueous environments. Therefore, it is sometimes necessary to supplement these metals to meet the physiological requirements of the fungi (Vrsanska & Langer, 2016).

In practical terms, incorporating different inducers and cofactors has proven essential for boosting the activities of lignin-degrading enzymes in fungi, being basidiomycetes the phylum more representative in the works reported in this review. Several cases show different roles of each metal added. Metals considered as inducers act as mediators, expediting the conversion of numerous compounds and thereby enhancing reaction kinetics through increased enzymatic activity (da Silva-Vilar *et al*., 2022). Otherwise, cofactors, operating at the transcriptional level, constitute integral components of enzyme architecture, serving diverse functions including electron transfer, O_2 binding, activation and reduction, NO_2^- and N_2O reduction, and substrate activation (Sekretaryova *et al*., 2019). The effectiveness of metal supplementation is typically evaluated based on the dosage employed. However, it's worth noting that the microbial response may be more influenced by bioavailable metal fractions or, if substrates contain a significant metal concentration, by the total metal concentration in the medium (Gustavsson *et al*., 2013; Pinto-Ibieta *et al*., 2016). Defining the mechanism of action of different metals in their interaction with fungal strains can help optimize the addition of metals as a strategy to improve fungal

enzymatic activity, being able to explain the processes in a deeper way than a cause-effect relationship.

2.2 Scope of the review

The review aims to provide information both on the effect on enzymatic activity and to offer a detailed view of the metal-fungi interaction mechanisms that justify the effects on enzymatic activity. Given their greatest relevance in the literature, the metals reviewed have been copper and manganese. The review also includes a discussion of the effect of metal addition depending on the type of substrate used, differentiating between synthetic models and lignocellulosic substrates.

This review also highlights the biotechnological potential of basidiomycete fungi in enzyme production under metal supplementation, which could significantly enhance the biodegradation and the obtaining of biomass-derived products. Additionally, it provides a summary of the research conducted over the last two decades, underscoring basidiomycetes as promising candidates for biotechnological applications.

2.3 Copper

Copper is an essential micronutrient for fungal growth, acting as a metal activator of fungal enzymes like oxidases. On the basis of its generally accessible I/II redox couple and bioavailability, copper plays a wide variety of functions in nature that mostly involve electron transfer, O_2 binding, activation, and reduction, NO_2^- and N_2O reduction, and substrate activation (Solomon *et al.*, 2014). Concretely in WRF, copper plays an important role in the lignin

degradation process serving as a cofactor in the laccase catalytic center (Baldrian, 2003). Laccases (EC 1.10.3.2) catalyze the oxidation of a wide variety of organic and inorganic substrates including phenols, ketones, phosphates, ascorbate, amines and lignin (Gianfreda *et al*., 1999). In fungi, laccases are transcriptional regulators and they carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen/host interaction, stress defense, and lignin degradation (Giardina *et al*., 2010; Mehra *et al*., 2018). Laccase (Lac) is considered an ideal *"green catalyst"* because of its oxidizing property is a vast variety of compounds using O_2 and liberating H_2O as the only by-product (Giardina *et al*., 2010).

Fungal laccases are extracellular and monomeric glycoproteins with ~520–550 amino acids and a typical weight of ~60–70 kDa in their glycosylated form (Giardina *et al*., 2010) and have accounted for the most important group of blue multicopper oxidases with respect to number and extent of characterization (Giardina *et al*., 2010). These enzymes are multi-copper oxidoreductases that catalyze the one-electron (e^-) oxidation of the substrates and sequentially transfer four electrons to the catalytic copper atoms, which are used to reduce O_2 to two water molecules (Mehra *et al*., 2018; Solomon *et al*., 2014). The copper architecture has two separate copper sites: a mononuclear site (T1 copper ion) and a trinuclear site (a cluster of T2, T3, and T3´ copper ions). Reducing substrates are oxidized close to the mononuclear site and then electrons are transferred through the Cys-His pathway into the trinuclear site where dioxygen is reduced to water molecules (Hakulinen & Rouvinen, 2015). These oxidases are unique in that they effectively perform this reaction in one step, indicating that the free energy barrier for the second two-electron reduction of the peroxide product of the first two-electron step is very low (Solomon *et al*., 2014).

According to the reviewed literature, the cooper addition in a range between $1.6x10^{-8}$ and 150 mM had permitted to enhance of ligninolytic enzyme activity, being the Lac, the main enzyme reported in the literature followed by manganese peroxidase (MnP) (Table 1). The supplementation of copper has yielded notable enhancements, ranging from 14.3% to 100% in Lac activity and 34.1% to 100% in MnP activity, as delineated in Table 1. However, the improvements are variable and depend on other parameters such as the strains, the culture medium and operational conditions (Table 1). Several studies have underscored the impact of different copper dosages added to culture media on the production of ligninolytic enzymes across a diverse array of fungi, particularly those within the basidiomycetes group. For instance, the addition of a minimal dose of $1.6x10^{-8}$ mM copper to Glucose-peptone culture media elicited a 20% improvement in Lac activity from *Grammothele fuligo* (Chauhan, 2019), while supplementation of lignocellulosic culture media with 0.007 mM copper resulted in a 37.4% enhancement in MnP activity from *Pleurotus eryngii* (Akpinar & Ozturk Urek, 2022). Likewise, the addition of 0.19 mM copper to the culture medium comprising Potato Dextrose Broth (PDB), wood chips, and rice bran led to a 61.3% increase in laccase activity by *Ganoderma lucidum* (Liu *et al*., 2020). These authors also demonstrated varying sensitivities to copper supplementation, highlighting that excess copper does not significantly contribute to laccase production. Additionally, Jain *et al*., (2019) demonstrated that the inclusion of 7 mM copper in malt extract broth culture medium resulted in a remarkable increase of up to 87.2% in laccase activity from *G. lucidum* compared to cultures without this metal.

Among the extensive array of fungi documented in the literature, Basidiomycetes stand out for their renowned capability to degrade highly recalcitrant wood constituents, including lignin. This group is also prominently investigated for enzyme production and agricultural waste treatment, with particular attention given to assessing the effects of copper, as outlined in Table

1. This review identified twelve important genera: *Pleurotus, Grammothele, Pycnoporus, Dichomitus, Ceriporiopsis, Lentinus, Ganoderma, Trametes, Coriolus, Daedaleopsis, Phlebia* and *Fomes.* Within this group, some fungi such as *Trametes versicolor* (Bucić-Kojić *et al*., 2017), *Tramates pubescens* (Galhaup & Haltrich, 2001), *Phlebia radiata* (Mäkelä *et al*., 2013), *Fomes fomentarius* (Vrsanska & Langer, 2016), *T. suaveolens* (Vrsanska & Langer, 2016), *G. lucidum* (Kuhar & Papinutti, 2014), *T. versicolor* (Vrsanska & Langer, 2016), *Daedaleopsis confragosa* (Vrsanska & Langer, 2016), *Trametes gibbosa* (Vrsanska & Langer, 2016), and *Trametes hirsuta* (Andriani *et al*., 2020) have demonstrated the highest enhancements in Lac activity, with improvements of more than 90% respect to the Lac activity without copper addition. Moreover, MnP activity has also been extensively enhanced, reaching improvements above 95%, for different fungal strains such as *T. hirsuta* (Andriani *et al*., 2020), *T. versicolor* (Vrsanska & Langer, 2016), *D. confragosa* (Vrsanska & Langer, 2016), *F. fomentarius* (Vrsanska & Langer, 2016), and *T. gibbosa* (Vrsanska & Langer, 2016).

Several authors have evaluated the supplementation of copper using complex lignocellulosic biomasses as substrates (Akpinar & Ozturk Urek, 2020; Andriani *et al*., 2020; Bucić-Kojić *et al*., 2017; Merino *et al*., 2019; Mishra *et al*., 2017; Slavens *et al*., 2019). In these studies, the effect of the addition of copper would be more limited in comparison to the use of synthetic mediums because i) enzymatic activity was already favored by the presence of the lignocellulosic fibers (Chepyala *et al*., 2024), and ii) the substrate would already contain relevant concentrations of copper that makes less necessary an external metal contribution. However, there are examples where the combination of synthetic mediums such as malt extract agar (MEA), glucose-yeast extract-peptone (GYP), malt extract broth (MEB), or potato dextrose broth (PDB) with lignocellulosic biomasses has led to large improvements in enzyme activity.

Relevant improvements in the fungal enzymatic activities by copper addition using lignocellulosic substrates has been reported in the literature. For example, cassava waste supplemented with 0.5 mM of copper (Kumar *et al*., 2022), corn silage supplemented with 100 mM of copper (Bucić-Kojić *et al*., 2017), and sorghum supplemented with 0.1 mM of copper (Andriani *et al*., 2020) lead to enhancements in Lac activity of more than 80% by *P. ostreatus*, *T versicolor* and *T. hirsuta*, respectively.

Despite the advantages mentioned above, it should be noted that fungal growth can be significantly hindered by high copper concentrations and, thus, lead to unfavorable reactions in the bioprocesses being carried out. For example, stress conditions due to copper toxicity can trigger a more prolonged adaptation phase in the microorganism, as demonstrated by studies on *T. pubescens* (Galhaup & Haltrich, 2001). That can be possibly explained by the fact that once the microorganism has colonized most of the substrate or culture medium, it undergoes, to a lesser extent, a growth inhibition due to copper consumption and bioadsorption in the mycelium (Galhaup & Haltrich, 2001). Moreover, the copper supplement in excess may delay the enzyme kinetics, due to a possible consequence of changes to the pH in the substrate or due to effects on microorganism growth on the substrate portion not colonized. Likewise, this addition can cause the production of other compounds like organic acids that may inhibit catalysis by ligninolytic enzymes (Milagres *et al*., 2002). In that sense, Mäkelä *et al*., (2013) reported that 0.5 mM L^{-1} of Cu suppressed the production of MnP from *P. radiata* grown under semi-solid conditions. Likewise, Gassara *et al*., (2010) associated *Phanerochaete chrysosporium* loss of viability with the inhibition of MnP as a consequence of the toxicity exerted by the addition of copper to a solid-state fermentation process.

These negative effects can be controlled in some cases thanks to microorganisms have developed different strategies to mitigate excess copper in the medium. For example, to avoid the long periods of adaptation that can occur, it has been shown that copper addition must occur at the beginning of the exponential phase to achieve satisfactory levels of Lac activity (Baldrian, 2003). Thus, the enzyme activity can be restored in the time, under conditions of solid-state fermentation, mainly, given the ability of WRF to use organic compounds generated in the process in various metabolic pathways, and consequently preventing the copper and new compounds accumulation (Milagres *et al*., 2002). According to Merino *et al*., (2019) in solid state fermentation using corn cob, the addition of copper on fungus adaptation phase can reduce the toxicity due to the microorganism has grown enough. In this way, the deleterious effects of copper on microorganism growth are prevented, and hence, copper act as an inducer in ligninolytic enzyme production during this time. Another reported natural strategy of the fungi to mitigate the toxic effect of copper is the natural pigments generation as melanin, which was demonstrated in *T. versicolor* to the see that progressively decreased its radial growth and biomass production in the time with the copper addition and the subsequent color changes in the fungus (Guillen & Machuca, 2008). However, this strategy can have a limited applicability in the biotechnology application of fungi.

Table 1. Enzyme activity in presence of copper as an inducer.

Strains	Culture medium/substrate	Added concentration (mM)	Doses defined as optimal (mM)	Lac (U/L) *ABTS **2,6DMP ***Other	Improvement with respect to control (%)	MnP (U/L) *ABTS **2,6DMP ***Other	Improvement with respect to control (%)	Reference
T. pubescens MB89	Glucose-based medium	1	1	47500	95.8	NA	ND	Galhaup and Haltrich, 2001
P. radiata	Peptone-yeast extract-glucose-succinate (PYGS) medium+milled alder	0.025, 0.1, 0.5, 1, 1.5, 2, 3		25	98	6.5	0	Makela, *et al*., 2013
P. eryngi F019	Agar nutriente	10	10	1500 *		250	ND	Hadibarata, 2013
Lentinus polychrous	Glucose Yeast Peptone (GYP) m edium	0.1,1.0, 2.0, 3.0	2	2000 *	85	NA	ND	Kammuamg *et al*. 2013
P. ostreatus	Mineral salts	0, 5, 10, 15, 20, 25, 30 and 50	15	NA	ND	NA	ND	Bhattacharya etal. 2014
Dichomitus squalens	1.2% glucose, 0.1% arabinose, 20 mM sodium nitrate, 0.5% casein	0.1, 0.31, 0.25, 0.4,0.5, 0.55	0.5	1815** U/g	65.1	NA	ND	K annaiyan *et al*., 2014
Ceriporiopsis subvermispora	2.5% glucose, 0.05% arabinose, 30 mM sodium nitrate, 0.5% casein	0.1, 0.31, 0.25, 0.4,0.5, 0.55	0.25	1242** U/g	56.5	NA	ND	K annaiyan *et al*., 2014
G. lucidum	Glucose-yeast extract	0, 0.5, 1, 1.5, 2	0.5	1** U/g	100	NA	ND	Kuhar and Papinutti, 2014
T. versicolor	Malt extract liquid medium	0.1, 0.05	0.05	NA		NA	ND	Sari *et al*., 2016
T. versicolor	Potato dextrose broth (PDB)	0.1, 0.3, 0.5 and 1.0	1	1291.7	100	0.09	100	Vrsanska *et al*., 2016
T. suaveolens	Potato dextrose broth (PDB)	0.1, 0.3, 0.5 and 1.0	1	2108.3	98.3	0.08	87.5	Vrsanska *et al*., 2016
D. confragosa	Potato dextrose broth (PDB)	0.1, 0.3, 0.5 and 1.0	1	750	100	0.38	100	Vrsanska *et al*., 2016
F. fomentarius	Potato dextrose broth (PDB)	0.1, 0.3, 0.5 and 1.0	1	1551.4	97.7	0.69	100	Vrsanska *et al*., 2016
T. gibbosa	Potato dextrose broth (PDB)	0.1, 0.3, 0.5 and 1.0	1	156.9	100	0.25	100	Vrsanska *et al*., 2016
Coriolus versicolor	Sweet sorghum bagasse	0, $5.5x10^{-4}$, $5.5x10^{-3}$, $1.1x10^{-2}$, $2.2x10^{-2}$, $4.4x10^{-2}$, 0.11	$1.1x10^{-2}$	NA	ND	NA	ND	Mihra and Jana, 2017
T. versicolor	Corn silage	50, 100, 150	100	1539.4	91	231.5	61.1	Bucic-Kojic *et al*., 2017
G. fuligo	Glucose-peptone medium	0, $1.6x10^{-8}$, $1.6x10^{-7}$, $1.6x10^{-6}$, $1.6x10^{-5}$, $1.6x10^{-4}$,	$1.6x10^{-8}$	653.5***	20	1.65	-50	Chauhan, 2019

		$1.6x10^{-3}$, $1.6x10^{-2}$, 0.16, 1.6, 6.3						
T. versicolor	Corn cob	0, 0.5, 1.0	0.5	18 U/g *	17	3.6 U/g	0	Merino *et al*., 2019
P. ostreatus	Switchgrass	1	1	0.21** U/g	14.3	0.7	-42	Slavens etal., 2019
G. lucidum	Malt extract broth (MEB)	7		0.705	89.2	NA	ND	Jain *et al*., 2020
G. lucidum	Potato dextrose broth+ wood chip + rice bran	0.038; 0.076; 0.114; 0.152; 0.19; 0.228; 0.304	0.19	574.9	61.3	NA	ND	Lui *et al*., 2020
P. eryngii	Cherry waste	0, 0.035, 0.07, 0.1, 0.5, 1, 2	1	3403	79.4	821.36	87.8	Akpinar and Ozturk, 2020
T. hirsuta	Sorghum 2,5%	0,1	0.1	19758.5	99.9	3315	97	Andriani *et al*, 2020
P. ostreatus	10 g of cassava waste moistened with 20 mL of MYG (1% malt extract, 0.4% yeast extract, and 0.4% glucose) and 100 mM acetate buffer (pH 6)	0, 0.25, 0.5, 0.75, 1, 1.25	0.5	15400	87.3	NA	ND	Kumar *et al*., 2022
Pycnoporus sanguineus 2512.	Glucose-casein medium	0, 3.9, 7.8	3.9	64580 *	79	80.72	34.1	De Souza *et al*., 2022
P. eryngii	Peach wastes+Stajic basal medium without Tween 80	0, 0.035, 0.07, 0.1, 0.5, 1, 2, 3, 5	0.07	NA		732.23	37.4	Akpinar and Ozturk, 2022

ND: not determined; NA: not available

2.4 Manganese

Manganese has important roles in fungal biology, i.e., mycelial growth, fruiting body and fungal biomass production being adsorbed and accumulated in different concentrations (Amiri-Sadeghan *et al*., 2023). In WRF, manganese has been shown to play the key role in the transcriptional phase as well as in the activation of the peroxidase enzyme and directly participates in the process of lignin degradation through the reaction cycle of Mn-dependent peroxidase (MnP) (Baldrian, 2003). Manganese peroxidase (E.C.1.11.1.13) is a heme-containing enzyme, which consists of Mn-binding sites, and catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Slavens *et al*., 2019). Manganese binding sites are constituted by three acidic amino acid residues that coordinate Mn^{2+} (i.e. Glu-35, Glu-39, and Asp-179), as revealed in *P. chrysosporium* (Asemoloye *et al*., 2021; Skellam, 2019). The highly reactive Mn^{3+} combines with organic chelating compounds such as oxalic acid and acts as low-molecular-weight, diffusible redox mediator targeting the oxidation of the phenolic components of lignin (Kannaiyan *et al*., 2015). In lignin degradation, chelated Mn^{3+} is diffused into cell wall lignin through the micropores, which are less accessible for larger enzymes (Kojiro *et al*., 2010; Skellam, 2019). MnP has lower redox potential, which was estimated around 1.0–1.2 V (Hofrichter *et al*., 2010). However, its action on non-phenolic lignin is generally mediated by MnP-lipid systems of lipid peroxidation, which generates reactive intermediates of peroxyl radicals (Asemoloye *et al*., 2021).

Manganese has an important role in the regulation of either laccase and/or peroxidases activity causing either induction or suppression of the enzyme activity, respectively, depending on the concentration of nutrients (Slavens *et al*., 2019). Therefore, is possible that, at higher Mn^{2+} concentrations, production of MnP increase and Lac decrease (Swamy & Ramsay, 1999) or the

other way around (Kannaiyan *et al*., 2015). In addition, manganese is demonstrated to play an important role on other physiological activities as the nutritional value during *G. lucidum* growth (Zhang *et al*., 2019).

The manganese addition in a range between 0.1 and 27 mM had permitted to enhance of ligninolytic enzyme activity, being the laccase and MnP, the main enzymes reported in the literature. However, most of the best results has been reported when doses lower than 4 mM were added (Table 2). However, similar to the copper, improvements can be variable and to depend on other parameters such as the strains, the culture medium and/or operational conditions (Table 2). For example, according to Kannaiyan *et al*., (2015), the effects were controlled by the type and relative ratios of carbon and nitrogen sources, as well as the prevailing enzyme in a fungal strain and its nutritional regulation are strain dependent, i.e., the highest peroxidase activity in *C. subvermispora* was enhanced to a greater level in the presence of arabinose and sodium nitrate along with $MnSO_4$.

Some authors have reported that minimal doses of manganese have influence on ligninolytic enzymes production, i.e., when 0.1 mM of manganese were added on sorghum straw, the laccase activity by *T. hirsuta* achieved a maximum value of 10215.6 U L^{-1} while Manganese peroxidase achieved 1280 U L^{-1} (Andriani *et al*., 2020). Likewise, when 0.12 mM of manganese were added at basal medium with 1% glucose and 1.1 mM ammonium tartrate, the values of MnP activity by *P. chrysosporium* were around of 176000 U L^{-1} (Pease & Tien, 1992). In addition, Scheel *et al*., (2000) reported an improvement of laccase activity between 50 and 80% and MnP activity between 93.3 and 100%, both effects with 0.13 mM of manganese added at Hofrichter medium, showing maximum enzyme activity values of 40000 U L^{-1} of laccase by i63-2

(unidentified strain) and 300000 U L^{-1} by *Clytocybula dusenii*. It is worth to note that there is not a consistency in literature respect to the relation between manganese addition and enzymatic response, whereas, usually, manganese addition excess does not significantly impact the enzyme production. From this perspective, when manganese was added on Corncob and Kirk´s medium in a dose of 18.2 mM, the Laccase and MnP activity by *P. chrysosporium* only achieve a value of 290 and 2400 U L^{-1}, respectively (Rodríguez-Couto *et al*., 1998) and to the added 27 mM at medium with glucose, peptone, yeast extract, the MnP activity by *P. ostreatus* (PC9) only achieve a value of 1300 U L^{-1} (Knop *et al*., 2014).

The main phylum reported to the enzyme production and the agricultural waste treatment induced by manganese was Basidiomycota. The most representative genus that stand out in this field are: *Phanerochaete, Pleurotus, Ganoderma, Trametes, Fomes, Nematoloma and Clytocybula* (Table 2). However, the best results to improvement laccase and MnP activity (100%) has been demonstrated using *P. ostreatus* (Slavens *et al*., 2019) and *P. chrysosporium* (Rodríguez-Couto *et al*., 1998). In regard to culture medium, their composition is variable and includes reagents that are very common in the preparation of culture media such as glucose, peptone, yeast extract, mineral salts, and other compounds to the use of complex matrices such as agricultural waste such as corncob, wheat straw, cherry waste, cottonseed hull, wheat bran, switchgrass, sorghum straw and peach waste has been used (Table 2). The last has been used as a growth substrate and supplementary inducer, i.e., when was used corncob supplemented with 18.20 mM of Mn, the laccase and MnP activity by *P. chrysosporium* were increased in a 100% (Rodríguez-Couto *et al*., 1998). Likewise, with switchgrass and 1mM of Mn, the laccase and MnP activity from *P. ostreatus* was increased in a 100% (Slavens *et al*., 2019).

Table 2. Enzyme activity in presence of manganese as an inducer.

Strains	Culture medium/substrate	Added concentration (mM)	Doses defined as optimal (mM)	Lac (U L^{-1}) *ABTS **2,6DMP ***Other	Improvement with respect to control (%)	MnP (U L^{-1}) *ABTS **2,6DMP ***Other	Improvement with respect to control (%)	References
P. chrysosporium	Basal medium with 1% glucose and 1.1 mM ammonium tartrate	0, 0.06,0.03, 0.119, 0.237	0.12	NA	NA	176000***	ND	Pease and Tien, 1992
P. chrysosporium	Basal medium	0, 0.06,0.03, 0.119, 0.237	0.24	NA	NA	90000***	ND	Pease and Tien, 1992
P. chrysosporium	Corncob and Kirk´s medium with modifications	18.2	18.20	290	100	2400	100	Rodriguez etal., 1998
C. dusenii	Hofrichter medium	0.13	0.13	25000	80	300000	96.7	Scheel *et al*, 2000
i63-2	Hofrichter medium	0.13	0.13	40000	75	130000	100	Scheel *et al*, 2000
Nematoloma frowardi	Hofrichter medium	0,133	0.13	10000	50	150000	93.3	Scheel *et al*, 2000
F. sclerodermeus	Glucose, tiamine, asparagine	0.4, 0.5, 0.6, 0.7, 08, 0.9, 1.0	0.70	2508000	ND	212600	ND	Papinutti and Fochiassin, 2003
P. ostreatus	Wheat straw	1	1.00	580000	ND	200000	ND	Aslam and Asgher, 2011
P. ostratus	Wheat straw Kirk´s medium with modifications	1	1.00	455110*	ND	210770**	ND	Slam, 2011
P. ostreatus (PC9)	Glucose, peptone, yeast extract	27	27.00	NA	ND	1300**	ND	Knop *et al*., 2014
P. ostreatus	GYP medium.	0.2, 0.5	0.20	159.1	ND	NA	ND	Rui *et al*., 2017
P. eryngii	Cherry waste +basal medium	0.18, 0.25, 0.5, 0.75, 1	0.75	43761,33	45,87	NA	ND	Akpinar *et al*., 2017
P. ostreatus HAUCC162	GPY medium	0.2 0.5	0.20	160*	93.75	NA	ND	Rui *et al*., 2017
G. lucidum	Cottonseed hull (90%), wheat bran (5%), cornfl our (4%), and gypsum (1%)	0.295, 0.591, 0.887, 1.183, 1.479, 1.775, 2.071	1.18	NA	ND	82.4***	62.8	Zhang *et al*., 2019
P. ostreatus	Switchgrass	1	1.00	6	100	26**	100	Slavens *et al*., 2019
P. eryngii	Cherry waste +basal medium	0.18, 0.25, 0.5, 0.75, 1	0.18	4677*	ND	2064**	ND	Akpinar *et al*., 2020
T. hirsuta	sorghum straw	0,1	0.10	10215.6	ND	1280	ND	Andriani *et al*., 2020
P. chrysosporium	Norfloxacin, glucose, ammonium tartrate and mineral salts	0, 0.21, 0.66, 1.5, 4.0	4.00	1249.6	95.8	7398.2	89.6	Chen *et al*., 2021
Microbial comunities from soil	Wheat straw, urea, and soil suspension	2.2, 8.7, 17.5	2.20	7000*	40	1900**	36.8	Jin *et al*., 2021
P. eryngii	Peach waste +basal medium	0.18, 0.25, 0.5, 0.75, 1	0.75	NA	ND	5036.82	ND	Akpinar *et al*., 2022

ND: not determined; NA: not available

2.5 Conclusions

The main conclusions of the reviewer literature are:

- Metals are a very interesting strategy to improve the enzymatic activity of ligninolytic fungi, reported especially in the phylum Basidiomycota, and can act through different interrelated mechanisms.
- It is difficult to define a single optimal metal addition range since it depends on many factors, among others the fungal strain or the type of substrate used.
- In this sense, enzymatic activity is favored by the use of substrates with lignocellulosic fibers, thus seeing that the effect of adding metals with respect to the control is not as significant as in the case of synthetic substrates.
- This may also be because the substrate would already contain relevant concentrations of copper that makes less necessary an external metal contribution. Thus, the quantification of the concentration of metals in the substrate is necessary for an accurate monitoring of bioavailable metals for fungi in these assays.
- Despite this, synergistic effects can be observed between the addition of metals and the use of lignocellulosic substrates in terms of improving enzymatic activity.

2.6 Author contributions

V. Benavides: Methodology, Writing - Original Draft. F. Pinto-Ibieta: Writing - Review and Editing. T. Robledo: Conceptualization, Visualization, Writing - Review and Editing. A. Serrano: Conceptualization, Visualization, Writing - Review and Editing. O. Rubilar: Writing - Review & Editing. G. Ciudad: Writing - Review & Editing.

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CHAPTER III

Use of *Anthracophyllum discolor* and *Stereum hirsutum* as a suitable strategy for delignification and phenolic removal of olive mill solid waste.

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Use of *Anthracophyllum discolor* and *Stereum hirsutum* as a suitable strategy for delignification and phenolic removal of olive mill solid waste

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Abstract

This study evaluated the use of the white-rot fungi (WRF) *Anthracophyllum discolor* and *Stereum hirsutum* as a biological pretreatment for olive mill solid mill waste (OMSW). The WRF strains proposed were added directly to OMSW. The assays consisted of determining the need to add supplementary nutrients, an exogenous carbon source or use agitation systems, and

evaluating WRF growth, enzyme activity, phenolic compound removal and lignin degradation. The highest ligninolytic enzyme activity was found at day 10, reaching 176.7 U/L of manganese-independent peroxidase (MniP) produced by *A. discolor*, and the highest phenolic removal (more than 80% with both strains) was reached after 24 days of incubation. The confocal laser scanning microscopy analysis (CLSM) confirmed lignin degradation through the drop in lignin relative fluorescence units (RFU) from 3967 for untreated OMSW to 235 and 221 RFU, showing a lignin relative degradation of 94.1% and 94.4% after 24 days of treatment by *A. discolor* and *S. hirsutum*, respectively. The results demonstrate for the first time that *A. discolor* and *S. hirsutum* were able to degrade lignin and remove phenolic compounds from OMSW using this as the sole substrate without adding other nutrients or using agitation systems. This work indicates that it could be possible to design an in-situ pretreatment of the valorization of OMSW, avoiding complex systems or transportation. In this sense, future research under non-sterile conditions is needed to evaluate the competition of WRF with other microorganisms present in the OMSW. The main drawbacks of this work are associated with both the low reaction time and the water addition. However, OMSW is seasonal waste produced in one season per year, being stored for a long time. In terms of water addition, the necessary optimization will be addressed in future research.

Keywords: white-rot fungi; OMSW; ligninolytic enzyme; pretreatment; agroindustrial waste valorization

3.1 Introduction

Every year, close to 1000 Gt of lignocellulosic biomass is generated worldwide, including wheat straw, sugarcane bagasse, corn stalks, and others (Al-Mallahi *et al*., 2016; Hermosilla *et al*., 2017a). This huge volume of biomass can be used as suitable feedstocks in biorefineries to obtain add-ed-value products derived from the polysaccharide fraction, including carbohydrate-enriched ruminant feed, chemical compounds, and biofuels (Parenti *et al*., 2013; Rouches *et al*., 2016; van Kuijk *et al*., 2016; Wan & Li, 2012). However, the molecular structure of lignin and its strong association with cellulose and hemicellulose strongly limit the acquisition of these added-value products (Hendriks & Zeeman, 2009; Taherzadeh & Karimi, 2008). To resolve this challenge, enzymatic pretreatments have been proposed to enhance the use of lignocellulosic biomass as feedstock in subsequent bioprocesses (Bilal & Iqbal, 2020). Some authors have reported the direct use of ligninolytic enzymes for the reduction of the lignin content in different lignocellulosic wastes (Salvachúa *et al*., 2011; Vivekanand *et al*., 2008; Wan & Li, 2011). Recently, the use of white-rot fungi (WRF) has been proposed as an alternative to the direct application of pure enzymes to delignify lignocellulosic residues (Mori *et al*., 2021; Shirkavand *et al*., 2017; Suryadi *et al*., 2022). WRF can produce extracellular oxidative enzymes such as laccase (Lac), manganese peroxidase (MnP) and manganese-independent peroxidase (MniP), which have been reported as being involved in the degradation of lignocellulosic fibers (Kissi *et al*., 2001; Ntougias *et al*., 2015). In this sense, different WRF strains have been proposed as pretreatments for different lignocellulosic residues, such as sweet sorghum bagasse, radiata pine and wheat straw, among others (Cui *et al*., 2021; Hermosilla *et al*., 2018; Shirkavand *et al*., 2017).

During olive oil extraction in two phase processes, a semi-solid byproduct called olive mill solid waste (OMSW) is generated, presenting a variable water content of 60–95% (Serrano *et al*., 2021).The incorrect management of OMSW has several deleterious environmental effects, such as coloration of natural waters, toxicity to aquatic life, degradation of soil quality by inhibiting plant germination and growth, phytotoxicity, and the generation of nuisance odors (Genethliou *et al*., 2020; Sounni *et al*., 2021). OMSW is composed of olive husk, olive pulp, and olive vegetation water, resulting in a substrate rich in lignocellulosic fibers and polyphenols (Caroca *et al*., 2021; Serrano *et al*., 2017a). Polyphenols in OMSW create a high antimicrobial activity that can hamper the valorization of this biomass through microbe-mediated processes (Fernández-Prior *et al*., 2020). In this context, few studies have described the use of WRF for the pretreatment and further valorization of OMSW. According to the literature, the effectiveness of WRF for phenol removal is strongly dependent on the selected fungi. For example, phenol removal in OMSW varied between 50% and 85% using *Phanerochaete chrysosporium* (Kissi *et al*., 2001) and *Phlebia* sp. (Sampedro *et al*., 2009), respectively. More recently, *Pleurotus citrinopileatus* and *Irpex lacteus* appeared as potent degraders of olive mill wastewater, while simultaneously producing biotechnologically relevant enzymes (Zerva *et al*., 2017). These cogenerated enzymes could be used to degrade other lignocellulosic waste or to bioremediate soil contaminated with pentachlorophenol (Rubilar *et al*., 2011; Schalchli *et al*., 2017; Zerva *et al*., 2017). Therefore, the application of WRF to OMSW might not only result in the phenolic removal of the biomass, but also in the acquisition of valuable ligninolytic enzymes. In particular, *Anthracophyllum discolor* and *Stereum hirsutum* are Chilean native WRF isolated from temperate forests in southern Chile that have been reported to generate lignocellulosic enzymes such as MnP and LiP (Tortella *et al*., 2008). Both WRF have been used in pentachlorophenol removal from soil (Rubilar *et al*., 2011) and used as pretreatment of agroindustrial waste such as wheat

straw (Hermosilla *et al*., 2017b). Therefore, *A. discolor* and *S. hirsutum* could be suitable strains for the pretreatment of lignocellulosic waste like OMSW. In this context, the novelties of this work are: (a) *A. discolor* and *S. hirsutum* have not been previously evaluated in this waste, (b) there are few studies oriented to OMSW treatment using WRF, with most focusing on olive mill wastewater treatment from three phase processes, all with other WRF strains(M. *et al*., 2001; Ntougias *et al*., 2015; Zerva *et al*., 2017), and (c) this work proposes applying the strains directly, in the static condition (without agitation or addition of an additional carbon source or nutrient); thus, the number of steps in this process is minimal and does not require any additional feedstock to culture the WRF. The use of local fungal strains could be very interesting because it would avoid the introduction of foreign organisms in the ecosystems, which would potentially result in several environmental impacts (Rossman, 2009). Furthermore, local fungal strains would already be adapted to the environmental conditions of the area where the fungal treatment will be carried out, which would be very desirable for the subsequent scalation and industrial implementation of the process. Therefore, the aim in this work was to evaluate the direct application of *A. discolor* and *S. hirsutum* as a biological pretreatment for OMSW.

3.2 Materials and methods

3.2.1 Microorganisms

The fungal strains *A. discolor* Sp4 and *S. hirsutum* were used, both isolated from decayed wood in the temperate forests of southern Chile belonging to the Culture Collection of the Environmental Nanobiotechnology Laboratory at the Universidad of La Frontera, Chile. The fungi were stored at 4 °C in Petri dishes with potato dextrose agar (PDA) medium until use. The

culture medium was prepared using 39 g of PDA in 1 L of distilled water and sterilized in an autoclave for 20 min at 121 °C. Then, the strains were activated in PDA medium using one disk of 6 mm as the initial inoculum and incubated at 25 °C for 7 days for the assays.

3.2.2 Olive mill solid waste (OMSW)

OMSW was collected from the olive mill "Olivares de Quepu". The olive tree variety is Arbequina for extra virgin olive oil production. OMSW was stored in self-sealing bags for refrigerated storage at −20 °C. For all tests, OMSW was used directly, without applying any pretreatment such as grinding or sieving. Characterization of OMSW included oxygen demand (COD), total solids (TS), volatile solids (VS), pH, total nitrogen (TN), and ammonia nitrogen (N-ammonia), all measured according to standard methods (Rice, E.W., Baird, R.B., 1999). Cu, Fe, and Mn present in OMSW were measured by EPA method 6020A (García *et al*., 2016). NO_2- and NO_3- anions were measured by ion chromatography (Rice, E.W., Baird, R.B., 1999).

Total phenol content in the liquid and solid phases were quantified by spectrophotometry with a gallic acid (GA) calibration curve, using the Folin-Ciocalteu method, expressing the results as mg GA/L and mg GA/100 g (García *et al*., 2016). For the determination of total phenol content in solid phases, previous extraction was carried out using 1 g of OMSW and 1 mL of methanol/water (80:20 v/v). The mixture was stirred for 1 min in a vortex apparatus and centrifuged at 1200 G for 10 min. The methanol layer was separated, and the extraction was repeated four times (García *et al*., 2016). For phenolic profile determination, samples were treated with a water/methanol/formic acid solution (24:25:1) and sonicated. They were allowed to stand for 24 h to sonicate again, centrifuged and finally filtered for further analysis. A Hitachi

Primaide high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD) was used, equipped with a Kromasil ® C18 column (Fregapane & Salvador, 2017).

The ash, total extractives, lignin, hemicellulose, and cellulose contents were measured by the Klason lignin method (TAPPI T 222 om-02) (Rice, E.W., Baird, R.B., 1999). Lipids were analyzed by the soxhlet extraction method described in the standard methods of the American Public Health Association APHA (Rice, E.W., Baird, R.B., 1999).

3.2.3 Qualitative detection of lignocellulolytic enzymes

Qualitative discoloration tests with Remazol Brilliant Blue R (RBBR) and staining with 2,2′-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) (Calbiochem) were performed. PDA culture medium enriched with sterile glucose aqueous solution 20% (w/v) and ABTS was used to qualitatively determine the presence of oxidase enzymes (laccases) in fungal strains and RBBR to determine peroxidase enzymes (manganese peroxidase and lignin peroxidase) (Wariishi *et al*., 1992a). The tests were performed in triplicate in Petri dishes, using uninoculated plates as controls. One agar disk (6 mm diameter) of active mycelium from a 7-day-old culture in PDA medium was inoculated in Petri dishes and then incubated at 25 °C in darkness. The green halo in culture medium with ABTS and the blue to yellow discoloration in culture medium with RBBR were periodically evaluated for 4, 7, and 14 days. The discoloration and coloration scale proposed by Tortella *et al*., (2008) was used in this assay.

3.2.4 Mycelial growth assays

The mycelial growth was evaluated in three different solid mediums: (a) PDA medium, (b) OMSW, and (c) OMSW+PDA. In the experiment, 20 g of OMSW were distributed on Petri dishes and in the combined conditions, OMSW was added to solidified PDA. OMSW (62% moisture content) was sterilized under the same conditions. Then, Petri dishes were inoculated with one agar disk (6 mm diameter) of active mycelium from a 7-day-old culture in PDA medium and were incubated at 25 °C for 12 days or until the dish was covered completely by the fungi. The experiment was carried out in triplicate. The mycelial growth was evaluated every 24 h for 12 days by photographic registration, so that the current limit of the mycelia growth could be visualized. The public domain program ImageJ v.1.52a was used for image processing.

The kinetics of the growth of *A. discolor* and *S. hirsutum* were evaluated by applying the modified Gompertz equation to the experimental data of mycelial growth monitored during the experimental period (Equation (1)). The application of the modified Gompertz equation was previously reported for the growth of *Fusarium verticillioides* and *Rhizopus stolonifera* by Ochoa-Velasco *et al*., (2018), and is defined as:

$$A = A_{max} * exp\left[-exp\left(\frac{r_{max} * e}{A_{max}}(\lambda - t) + 1\right)\right] \quad 1)$$

where A is the average fungal colony size in the Petri dish (mm) at time t (d), A_{max} is the maximal fungal colony size (mm) , r_{max} is the maximum specific growth rate (1/d), λ is the lag time (d), and e = exp(1), equal to 2.7183. Additionally, r^2 was determined to evaluate the goodness-of-fit of the experimental data to the selected modified Gompertz equation. The kinetic

parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 10.0).

3.2.5 OMSW pretreatment assays

For this assay, 12 samples of 30 g of sterilized OMSW (49.7% moisture content) from self-sealing bags refrigerated at −20 °C and without any mechanical pretreatment were added to Erlenmeyer flasks (500 mL) according to conditions defined in Table 1. The OMSW final weight on a wet basis in the flasks after sterilization was 28.8 g. Modified Kirk's medium (MKM) was used to evaluate the need to add supplementary media to improve the enzymatic activity. This medium is widely used for the growth and stimulation of the ligninolytic activity of WRF (Acevedo *et al*., 2011; Wariishi *et al*., 1992b). Distilled water was added to the OMSW assays to facilitate the comparison among the different culture media, avoiding factors such as differences in the mass transference or total solid concentration that would cover up the differences due to the different assayed substrates. Finally, the flasks were inoculated with five disks (6 mm diameter) of active mycelium from 7-day-old culture plates. The flasks were incubated at 25 °C under static conditions for 24 days. Control flasks without OMSW were evaluated at the same time.

Table 1. Culture conditions for enzymatic extract production using OMSW.

Strain	Culture Media
S. hirsutum	100 mL MKM
A. discolor	100 mL MKM

S. hirsutum	100 mL MKM + 30 g OMSW
A. discolor	100 mL MKM + 30 g OMSW
S. hirsutum	100 mL distilled water + 30 g OMSW
A. discolor	100 mL distilled water + 30 g OMSW

OMSW: Olive mill solid waste; MKM: Modified Kirk's medium.

3.2.6 Enzymatic activity quantification

Extract recovery and enzymatic activity quantification were performed according to Acevedo *et al*., (2011) and Hermosilla *et al*., (2017b). The crude extracts were collected by filtration through Whatman Nº 1 filter paper (pore size 11 μm). Enzymatic activity was periodically monitored every two days for 24 days. The enzyme activity of laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP) was determined through the 2,6-DMP assay, where the reaction mixture contained 200 μL of 50 mM of sodium malonate (pH 4.5), 50 μL of 20 mM 2,6-DMP, 50 μL of 20 mM $MnSO_4.H_2O$, and 50 μL of supernatant. The reaction was initiated by adding 100 μL of 4 mM H_2O_2, and the absorbance of the colored product was measured at 468 nm using a UV-vis spectrophotometer (Thermo Scientific Evolution™ 60S) at 30 °C and corrected for the Lac activity (Wariishi *et al*., 1992b). One MnP activity unit (U) was defined as the amount of enzyme transforming 1 μmol 2,6-DMP per minute at pH 4.5 and 30 °C (Wariishi *et al*., 1992b). Total enzymatic activity of WRF was represented as the sum of MniP, Lac and MnP, which were individually determined at the time.

3.2.7 Microstructure analysis

Structural changes between untreated and pretreated OMSW were appraised by a confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). For CLSM and SEM, representative samples of untreated OMSW and pretreated with both WRF for days 12 and 24 were taken, respectively. In particular, for CLSM the samples were incubated with two markers in the dark for 30 min at room temperature. The markers used were Calcofluor at 20% *v/v* for cell wall staining (blue) ʎ emission/excitation 405/450 nm, and Safranin O at 1% *w/v* for lignin staining (green) ʎ emission/excitation 546/590 nm. Then, the samples were centrifuged and washed with PBS 1X, and mounted for visualization on a fluodish plate. The samples were visualized in three fluorescence channels using the FV1000 confocal laser microscope (Olympus, Japan), and then the images were analyzed (including the relative fluorescence unit (RFU) quantification) using the FV10 Olympus var 0.2c software. For scanning electron microscopy (SEM), the samples were dried at 25 °C for 4 days and mounted on an aluminum stub and adhered with double-sided carbon tape. After this, samples were outlined using silver paint. The visualization was carried out within the following parameters: 10 KV, WD 10 mm, BSE (chemical contrast), and UVD (topography) detectors in SEM (Hitachi SU3500, Japan).

3.3 Results and discussion

3.3.1 Olive mill solid waste characterization

According to Table 2, OMSW had a lignin, hemicellulose, and cellulose content of 33.4 ± 4%, 45.5 ± 8%, and 35.2 ± 3% of the total weight of OMSW, respectively. These values are within the ranges reported in the literature for two-phase OMSW (Kaouachi *et al*., 2015). The

organic carbon content of OMSW for the study variety Arbequina is within the range reported in other studies, between 20.5 to 58.5% (Kaouachi *et al*., 2015; Sampedro *et al*., 2009). The determined C/N ratio for the used OMSW was 48.4/0.84 (Table 2). In fact, the much higher concentration of carbon compared to nitrogen is a feature of OMSW that has also been defined as limiting the biological treatment of this biomass with supplementation with a co-substrate being recommended (Fernández-Rodríguez *et al*., 2019; Messineo *et al*., 2020).

OMSW presented a pH value of around 5.0–5.1, which is within the desirable range for enzyme activity. For example, Bustamante *et al*., (2011) determined that enzyme activity for *A. discolor* in modified Kirk's medium (MKM) at 25 °C incubation was enhanced at a pH between 5 and 6. On the other hand, some metals from OMSW were determined, giving values for Fe (<13 mg/kg), Mn (<140 mg/kg), and Cu (8.48 mg/kg). The presence of copper and manganese is important due to their role as inducers. Jain *et al*., (2019) showed that with copper addition in the culture media, the laccase activity can be increased up to eight times over culture media without this metal. In the case of Mn, enhancement of MnP production and stimulation of enzymatic activity by WRF has been observed (Acevedo *et al*., 2011; Gill & Arora, 2003), showing that expression of MnP in the fungal cultures is dependent on Mn. In addition, Papinutti *et al*., (2003) indicated that specific MnP activity increases with increasing concentrations of Mn^{2+}, reaching a maximum, from which point the activity decreases.

Other analyses have shown a total polyphenol content equivalent to 1.49 g/kg. Compared to other reports, this value is in the low range of total polyphenol content that varies between 1.4 and 10.7 g/kg (Ntougias *et al*., 2015). OMSW has an antioxidant capacity of 214.4 mg Trolox/L

(Table 2). In addition, a phenolic profile analysis was carried out, identifying phenolic acids and flavonols, such as catechin, epicatechin, quercetin, and kaempferol, as shown in Table 2.

Table 2. Physicochemical characteristics of olive mill solid waste.

Characteristics	Value
α-cellulose (% w/w)	35.2 ± 3
Lignin (% w/w)	33.4 ± 4
Hemicellulose (% w/w)	45.5 ± 8
Elemental analysis (C/N) (% w/w)	48.4/0.84
Ash (% w/w)	2.96
Total nitrogen (mg/kg)	134.2
Nitrate (mg/kg)	<1.0
Nitrite (mg/kg)	<0.6
Total copper (mg/kg)	8.5
Total iron (mg/kg)	<140.0
Total manganese (mg/kg)	<13.0
Chemical oxygen demand (mg O_2/L)	87.9
Total solids (% w/w)	38.5
Volatile solids (% of TS)	89.7
pH	5.08
Total polyphenols (mg GA/100 g)	149.0
Antioxidant capacity (mg Trolox/L)	214.4
Gallic acid hexoside (mg/100 g)	0.9
Gallic acid protocatechuic acid hexoside (mg/100 g)	0.5

Hexoside ferulic acid (mg/100 g)	0.3
Cautaric acid (mg/100 g)	1.0
Caftaric acid (mg/100 g)	0.9
Catechin (mg/100 g)	1.6
Epicatechin (mg/100 g)	1.9
Quercetin-3-rutinoside (mg/100 g)	1.3
Quercetin-3-hexoside (mg/100 g)	1.7
Quercetin-3-glucuronide (mg/100 g)	2.0
Kaempferol-3-glucoside (mg/100 g)	3.5
Kaempferol-3-hexoside (mg/100 g)	10.2
Lipids (% w/w)	13.23

3.3.2 Qualitative detection of lignocellulolytic enzymes

The qualitative assay to identify the ligninolytic enzymatic potential of *A. discolor* and *S. hirsutum* was carried out for 14 days. The tested strains of WRF showed enzymatic activities. Specifically, the extent of decoloration with PDA+RBBR resulted in a decoloration ratio between 67 and 90 mm for *A. discolor* (Figure 1, Supplementary file 1), and from 22 mm and 45 mm for *S. hirsutum* (Table 3). However, both strains showed a similar activity for PDA+ABTS, resulting in a coloration diameter ranging from 67 to 90 mm. Similar results were published by Tortella *et al*., (2005) for these strains, which indicated that the fungal strains maintained their enzymatic potentials after subcultures since they were isolated. Both strains were selected for further assays in OMSW because they produced the highest reaction intensity with all enzymatic indicators in

the media tested, which indicates that they have enzymatic mechanisms to degrade lignocellulosic components of OMSW.

Table 3. Qualitative detection by RBBR decoloration and ABTS coloration at evaluation day 14.

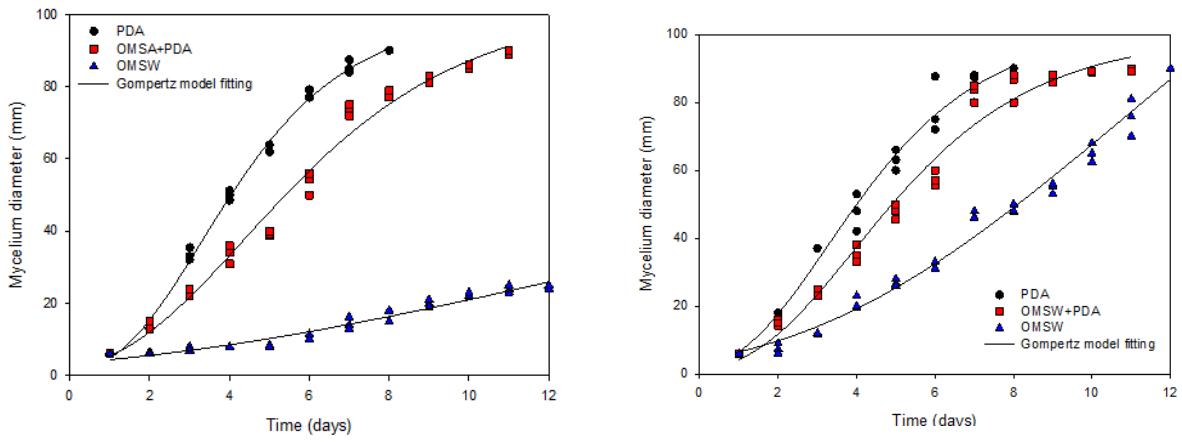
Strain	PDA+RBBR (Decoloration)	PDA+ABTS (Coloration)
A. discolor	++++	++++
S. hirsutum	++	++++

Decoloration and coloration scale at 14 days of cultivation. +: decolored or colored area diameter > 1 mm and ≤ 22 mm, ++: diameter> 22 mm and ≤45 mm, +++: diameter> 45 mm and ≤ 67 mm, ++++: diameter> 67 mm and ≤ 90 mm and 0: no effect.

3.3.3 Mycelial growth assays

Once the enzymatic activity of the selected WRF strains had been corroborated, the suitability of OMSW as substrate was studied. Figure 1 shows the mycelial growth for *A. discolor* (A) and *S. hirsutum* (B) as a function of culture time using PDA, OMSW, and PDA+OMSW as the substrate. *A. discolor* and *S. hirsutum* showed higher growth using PDA followed by PDA+OMSW and OMSW. When the two strains were cultivated in PDA and PDA+OMSW, shorter latency states (lag phases) were observed than when the OMSW was used as the substrate (Table 4). This behavior may be due to the easier biodegradability of the PDA, which is composed of simple sugars and nitrogen sources more accessible for the fungus than

OMSW (Koranda *et al*., 2014). In particular, *A. discolor* showed growth from day 6, reaching a maximum mycelial diameter of 20 mm in OMSW, while *S. hirsutum* left the latency state on day 2, reaching a maximum mycelial diameter of 90 mm (Figure 1). In detail, Table 4 shows the mycelial growth rate (mm/d) determined by fitting the modified Gompertz model. In every case, the correlation coefficient (r^2) values were greater than 0.95, evidencing a statistically significant relationship. However, the value obtained for the maximum mycelial diameter for *S. hirsutum* in OMSW may be overestimated by the proposed model adjustment due to the more complex shape of the curve (Figure 1b, Table 4). As expected, in both strains the highest mycelial growth rate was achieved using PDA, followed by PDA+OMSW and OMSW (Table 4), probably due to the readily biodegradable compounds provided by the PDA (Jain *et al*., 2019). When the substrate was OMSW, *A. discolor* showed a mycelial growth rate of 2.5 ± 0.5 mm/d, while *S. hirsutum* reached 9.7 ± 0.7 mm/d. Although both strains were able to grow in OMSW, the higher mycelial growth rate of *S. hirsutum* compared to *A. discolor* indicated a better adaptability of *S. hirsutum* to grow in this substrate.



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

Figure 1. Evolution of fungal growth, equivalent diameters: (**a**) *A. discolor*; (**b**) *S. hirsutum.*

Table 4. Kinetics results of the modified Gompertz model applied to the fungal growth studied.

Culture Conditions	Maximum Mycelial Diameter (mm) (A_{max})	Mycelial Growth Rate (mm/day) (r_{max})	Lag Phase (d) (λ)	e	R^2
PDA medium					
A. discolor	100 ± 2	17.9 ± 0.5	1.2 ± 0.1	2.7183	0.9968
S. hirsutum	102 ± 5	16.8 ± 1	1.0 ± 0.2	2.7183	0.9838
OMSW					
A. discolor	78 ± 39	2.5 ± 0.5	1.6 ± 1.9	2.7183	0.9585
S. hirsutum	212 ± 46	9.7 ± 0.7	3.0 ± 0.6	2.7183	0.9857
PDA and OMSW					
A. discolor	103 ± 4	12.1 ± 0.5	1.3 ± 0.1	2.7183	0.9864
S. hirsutum	100 ± 3	14.3 ± 0.9	1.4 ± 0.2	2.7183	0.9793

PDA: potato dextrose agar; OMSW: olive mill solid waste.

3.3.4 Enzymatic extract production using OMSW

Figure 2 shows the total activity of the ligninolytic enzymes (represented as the sum of Lac, MnP, and MniP) as a function of time for both strains studied. *A. discolor* was able to produce ligninolytic enzymes in the presence of OMSW, regardless of the supplementation with modified Kirk's medium. Specifically, *A. discolor* cultivated in OMSW supplemented and not supplemented with modified Kirk's medium (MKM) reached maximum enzymatic activity as from day 10. By contrast, Schalchli *et al*., (2017) reported maximum enzymatic activity at 15

days using *A. discolor* cultivated in potato peel waste. The same maximum ligninolytic enzyme produced by *Ganoderma lobatum* using wheat straw was obtained after 40 days of incubation (Hermosilla *et al*., 2017b). The time difference may be due to the different composition of the substrates used in each study, since the OMSW presented a lignocellulosic content of 33.6% (Table 2), markedly lower than the other reported substrates, e.g., wheat straw contains around 35–45% cellulose, 20–30% hemicellulose, and around 15% lignin (del Río *et al*., 2012). On the other hand, *S. hirsutum* (Figure 2) showed ligninolytic enzyme activity using OMSW, but not for OMSW supplemented with Kirk's medium or with Kirk's medium alone. Similarly to *A. discolor*, *S. hirsutum* also showed an increase in enzymatic activity at around days 8–10 (Figure 2).

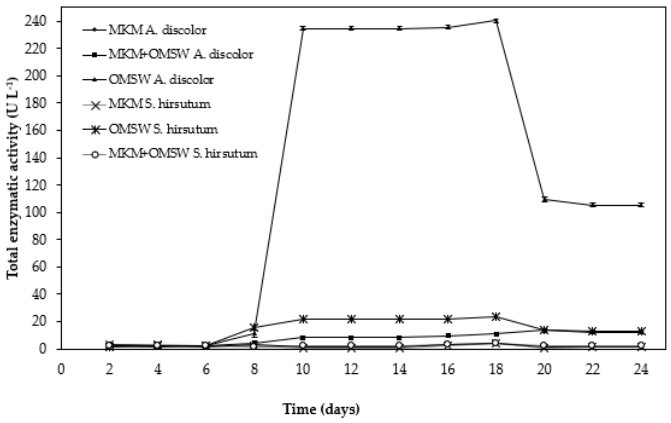


Figure 2. Ligninolytic enzyme activity (represented as sum of Lac, MnP, and MniP), as a function of time using olive mill solid waste (OMSW), OMSW + modified Kirk's medium (MKM) and modified Kirk's medium as a substrate for *A. discolor* and *S. hirsutum*.

Comparing the three conditions studied (OMSW, OMSW+MKM, and MKM), both strains produced the highest amount of ligninolytic enzymes using OMSW, reaching 234.8 U/L for *A. discolor* and 22.1 U/L for *S. hirsutum* on day 10 (Figure 2). By contrast, no strain produced enzymes using MKM. This behavior was likely a consequence of the lignocellulosic waste acting

as a support for WRF growth, providing conditions similar to their natural habitat and possibly containing substances that promote the production of the desired enzymes (Chmelová *et al*., 2022; Elisashvili *et al*., 2008). The most likely reason for the increase in their production is the presence of phenols such as ferulic, caffeic, coumaric, and chlorogenic acids (González Bautista *et al*., 2019; Torres-Mancera *et al*., n.d.). Furthermore, the fungal strains would not be pushed to degrade the OMSW to obtain energy in the presence of the MKM due to the availability of enough easily biodegradable soluble organic matter, making the metabolic cost of producing ligninolytic enzymes unnecessary for the fungus. Therefore, although the fungal growth was lower for both strains when cultured in OMSW (Figure 1, Table 4), this condition was the optimal to maximize the enzymatic activity (Figure 3). In addition, white-rot fungi have the ability to secrete extracellular nonspecific ligninolytic enzymes during their secondary metabolism, mainly triggered by the exhaustion of nutrients such as nitrogen (Rodríguez-Couto, 2017; Tien & Myer, 1990; Wu Feng, Ozaki Hiroaki, Terashima Yutaka, 1996; Xiaoping *et al*., 2008), carbon (Couto *et al*., 1998; Tien & Myer, 1990) or sulfur (Tien & Myer, 1990). Thus, when growing on a biomass such as OMSW, there is a period of 8 days that corresponds to fungi primary metabolism, which explains the low enzyme activity found in this work. Then, limited growth conditions are generated at 8 days (Figure 2) due to the restricted availability of nutrients and possibly to the presence of copper in the substrate (8.5 mg/kg) (Table 2) that can limit fungal growth (Ntougias *et al*., 2015). These conditions allow activation of the extracellular enzymatic machinery of the fungi, and, consequently, the fungi obtain compounds that can be assimilated for fungal nutrition (Rodríguez-Couto, 2017; Tien & Myer, 1990; Wu Feng, Ozaki Hiroaki, Terashima Yutaka, 1996; Xiaoping *et al*., 2008). These results are in agreement with those obtained by Gassara *et al*., (2012) who evaluated the ligninolytic enzyme activity using apple pomace in liquid fermentation. On the other hand, OMSW contains Mn (<13 mg/kg) (Table 2),

which may have functioned as an enzyme inducer. In this sense, studies carried out by Acevedo *et al*., (2011); Gill & Arora, (2003); Salvachúa, Prieto, López-Abelairas, *et al*., (2011); van Kuijk *et al*., (2016) who used Mn to enhance MnP production and stimulate enzymatic activity by the white-rot fungi, showed that the presence of MnP in the fungal cultures is Mn-dependent.

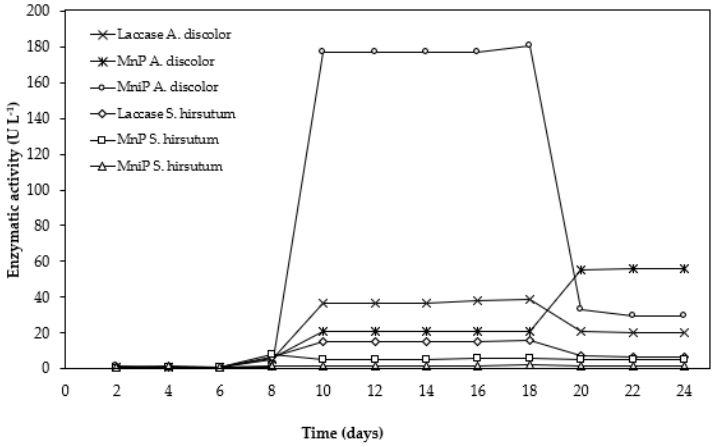


Figure 3. Lac, MnP, and MniP activity variation as a function of culture time using OMSW as the substrate for both strains studied; *A. discolor* and *S. hirsutum.*

A different case occurs when MKM is used because it is a medium rich in nutrients (Rollag *et al*., 1982), where the main carbon source is glucose, which, being easily assimilated, maintains the nutritional conditions for the fungus over time, and this condition limits or prolongs the activation of the ligninolytic enzyme. In the results, it is noted that low enzymatic activity can be associated with the previously mentioned conditions. However, it may be possible that with more evaluation time (up to 24 days), the enzymatic production will increase due to the limited nutrient conditions in the culture media.

Figure 3 shows the individual enzyme activity for Lac, MnP, and MniP using *A. discolor* and *S. hirsutum* cultivated in OMSW. *A. discolor* was able to produce the highest amount of MniP, followed by Lac and MnP, reaching a maximum of 176.7, 36.8, and 21.2 U/L,

respectively, for each enzyme at day 10, i.e., MniP was produced by *A. discolor* three and eight times more than MnP and Lac, respectively. *S. hirsutum* reported higher Lac productivity over MnP and MniP, reaching 15.3, 5.2, and 1.5 U/L, respectively. The higher presence of MniP may be related to its high selectivity in the degradation of lignin, and also due to the low concentration of manganese in the OMSW (<13 mg/kg OMSW, Table 3), which would have limited the MnP activity in favor of the MniP activity (Ansari *et al*., 2016; Knežević *et al*., 2014).

The increase in the activity of MniP and MnP enzymes was previously reported by Reina *et al*., (2013), who described an increase in peroxidase activity from 0% to 19% in 14 days for *Auricularia auricula-judae*, *Bjerkandera adusta*, and *Coprinellus radians* during the solid-state fermentation of olive mill residues. Likewise, the presence of phenolic compounds from the OMSW would have induced the production of Lac and MnP (Fenice & D'Annibale, 2003; Reina *et al*., 2013b). Although Lac activity was detected, its low production may be due to the C/N ratio of the OMSW, i.e., 60 (Table 2), since some experimental works suggested that substrates with a C/N ratio of less than 16 are the most suitable condition for Lac production (Chmelová *et al*., 2022). Additionally, substrates with a ratio greater than 40 can strongly limit Lac production by WRF (Ansari *et al*., 2016; Rollag *et al*., 1982).

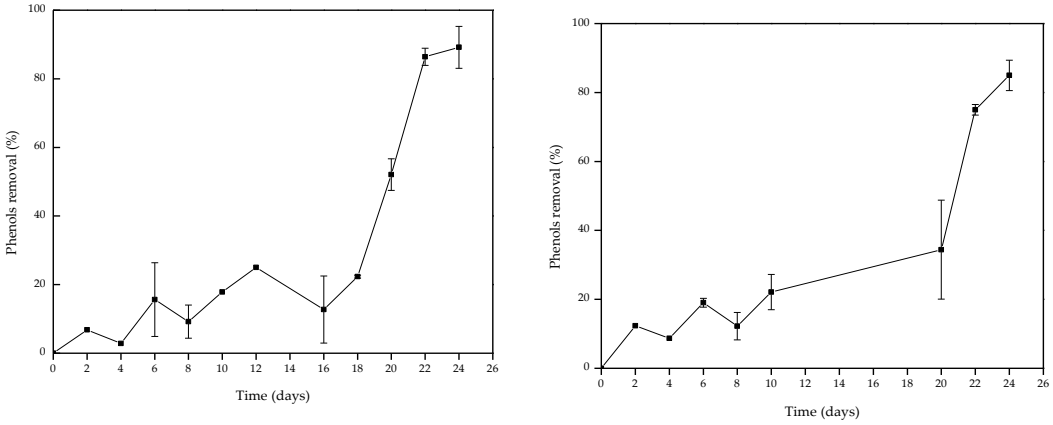
Although the results obtained are interesting, the Lac and MnP values reported in the present work were low compared to 600 and 163 U/L of Lac and MnP, respectively, produced at day 15 for *A. discolor* using potato peel waste as the substrate, reported by Schalchli *et al*., (2017). However, further research on strategies for enhancing the enzymatic activity would be interesting, considering that culture supplementation with agroindustrial wastes rich in lignin compounds such as OMSW can generate many inducers during fermentation processes,

significantly improve the potential of synergic of inducers-enzymes for enzyme extract production, degradation of recalcitrant organic pollutants, removal of recalcitrant lignin, and transformation of its structural polysaccharides into fermentable sugars (Atilano-Camino *et al*., 2020; Mishra *et al*., 2017).

3.3.5 OMSW phenol removal using *A. discolor* and *S. hirsutum*

The potential of *A. discolor* and *S. hirsutum* in the phenolic detoxification of OMSW was evaluated by monitoring the phenol removal percentage throughout the experimental period (Figure 4). As can be seen, both strains were highly effective at removing phenolic compounds, reaching similar values above 80% at day 24. The highest phenol removal efficiencies being reached at the end of the experimental period is related to the release of new phenolic compounds from complex lignocellulosic structures during the OMSW degradation (Liang & McDonald, 2014). The phenol removal efficiencies achieved in the present work were slightly higher than those reported by other authors and were also achieved in a shorter time. In particular, Reina *et al*., (2013) described an efficiency in phenol compounds removal between 60 to 80% for *Auricularia auricula-judae*, *Bjerkandera adusta*, and *Coprinellus radians* after 28 days of OMSW pretreatment. On the other hand, Sampedro *et al*., (2009) found that in OMSW pretreated by *Coriolopsis rigida* the phenolic compound removal was about 36% after 14 days of incubation. By contrast, the phenolic compounds removal was higher than 80% in OMSW pretreated for 140 days, the highest removal percentage being attained with other WRF as *Phlebia radiata* (95.8%), *C. rigida* (89.2%) and *Pycnoporus cinnabarinus* (88.7%).

The phenolic compound removal achieved in the present study stands out over other pretreatments previously proposed for OMSW; for example, some thermal pretreatments have achieved between 58 and 70% removal of phenolic compounds from OMSW. Thus, Serrano *et al*. (Serrano *et al*., 2017b) found that using a thermal pretreatment with vapor injection at 170 °C for 60 min allowed the separation of OMSW into two phases, solid and liquid, and subsequent phenolic compound removal of 69.5%. Another study by Serrano *et al*. (Serrano *et al*., 2017c) demonstrated that applying a steam explosion pretreatment with a maximal operating pressure of 42 kg/cm^2 for 5 min at 200 °C prior to a rapid decompression (explosion) and then centrifugation at 4700× *g* for solid and liquid separation achieved a phenolic compound removal of 58.3%. However, these pretreatments were composed of more stages and high energy requirements compared to the treatment proposed in this study, and therefore, the proposed fungal pretreatments using *A. discolor* and *S. hirsutum* would be a suitable strategy for the detoxification of OMSW in soft conditions (lower temperature (24 °C), not adding other nutrients or agitation systems), allowing the subsequent valorization of this biomass without a high energy cost.



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

Figure 4. Phenol removal as a function of culture time using OMSW as substrate for both strains studied: *A. discolor* (**a**) *S. hirsutum* (**b**).

3.3.6 Microstructure analysis

The morphology of the untreated OMSW and fungal pretreated OMSW at 12 and 24 days of degradation is shown in Figure 5. The untreated OMSW (Figure 5a) shows clearly defined fibers. By contrast, in Figure 5b–g, fibers are not distinguished. Particularly in Figure 5b, for day 12 of treatment with *A. discolor*, a greater number of pores are observed than in the untreated OMSW. These results are according to Mishra *et al*., (2017), who reported the formation of holes and crevices on the biomass surfaces in pretreated sweet sorghum bagasse.

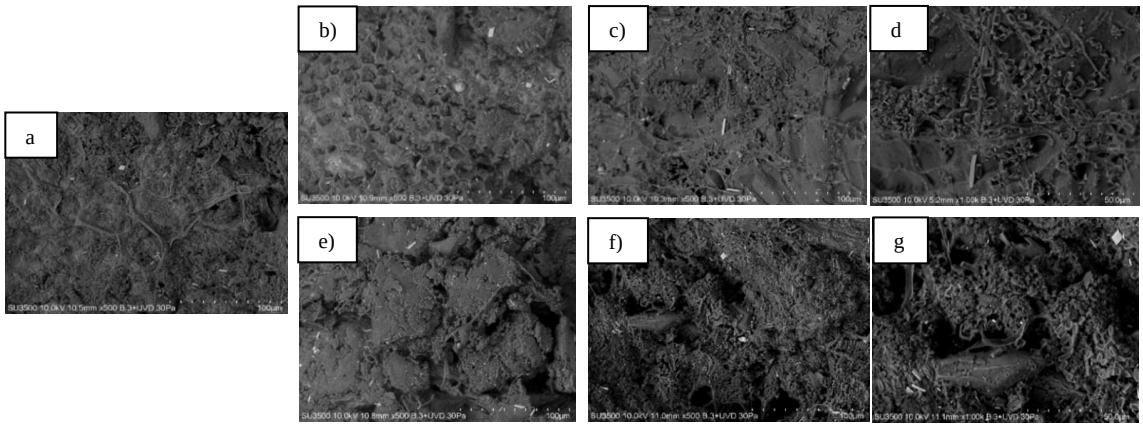


Figure 5. SEM images of: (**a**) untreated OMSW; (**b**) pretreated OMSW with *A. discolor* at 12 (**b**) and 24 (**c**, **d**) days; pretreated OMSW with *S. hirsutum* at 12 (**e**) and 24 (**f**, **g**) days.

The lignocellulosic structure of the untreated OMSW and pretreated OMSW at 12 and 24 days of degradation is shown in Figure 6. In particular, Figure 6a–c shows the untreated OMSW; the green staining is selective for lignin and blue for WRF, clearly showing the OMSW lignin composition. Figure 6d–i and j–o shows the OMSW after 12 and 24 days of treatment using *A. discolor* and *S. hirsutum*, respectively. It can be seen as green in untreated biomass, which could be due to lignin degradation using both WRF. On the other hand, blue indicates the growth of both fungi in the OMSW.

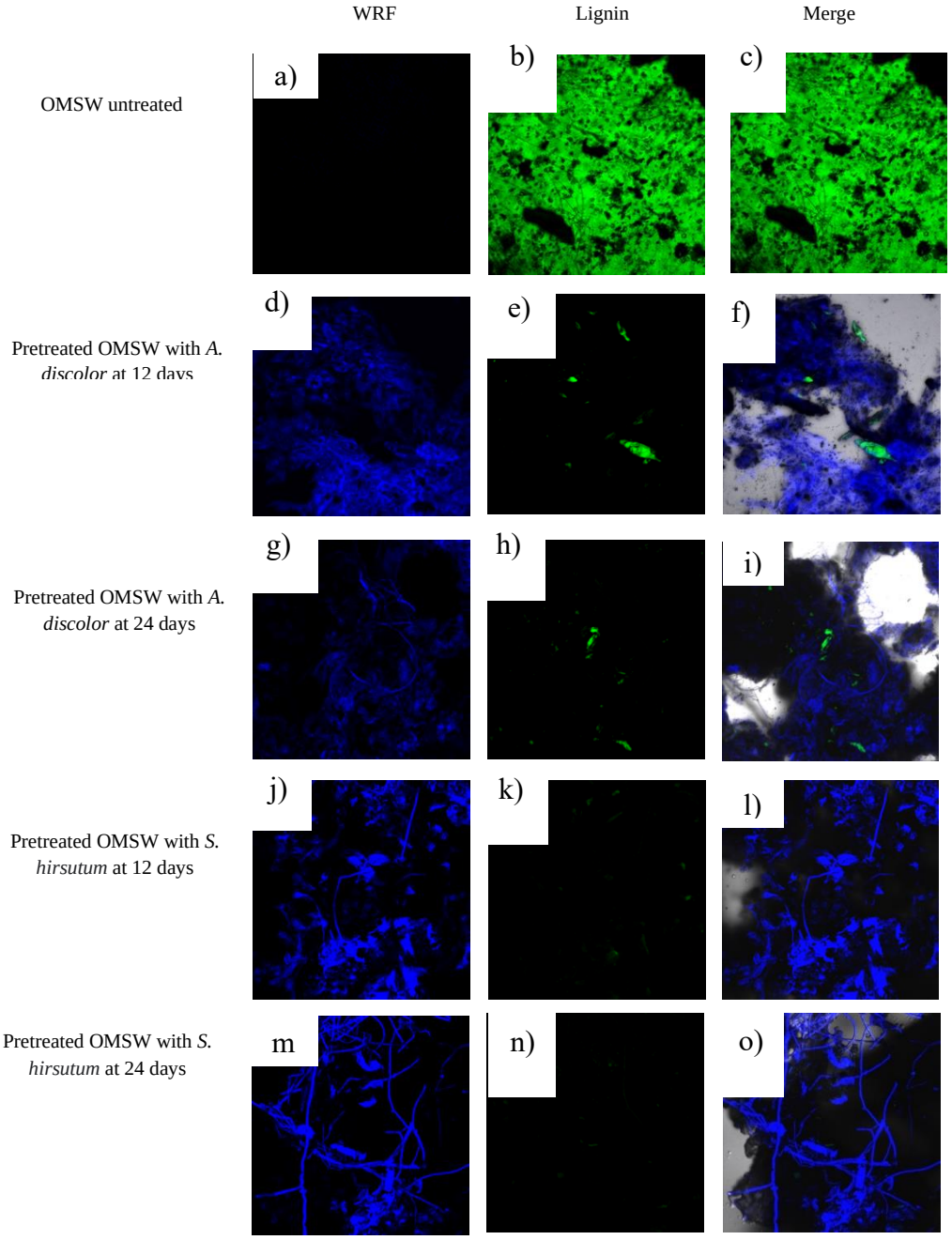


Figure 6. Confocal images of untreated OMSW (**a**–**c**); pretreated OMSW with *A. discolor* at 12 (**d**–**f**) and 24 (**g**–**i**) days; pretreated OMSW with *S. hirsutum* at 12 (**j**–**l**) and 24 (**m**,**o**) days.

The relative fluorescence units (RFU) obtained from the CLSM analysis showed that lignin fluorescence decreased from 3967 RFU for untreated OMSW to 235 and 221 RFU, showing a lignin relative degradation of 94.1% and 94.4% after 24 days of treatment using *A.*

discolor and *S. hirsutum*, respectively (Table 5). At the same time, the fungi fluorescence increased from 291 to 3548 and 3423 at 12 and 24 treatment days for *A. discolor* and *S. hirsutum*, indicating WRF growth while lignin is degrading.

Although the present work did not exhibit high ligninolytic enzyme activity (see point 3.4) compared to results that have been reported using *A. discolor* for pretreatment of wheat straw (Hermosilla *et al*., 2017a) and potato peel (Schalchli *et al*., 2017), our results demonstrate that the enzyme activities obtained are sufficient to remove phenolic compound (point 3.3) and lignin degradation. Despite *S. hirsutum* showing a low ligninolytic enzymatic activity compared to *A. discolor*, both fungi demonstrated the ability to remove phenolic compounds and lignin degradation from OMSW, thereby proving for the first time the applications of this strain for OMSW pretreatment. To the best of our knowledge, *S. hirsutum* has never been used for pretreatment of OMSW. As shown in Table 5, there are no reports about lignin degradation in OMSW using WRF.

Table 5. Comparison with similar works.

Strain	Phenolic Compounds Removal (%)	Reaction Time (days)	Specific Conditions		OMSW (g)/Water (mL) Proportion	References
			Temperature (°C)	Others		
Phanerochaete flavido-alba	70	60	30	Aeration: sterile O_2 (3 L/min for 1 min) every 24 h	1:0.3	(Linares *et al*., 2003)
P. radiata *C. rigida* *P. cinnabarinus*	95.8 89.2 88.7	140	30	-	1:0.1	(Sampedro *et al*., 2007)
Phlebia sp. *Panus tigrinus* *Phlebia sp.*	85 36 43	30	28	Immobilization in Polyurethane sponge	1:0.1	(Sampedro *et al*., 2009)
A. auricula-judae	60	28	24	-	1:4.5	(Reina *et*

B. adusta	80					*al*., 2013a)
C. radians	75					
A discolor	90	24	25	-	1:3.3	This work
S. hirsutum	85					

-: not reported.

Further characterization (such as glucose, fatty acids, chemical oxygen demand) of OMSW pretreated with WRF are needed since it could subsequently be fermented into ethanol or used for the production of other chemicals (for example biogas) in a biorefinery concept, it being feasible to apply a biological pretreatment using *A. discolor* or *S. hirsutum*. For example, (Hermosilla *et al*., 2018) reported that *Ganoderma lobatum* showed a 50.3% lignin degradation after 40 days. This fungal pretreatment significantly increased the amount of glucose released from wheat straw, reaching 97.4 mg of glucose/g of wheat straw.

The results of this work demonstrate that *A. discolor* and *S. hirsutum* have the phenolic compound removal and lignin degradative abilities for a pretreatment of OMSW, applying WRF directly without the need to add nutrients, exogenous carbon source, or use agitation systems. Moreover, the main issues found are associated with: (1) high reaction time (24 days) compared with thermal treatments (no more than 1 h), and (2) water addition (OMSW/water of 1: 3.3) (Table 5).

However, OMSW is seasonal waste produced one season per year, being stored for a long time (Khdair, Adnan; Abu-Rumman, 2020). Therefore, the low reaction might not be a problem since the application of the proposed pretreatment could be carried out in situ. In terms of the water addition, the necessary optimization will be addressed in future research.

3.4 Conclusions

The WRF *A. discolor* and *S. hirsutum* were able to grow under static conditions using OMSW as the sole substrate without supplementing other nutrients, reaching more than 80% of total phenol removal, with clear evidence of lignin degradation after 24 days. These results indicate that it could be possible to design an in-situ pretreatment of the valorization of OMSW, avoiding complex systems or transportation. In this sense, future research under non-sterile conditions is needed to evaluate the competition of WRF with other microorganisms present in the OMSW. In addition, water use must be optimized in order to minimize it.

Supplementary Materials: The following figures and tables are available online at www.mdpi.com/xxx/s1, Figure S1: Qualitative detection of lignocellulosic enzymes. (a) control with ABTS and PDA, (b) *A. discolor* in PDA with ABTS day 1, (c) *A. discolor* in PDA with ABTS day 14, (d) *S. hirsutum* in PDA with ABTS day 1, (e) *S. hirsutum* in PDA with ABTS day 14, (f) control with RBBR and PDA, (g) *A. discolor* in PDA with RBBR day 1, (h) *A. discolor* in PDA with RBBR day 14, (i) *S. hirsutum* in PDA with RBBR day 1, (j) *S. hirsutum* in PDA with RBBR day 14.

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CHAPTER IV

Biodegradation of olive mill solid waste by *Anthracophyllum discolor* and *Stereum hirsutum*: Effect of copper and manganese supplementation

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Biodegradation of olive mill solid waste by *Anthracophyllum discolor* and *Stereum hirsutum*: Effect of copper and manganese supplementation

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Abstract

Anthracophyllum discolor and *Stereum hirsutum* produce manganese peroxidase (MnP) and lacasse to break down lignin, being a potential biological pretreatment of lignocellulosic biomass. An adequate dosage of manganese and copper could be used for increasing the production of MnP and lacasse according to literature. Hence, the aim of this work was to evaluate the effect of copper and manganese added to olive mill solid waste (OMSW) inoculated with *A. discolor* and *S. hirsutum* on ligninolytic enzyme activity, lignin degradation, and phenolic compound removal. Optimal dosages of metals increased the enzyme activity depending on the

strain. A positive effect associated with both strains of Mn could be seen as a direct consequence of improving the MnP activity. The highest level of lignin degradation was also associated with the dosage added, and the strain, *S. hirsutum*, with 2.5 mg Kg^{-1} of Cu and 7.3 mg Kg^{-1} of Mn, added the best condition. This strain also achieved a stable phenol removal efficiency of 65-75% in OMSW at 25 days, while in its leachate, the values were 80-95% at 30 days. However, at 20 days, 80-90% removal values were achieved by both strains. Therefore, they could be good candidates for future mycoremediation processes.

Keywords. Biological pretreatment, inducers, phenols, lignin, white-rot fungi.

4.1. Introduction

Around 1000 Gt of lignocellulosic biomass, including wheat straw, sugarcane bagasse, and corn stalks, are generated annually (Al-Mallahi *et al*., 2016). The molecular structure of these biomasses is complex due to the presence of lignin, which forms a strong bond with cellulose and hemicellulose. This bond significantly restricts their processing and subsequent utilization (Ning *et al*., 2021). To break the compact structure of the lignocellulosic fibers, physicochemical treatments have been widely proposed for treating lignocellulosic waste due to the combination of chemicals and severe conditions (high temperature and or pressure). Nowadays, the use of a wide variety of biocatalysts from fungi in the form of crude enzymatic extracts enriched in laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP) is gaining attention as an alternative to physicochemical treatments (Chaudhary *et al*., 2023; Khalil, 2023). The main advantages of fungal treatments include that they do not imply the use of toxic organic solvents (Sheldon and Woodley, 2018), do not require a high energy consumption (El-Gendi *et al*., 2022), and, usually, a crude enzymatic extract can be easily obtained during the treatment for further application, like xenobiotic removal (Bilal and Iqbal, 2020; Corbu *et al*., 2023). Thanks to these advantages, several authors have proposed the application of fungal treatments for the biodegradation and stabilization of lignocellulosic biomasses, such as *Stereum hirsutum* and *Trametes versicolor* for radiata pine wood chips (Shirkavand *et al*., 2017); *Ceriporiopsis subvermispora* for sugarcane bagasse (Machado and Ferraz, 2017); *Ganoderma lobatum* and *Gloeophyllum trabeum* for wheat straw (Hermosilla *et al*., 2018); *Irpex lacteus* for

olive tree biomass (Martínez-Patiño *et al*., 2018); *T. versicolor* for manure with Triticale (Akyol *et al*., 2019); or *S. hirsutum* and *A. discolor* for olive mill solid waste (Benavides *et al*., 2022).

Despite the potential interest in fungal treatments, enhancing their application to biodegradation processes and improving the obtained enzymatic extracts is still challenging. To achieve this, many strategies can be implemented, including enhancing the nutrient balance through the careful selection of substrates (Atilano-Camino *et al*., 2020; Henske *et al*., 2018; Mishra *et al*., 2017), adding metals as inducers and or cofactors (Baldrian, 2003; Vrsanska and Langer, 2016), or choosing an appropriate fungal strain (Tortella *et al*., 2005), etc., which can positively integrate into the bioprocess. In concrete terms, the addition of a wide variety of inducers and cofactors, such as Cu, Mn or Fe, are involved in essential mechanisms for improving the ligninolytic enzymatic activity in white-rot fungi (WRF) (Acevedo *et al*., 2011; Baldrian, 2003; Hermosilla *et al*., 2017; Reddy and Kanwal, 2022; Rubilar *et al*., 2011b). Inducers serve as mediators to assist the bioconversion of several compounds, and their presence can increase the kinetics of reactions by enhancing the enzymatic activity (da Silva Vilar *et al*., 2022). Cofactors act on the transcriptional level, and they are part of the architecture of enzymes with a wide variety of functions that mainly involve electron transfer, O_2 binding, activation, and reduction, NO_2^- and N_2O reduction, and substrate activation (Sekretaryova *et al*., 2019). The effect of metal addition has been usually evaluated based on the added doses. For example, Cu addition of 0.25 mM in the culture medium can increase the laccase activity by *Ganoderma lucidum* up to eight times compared to culture media without this metal (Kuhar and Papinutti, 2014). Mn dosage has been reported to promote MnP from *A. discolor* and *Fomes sclerodermeus* in a wide range from 0.2 to 2.0 mM (Acevedo *et al*., 2011; Papinutti *et al*., 2003). However, it is worth noting that the microbial response could be more related to bioavailable metal fractions or, if the substrates contain a notable concentration of metals, the total metal concentration in the medium (Gustavsson *et al*., 2013; Pinto-Ibieta *et al*., 2016).

On the other hand, agricultural biomasses may be used as growth substrates, such as wheat straw and wood chips (Rubilar *et al*., 2011a), banana peel (Vivekanand *et al*., 2011), corn stover, switchgrass, wheat straw, soybean stalk, hardwood (Wan and Li, 2012), rice straw, kapok fibers, wood meal, or pulp waste (Andriani and Tachibana, 2016) or olive mill solid waste (OMSW) (Benavides *et al*., 2022). The presence of lignocellulosic fibers in these agricultural

biomasses can promote the production of ligninolytic enzymes during fermentation processes, thereby boosting fungal growth and enzymatic extract potential (Atilano-Camino *et al*., 2020; Majul *et al*., 2020). Andriani and Tachibana (2016) demonstrated that ligninolytic enzyme production (laccase and lignin peroxidase) from *Bjerkandera adusta* SM46 depends on the type of lignocellulosic material used, achieving the highest laccase and manganese peroxidase activities (1.08 and 0.42 U g, respectively) using rice straw in comparison with kapok fibers, wood meal, or pulp waste. Benavides *et al*. (2022) evaluated the ligninolytic enzyme activity of *A. discolor* and *S. hirsutum* using OMSW as substrate. The OMSW acted as growth support and substrate to the fungi and promoted the secretion of extracellular ligninolytic enzymes, with both WRF strains reaching a high OMSW biodegradability. Applying both strategies, i.e., metal addition and the use of the lignocellulosic substrate, to WRF could result in synergistic effects for the degradation of recalcitrant organic pollutants and the production of enzyme extracts (Atilano-Camino *et al*., 2020). However, to our knowledge, the joint evaluation of the two strategies has not yet been properly evaluated. Therefore, this work aimed to assess the increase in the enzymatic activity from WRF through 1) Cu and Mn addition in liquid-state fermentation by *A. discolor* in culture medium, and 2) a combination of Cu and Mn addition and OMSW as substrate in solid-state fermentation (SSF) by *A. discolor* and *S. hirsutum*, separately. The processes were evaluated according to the enzyme production (Lac, MnP, and MniP), lignin degradation, and phenol removal.

4.2. Materials and methods

4.2.1 Microorganisms

The present research was carried out using *A. discolor* Sp4 and *S. hirsutum* as fungal strains. The selection, isolation and storage of the fungi was previously described in Benavides *et al*. (2023) (more details are also provided in supplementary material). Prior to the experiment phases, the strains were activated in potato dextrose agar medium (39 g of PDA in 1 L of distilled water and sterilized in an autoclave for 20 min at 121 °C). From that, a 6 mm disk of fungi was used as initial inoculum for the assays, obtained after an incubation during 7 days at 28ºC (Benavides *et al*., 2023).

4.2.2 Olive Mill Solid Waste (OMSW)

The olive mill solid waste (OMSW) used as substrate for the fungi growth was collected from the olive mill "Olivares de Quepu", located in Pencahue, Chile. To avoid undesirable fermentation processes, the OMSW was stored at −20 °C prior its use.

4.2.3 Effect of copper and manganese on enzymatic activity by A. discolor in liquid medium

The effect of copper and manganese on ligninolytic enzyme production was evaluated by using various concentrations from stock solutions of $CuSO_4.5H_2O$ and $MnSO_4.H_2O$ in a medium with glucose (15 g L^{-1}), peptone from potatoes (5 g L^{-1}) and yeast extract (2.5 g L^{-1}) (pH 5.5) (Table 1). 100 mL of culture medium in 250 mL Erlenmeyer flasks were inoculated with five 6 mm agar disks of *A. discolor* (details described in 2.1) and incubated for 30 days at 28ºC.

Table 1. Conditions for evaluation of the effect of inducer cofactors on enzymatic activity from *A. discolor*.

Trial No.	Strain	Cu (mg L^{-1})	Mn (mg L^{-1})
1		0.0	0.0
2		25.5	0.0
3		0.0	72.8
4	*A. discolor*	25.5	72.8
5		30.7	36.4
6		12.7	87.8
7		12.7	36.4

4.2.4 Solid-state fermentation

For inoculum preparation, a 250 mL Erlenmeyer flask containing 100 mL of culture medium with glucose, peptone from potatoes, and yeast extract at pH 5.5 (described in section 2.3) was autoclaved at 121 °C for 20 min. Each flask was then initially inoculated with five 6 mm agar disks of active mycelia (section 2.1) and then incubated for 14 days at 28 °C. Finally, a

liquid inoculum was obtained by blending the previous fungal cultures for 1 minute, according to the methodology described in Rubilar *et al*. (2007).

For the solid-state fermentation (SSF) assay, 10 g of sterilized OMSW at 121 °C for 20 min in Erlenmeyer flasks (100 mL) were used, adjusting moisture to 80%. Moreover, the different metal concentrations selected for each experiment (Table 2) were added in 2 mL of sterile water. The flasks were then autoclaved and, after cooling, inoculated with 1 mL of blended fungal mycelia (Rubilar *et al*., 2007). Negative controls were included as flasks without fungal inoculum. The SSF experiments were run during 30 days in the dark at 28 °C.

Table 2. Culture conditions for OMSW treatment in solid-state fermentation (SSF) using *A. discolor* and *S. hirsutum*

Trial No	Metal added		Total metal content	
	Cu (mg Kg^{-1})	Mn (mg Kg^{-1})	Cu (mg Kg^{-1})	Mn (mg Kg^{-1})
1	0.0	0.0	8.48	13.0
2	5.1	0.0	13.58	13.0
3	0.0	14.6	8.48	27.56
4	5.1	14.6	13.58	27.56
5	6.1	7.3	14.62	20.28
6	2.5	17.6	11.02	30.56
7	2.5	7.3	11.02	20.28

4.2.5 Analytical methods

Previously to the experiment, the total content of Cu and Mn in OMSW was analyzed from 0.5 g of sample digested with 10 mL of HNO_3 5% (Microwave Digestion of US EPA 3051-Solid Sample) and measured by inductively coupled plasma - mass spectrometry (EPA Method 6020B -SW-846) (US EPA, 2014). Extract recovery and enzymatic activity quantification were performed by the spectrophotometric methodology described by Acevedo *et al*. (2011) and

Hermosilla *et al*. (2017). More details about the determination of Lac, MnP and MniP can be found in Benavides *et al*. (2023). Total phenolic content was quantified by spectrophotometry with a gallic acid (GA) calibration curve, using the Folin-Ciocalteu method. the results were expressed as mg GAE L^{-1} to leachate and mg GAE 100 g^{-1} of OMSW after treatment in SSF. More details about the extraction process before the phenolic quantification can be found in the supplementary material. The phenolic profile determination was carried out by a Hitachi Primaide high-performance liquid chromatograph was used, coupled to a diode array detector (HPLC-DAD) equipped with a Kromasil® C18 column (more details in supplementary material). Lignin content in the OMSW was quantified as acid-insoluble Klason lignin (TAPPI T222-om02) and acid-soluble lignin (TAPPI UM 250), according to the methodology described by Dence and Lin (1992). The difference between the lignin content prior and after the fungal-treatment was used to calculate the percentage of lignin degradation according to Hermosilla *et al*. (2018) (more details in supplementary material).

4.3. Results and discussion

4.3.1 Effect of Cu and Mn addition on enzymatic activity by *A. discolor* in liquid state fermentation.

The variations of Lac, MnP, and MniP were monitored in relation to the different metal doses using *A. discolor* in liquid-state fermentation using a culture medium as substrate (Figure 1). MnP was the enzyme that reached a higher production than Lac and MniP, with concentrations below 2 U L^{-1} and 8 U L^{-1}, respectively. Similar results were reported by Hermosilla *et al*. (2017) using WRF with metal addition as Fe and Mn in a substrate with wheat straw and Kirk's media, where the enzymatic activity of MniP was under 5 U L^{-1}. The highest production of MnP was detected at 20 days of treatment for a dose of 2 mM Mn, reaching a value of 34.9 ± 1.0 U L^{-1} (Figure 1a). This value was around 9 times higher than the control (no metal addition), i.e., 3.9 ± 0.3 U L^{-1} at the same 20 days. The positive effect of Mn dosage on MnP activity in *A. discolor* has been previously reported by Acevedo *et al*. (2011), where adding 0.25 mM of Mn showed the best results, achieving 1354 U L^{-1} of MnP activity.

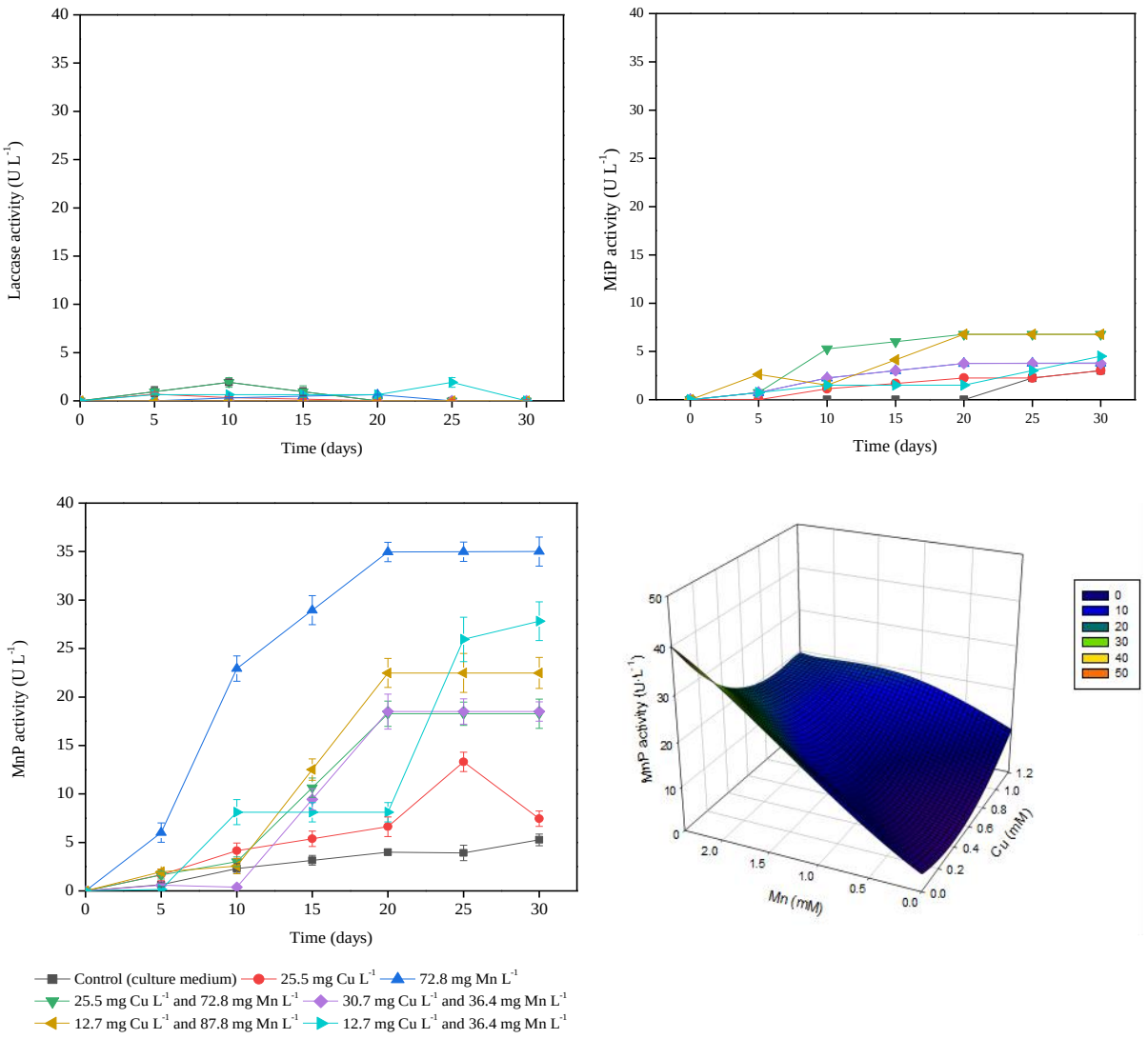

Figure 1. Effect of Mn and Cu on laccase (a), manganese-independent peroxidase (b), and manganese peroxidase activity (c) produced by *A. discolor* in liquid medium, and response surface plot of manganese peroxidase activity at 20-day (d).

The response surface plot for the values obtained at 20 days showed a positive effect of Mn dose on MnP activity when the total concentration of this metal increased from 20.4 to 30.6 mg L^{-1} (Figure 1b). This is possible because MnP is a heme-containing enzyme consisting of Mn-binding sites, and it catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Kannaiyan *et al*., 2015). Thus, Mn^{2+} plays a key role in the transcriptional phase and the activation of the peroxidase enzyme (Baldrian, 2003; Slavens *et al*., 2019). On the other hand, adding Cu slightly favored the enzyme activity at increasing Cu concentrations, although to a more limited extent

than Mn (Figure 1b). This increase could be associated with Cu having an important role in regulating peroxidase activity, causing induction or suppression of the enzyme activity depending on the concentration of nutrients (Slavens *et al*., 2019).

The combined addition of Cu and Mn negatively affected the MnP activity compared to the individual addition of the metals (Figure 1b). Concretely, MnP activity decreased up to 50% compared to the individual addition of Mn, but it was better than when only copper was added (Figure 1b). These results were consistent with the values obtained on day 20, where the MnP activity in the treatments with both metals in different concentrations was in the range of 7.0 ± 0.3 U L^{-1} to 22.0 ± 1.0 U L^{-1} (Figure 1a), three times higher than the control, but 17% lower than the optimum seen for the Mn dose of 2.41 mM (34.9 ± 1.0 U L^{-1} of MnP). These results may be associated with 1) a high concentration of metals in the extracellular environment that limits the development of the ligninolytic enzyme system (Baldrian, 2003); 2) under this condition, metal excess, probably copper, can also cause oxidative damage of proteins by the induction of oxidative stress associated with the production of reactive oxygen species like hydroxyl or superoxide radicals (Stohs and Bagchi, 1995), while Mn accumulation can be generating consequences on the cellular level (vacuoles for storage and degradation and Mn export), restricting the associated process to Mn (Robinson *et al*., 2021); and 3) enzymes are not protected by cell-associated metal-detoxication mechanisms (Baldrian, 2003).

4.3.2. Effect of Cu and Mn addition on enzymatic activity by *A. discolor* and *S. hirsutum* in solid-state fermentation.

4.3.2.1. *A. discolor*

Lac was the main ligninolytic enzyme produced by *A. discolor*. The highest Lac activity was determined for the combined dose added of 7.3 mg Mn Kg^{-1} and 2.5 mg Cu Kg^{-1}, reaching a mean value of 54.8 ± 6.8 U L^{-1} from days 10 to 20 (Figure 2a). This value was 2.2 times higher than the determined for the control (no metal addition). However, metal doses of 17.6 mg Mn Kg^{-1} and 2.5 mg Cu Kg^{-1} decrease the Lac activity by around 50% compared to the above-mentioned optimal activity. Similarly, individual Cu addition strongly affected the Lac activity at days 10-20, i.e., 90-100% reduction compared to the optimal value. Therefore, these results explain that there is also an optimal dosage of metals that, added together and in appropriate concentrations,

may be an alternative to increasing the lac activity from *A. discolor*. However, these compounds added in an exceeding concentration can result in an inhibition of enzyme production.

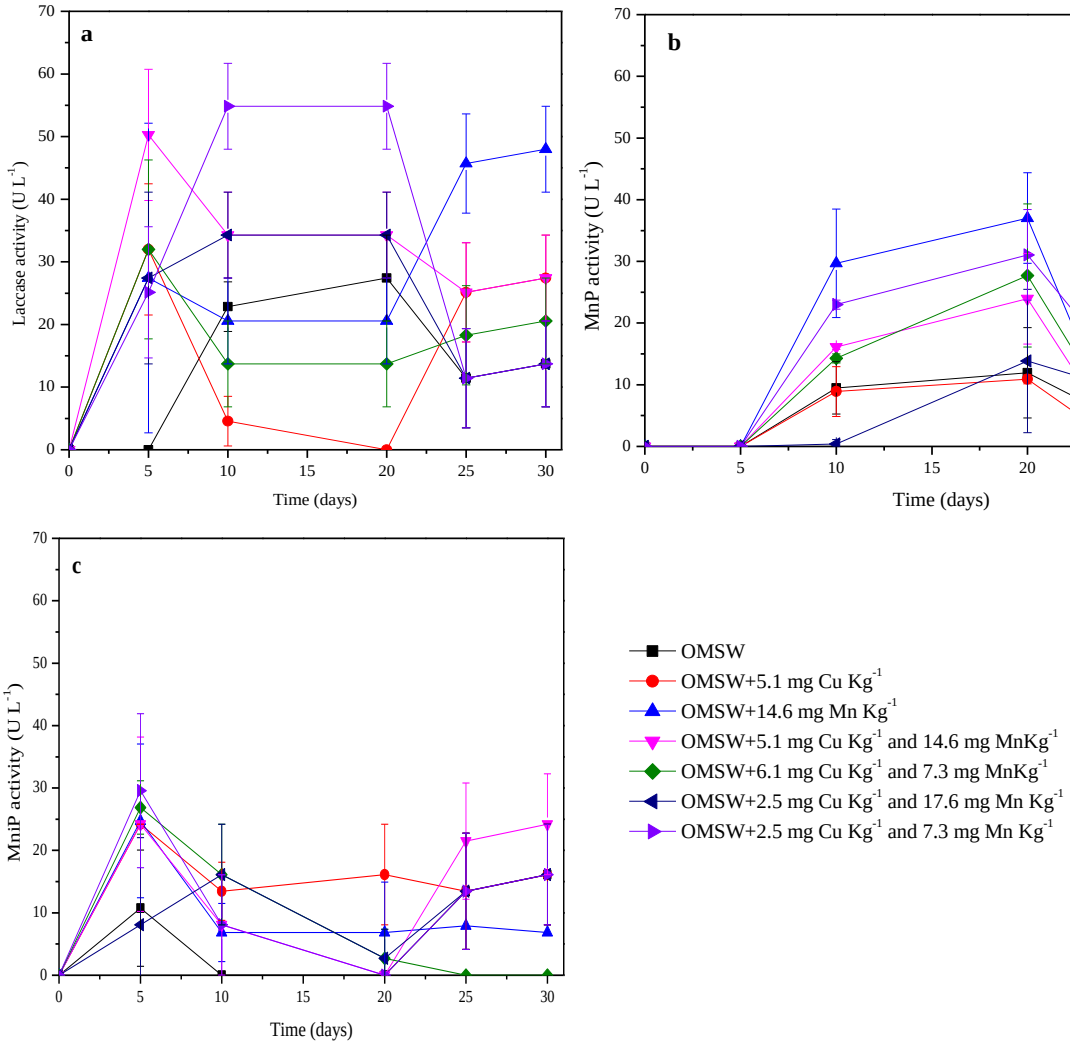

Figure 2. Laccase (a), manganese peroxidase (b), and manganese-independent peroxidase activity variation (c) as a function of different doses of copper and manganese for A. discolor in solid-state fermentation using OMSW as substrate.

The observed positive effects of Cu in combination with Mn, but its inhibitory behavior when it is added alone concerning the control, was related by Kannaiyan *et al.* (2015) to cross-regulation phenomena, reporting that manganese can act as an inducer to Lac activity. That would explain why the improvement in the enzymatic activity was higher by adding combined doses than by adding individual metals. Likewise, the lack of Lac activity with the individual Cu dose could be because, without the action of the Mn, the MnP was inactive, and, therefore, the

lignocellulosic degradation could not be enough to release phenolic compounds. Considering that Lac activity has been closely related to phenol degradation (Gianfreda *et al*., 1999; Giardina *et al*., 2010; Mehra *et al*., 2018), the fungi may not need it to activate Lac production mechanisms.

Despite the negative effects of some metal doses compared to the control, the results were markedly higher than the Lac values determined in the previous liquid-state fermentation assays, i.e., less than 2 U L^{-1} (Figure 1a). Similarly, the results of MnP activity at days 10 and 20 were higher than those obtained with liquid-state culture media (section 3.1) in the same period, increasing 5 U L^{-1} at day 10 and 3 U L^{-1} at day 20 (Figure 1b). These enhancements may be associated with the use of a complex matrix as substrate, with high lignocellulosic contents (35.2 ± 3% cellulose, 33.4 ± 4% lignin, and 45.5 ± 8% of hemicellulose) that could act as enzyme inducer, favoring the secretion of both enzymes by the fungi (Andriani and Tachibana, 2016; Benavides *et al*., 2022; Kheirkhah *et al*., 2020; Majul *et al*., 2020; Salvachúa *et al*., 2011; Vivekanand *et al*., 2011; Wan and Li, 2012).

The highest value of MnP activity in solid-state fermentation with *A. discolor* was determined for a metal dose of 14.6 mg of manganese Kg^{-1}, reaching a value of 37 ± 7.3 U L^{-1} at day 20 (Figure 2b). This value was more than 3 times higher than the determined for the control, which reached a maximum MnP activity of 11.9 ± 7.3 U L^{-1} on day 20. The individual dose of Cu did not show any difference in the MnP activity compared to the control without metal dose. The combined doses of Cu and Mn increased the MnP activity compared to the control, where the dose of 2.5 mg Kg^{-1} of Cu and 7.3 mg Kg^{-1} of Mn showed the best results with a value of 29.5 ± 12.3 U L^{-1}. This value was similar to the obtained for the individual dose of 14.6 mg Kg^{-1} of manganese (Figure 2b). The positive effect of the Mn dose was expected since this metal has been shown to play a key role in the transcriptional phase as well as in the regulation of either laccase or peroxidase activity, causing either induction or suppression of the enzyme activity, respectively, depending on the nutrient concentration (Slavens *et al*., 2019). Manganese directly participates in lignin degradation in white-rot fungi through the reaction cycle of Mn-dependent peroxidase (MnP) (Baldrian, 2003).

A positive effect was also observed for the MniP at the different metal doses compared to the control without metal addition (Figure 2c). This improvement could be due to the

enhancement observed for the Lac and MnP activities, which release organic compounds initially comprised of the lignocellulosic structures and phenols. As a result, these compounds become more bioavailable for fungi biodegradation, which activates more enzymatic mechanisms including MniP (Giardina *et al*., 2000; Martinez *et al*., 1996; Moreira *et al*., 2006; Petruccioli *et al*., 2009).

The response surface plot for Lac, MnP, and MniP activities at day 20 by *A. discolor* is shown in Figure 3. Lac activity showed optimal values at intermediate doses of Mn and Cu, i.e., between 8.0 and 14 mg Kg^{-1} of Mn and 2.0 to 4.0 mg Kg^{-1} of Cu. Increasing the individual metal amounts decreased the Lac activity (Figure 3a). This improvement in Lac activity obtained with individual doses was enhanced even when the metals were added together in the same concentration ranges, reaching activity values above 40 U L^{-1}, around 10 U L^{-1} higher than that predicted for the control. The individual effect of manganese on MnP activity was favorable at increasing concentrations (Figure 3b); however, the MnP activity was not influenced by the individual addition of Cu at the assessed range (Figure 3b). The combined dose of Cu and Mn can improve the MnP activity at doses below 14.0 mg Kg^{-1} of Mn. At doses higher than 14.0 mg Kg^{-1} of Mn, however, the co-addition of even low concentrations of Cu, i.e., 1-3 mg Kg^{-1}, resulted in a marked decrease in the MnP activity (Figure 3b). The individual doses of metals enhanced the MniP activity compared to the control, while the combined effect was negative in every case (Figure 3c). This trend should be carefully considered because these results correspond to the values obtained at 20 days, which was considered optimal for all the enzymatic activities. However, MniP activity might not be representative of the process behavior. In fact, according to Figure 1c, positive effects were observed in the combined metal doses in the other sampling days. Given the results, perhaps the optimal values to improve the enzymatic activity of the three types of enzymes would be the addition of medium concentrations of Mn (6-12 mg Kg^{-1}) and low concentrations of Cu (1-2 mg Kg^{-1}).

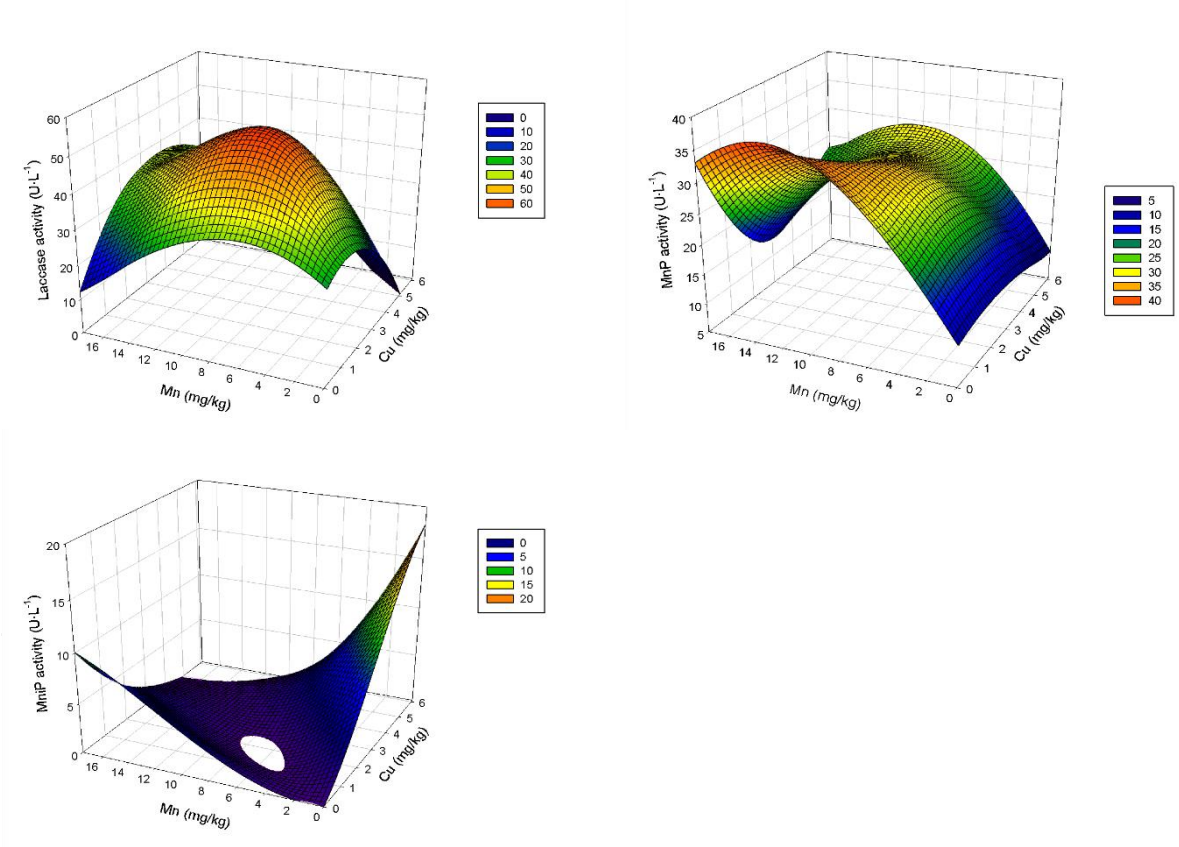

Figure 3. Three-dimensional surface plots of manganese peroxidase activity produced by *A. discolor* at 20-day in solid-state fermentation using OMSW as substrate. a) Plot of Lac activity as a function of copper and manganese; b) Plot of MnP activity as a function of copper and manganese; c) Plot of MniP activity as a function of copper and manganese.

The evolution of enzymatic activities determined for each metal dose to *S. hirsutum* using OMSW as substrate shows that the highest Lac activity was determined on day 5 at the control, obtaining 120.0 ± 10.5 U L^{-1} (Figure 4a). This peak in the Lac activity rapidly decreased to values below 30 U L^{-1}, i.e., around 77.2% lower than at day 5. However, the metal dose of 2.5 mg Kg^{-1} of Cu and 17.6 mg Kg^{-1} of Mn maintained the Lac activity at values higher than 60 U L^{-1} up to day 20 (Figure 4a). The metal concentration in the OMSW was enough to result in higher Lac activity in the control than at the other evaluated metal doses. The highest individual dose of Cu, i.e., 5.1 mg Cu Kg^{-1}, strongly affected the Lac activity, showing almost negligible values at most of the experimental times. This inhibition caused by excessive copper may be linked to the direct toxicity of free copper ions as well as the production of a toxic compound, which could result in oxidative stress at an advanced stage of fungal growth and could be responsible for limited

transcriptional induction (Palmieri *et al*., 2000). The alteration of the target protein to inhibit metal binding, metal-restricted uptake to prevent cellular interference, and overproduction of targeted proteins to control or compensate for loss of functions directly related to the level toxicity of metal are some other resistance mechanisms developed by fungi under conditions of copper excess. These strategies may ultimately have an impact on the secondary metabolism of the fungus (Robinson *et al*., 2021).

The highest value of MnP activity by far was determined for a metal dose of 6.1 mg Kg^{-1} of Cu and 7.3 mg Kg^{-1} of Mn, reaching a value of 140.8 ± 2.6 U L^{-1} at day 10 and 160.8 ± 20.6 U L^{-1} at day 20 (Figure 4b). These values were 25 times higher than the determined for the control, which reached a maximum MnP activity of 2.2 ± 0.6 U L^{-1} on day 10 and 6.2 ± 3.6 U L^{-1} on day 20. The individual dose of the metal also resulted in a positive effect compared to the control, reaching a value of 20 ± 2.6 U L^{-1} with the addition of 5.1 mg Kg^{-1} of Cu and 19 ± 2.6 U L^{-1} with the addition of 14.6 mg Kg^{-1} of Mn, i.e., around 40% compared to the control with no metal addition (Figure 4b). These values are higher than the results obtained for *A. discolor* in the same experimental period in comparison to the control (without Cu or Mn), showing that the improvements depend on other parameters despite the dosed metal concentration, such as the requirement of the used strains and/or the metals provided by the substrate itself (Liu *et al*., 2020).

For MniP activity, no significant differences were observed with the addition of metals compared to the control, showing a range of values between 10.0 ± 3.9 U L^{-1} and 54.5 ± 12.3 U L^{-1} (Figure 4c). MniP activity produced by *S. hirsutum* was lower than those obtained in the treatments under solid-state fermentation with *A. discolor* (Figure 2c). However, *S. hirsutum* allowed the increase of enzymatic activity by at least 50% compared to the results obtained under liquid fermentation conditions using commercial media, which were always under 8 U L^{-1} (Figure 1b). This suggests that using lignocellulosic waste as a growth substrate and supplementary inducer is a valid strategy to enhance enzymatic extract production (Merino *et al*., 2019; Slavens *et al*., 2019).

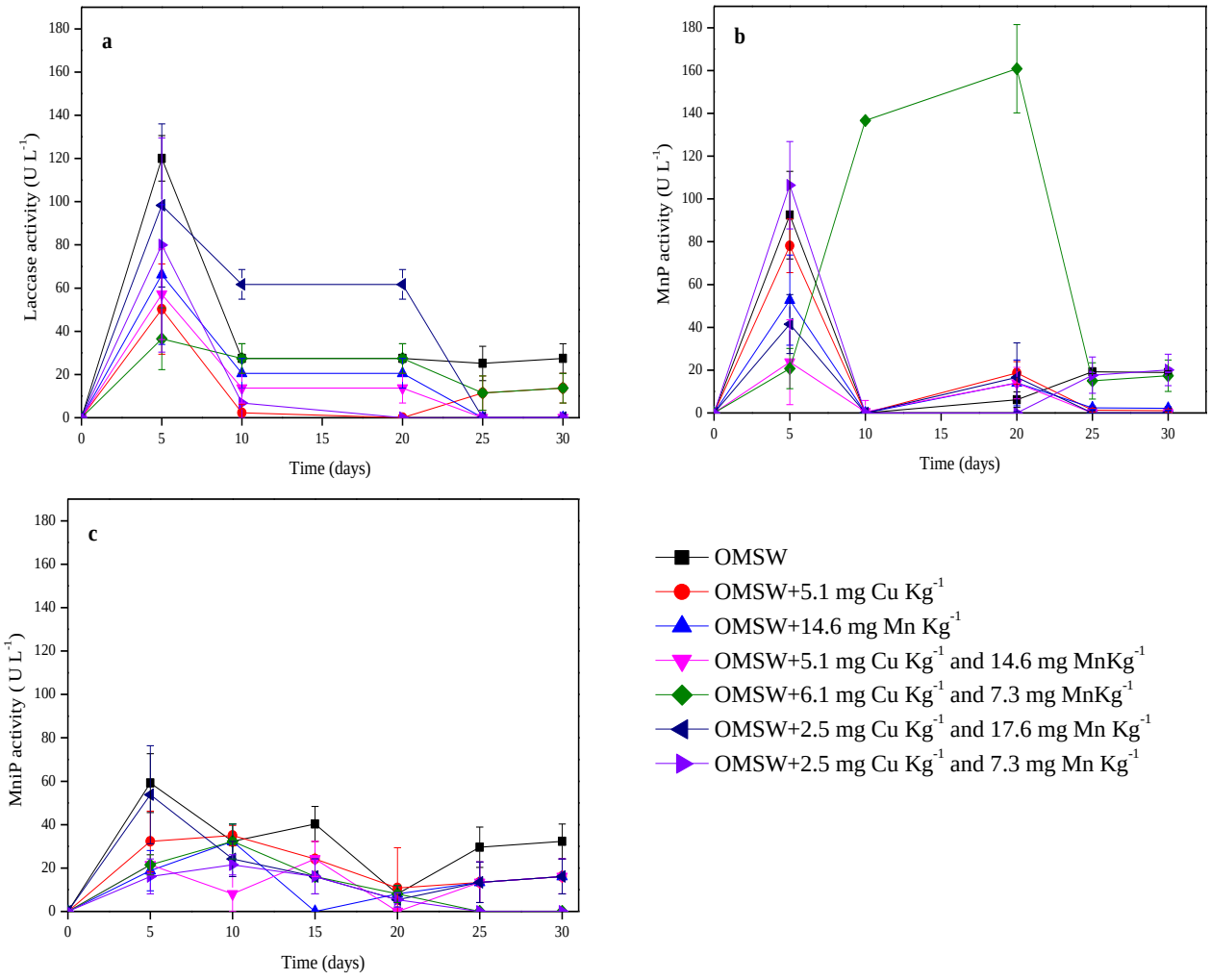

Figure 4. Laccase (a), manganese peroxidase (b), and manganese-independent peroxidase activity variation (c) as a function of different doses of copper and manganese for *S. hirsutum* in solid-state fermentation using OMSW as substrate.

The response surface plots for Lac, MnP, and MniP activities at day 20 by *S. hirsutum* are shown in Figure 5. The Lac activity showed the highest values at the combination of the highest Mn concentrations and intermediate Cu concentrations (Figure 5a). However, MnP activity was favored at high Cu and intermediate Mn concentrations. The Cu dose strongly influenced MnP activity, the values of which were almost constant at Cu concentrations below 4 mg Kg^{-1} regardless of the dosed Mn (Figure 5b). The individual dose of Cu favored MniP activity. However, the individual dose of Mn did not substantially impact the MniP activity. Furthermore,

MniP activity was negatively affected by increasing the concentration of both metals when dosed together. Therefore, according to the response surface plots, it is not possible to determine an optimal dose concentration of both metals for all the enzyme activities, showing the contradictory requirements Lac and MnP activities. That would imply that the metal dose strategy needs to be adjusted to the concrete enzyme activity, the enhancement of which is more desirable at each time.

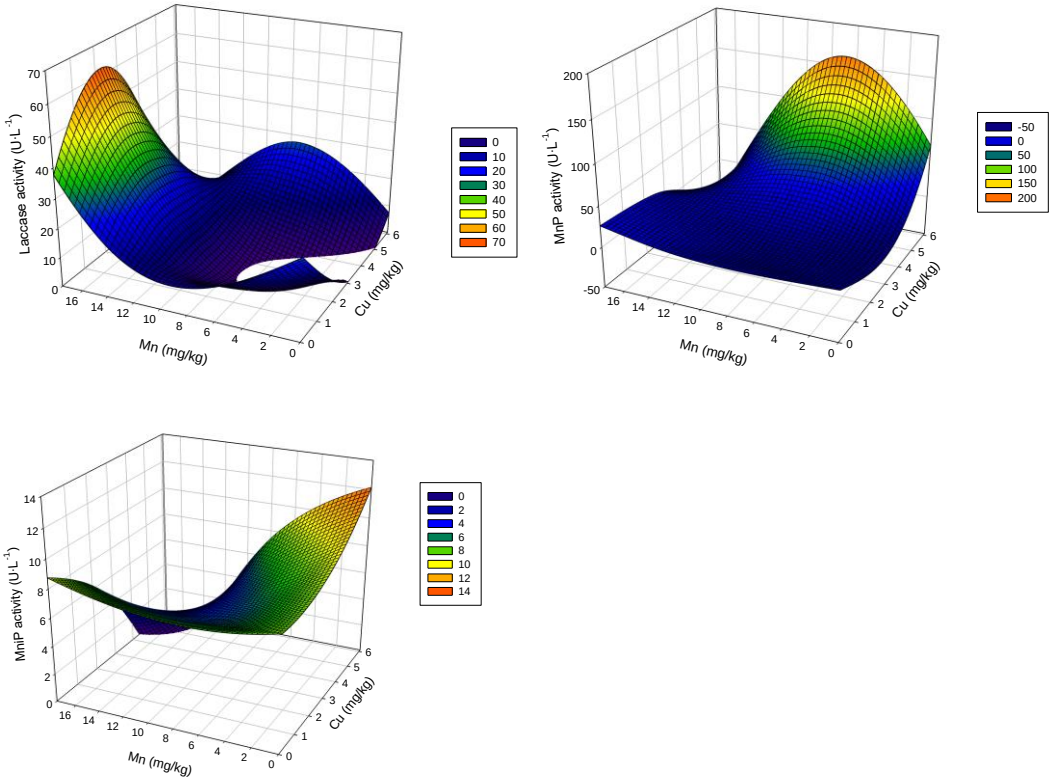

Figure 5. Three-dimensional surface plots of enzyme activity produced by *S. hirsutum* at 20-day in solid-state fermentation using OMSW as substrate. a) Plot of Lac activity as a function of Mn and Cu; b) Plot of MnP activity as a function of Mn and Cu; c) Plot of MniP activity as a function of Mn and Cu.

4.3.3. Effect of Cu and Mn on lignin degradation by *A. discolor* and *S. hirsutum* in solid-state fermentation.

The highest level of lignin degradation, 41.5 ± 3.2% at 20-day, was obtained with the lowest dose of Cu (2.5 mg Kg^{-1}) and intermediate dose of Mn (7.3 mg Kg^{-1}) in the treatment with *S. hirsutum* (Figure 6). For *A. discolor*, the highest level of lignin degradation (37.7 ± 6.9%) after

20 days was obtained with the maximum individual Mn dose (14.6 mg Kg^{-1}). That positive effect associated with both strains to Mn dose can be a direct consequence of the improvement of the MnP activity described in section 3.2. Moreover, Sari *et al*. (2016) reported that improving the MnP activity favors the oxidizing of Mn^{2+} to Mn^{3+} by the MnP. The highly reactive Mn^{3+} can combine with organic chelating compounds and acts as a low-molecular-weight, diffusible redox mediator targeting the oxidation of the phenolic components of lignin and, thus, enhancing the lignin degradation (Kannaiyan *et al*., 2015).

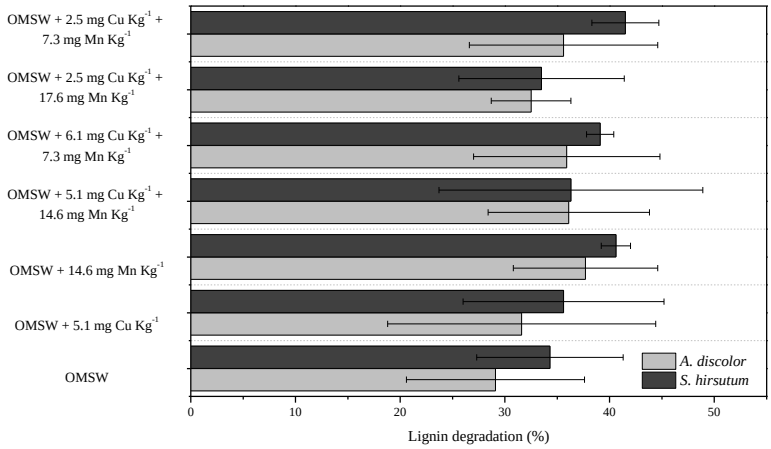

Figure 6. Lignin degradation (%) by *A. discolor* and *S. hirsutum* in solid-state fermentation with different metal doses at day 20.

4.3.4. Effect of Cu and Mn on phenolic compounds degradation from OMSW using *A. discolor* and *S. hirsutum* in solid-state fermentation.

The effect of copper and manganese on OMSW treatment by *A. discolor* and S. *hirsutum* and the behavior of phenolic compounds was evaluated by monitoring the total phenolic compound removal percentage throughout the experimental period in the OMSW (Figure 7a and 7b) and its leachate (Figure 7c and 7d). As noted, in most cases, no significant differences were found in the treatments with different individual and combined metal doses. *A. discolor* removed phenolic compounds effectively from OMSW, reaching stable values of 45%-60% at 5 days (Figure 7a), while leachate phenol removal values were 85-95% at 10 days (Figure 7b). *S. hirsutum* achieved a stable removal efficiency of 65-75% of phenols in OMSW at 25 days (Figure 7c), while in its leachate, the highest removal values were between 80-95% at 30 days. However, at 20 days, it is possible to observe an 80-90% removal of phenol (Figure 7d).

Therefore, both strains could be good candidates in future mycoremediation processes for OMSW thanks to their oxidative ligninolytic system that allows the lignin degradation and opening of phenyl rings (Benavides *et al*., 2022; Naraian, 2019). The phenol removal efficiencies achieved in this work were close to those reported by other authors, such as Reina *et al*. (2013), who described an efficiency in phenol compound removal from OMSW of 90% by *Auricularia auricula-judae*, 80% by *Bjerkandera adusta* and 100% by *Coprinellus radians*, all at 14-day under SSF without the addition of metals.

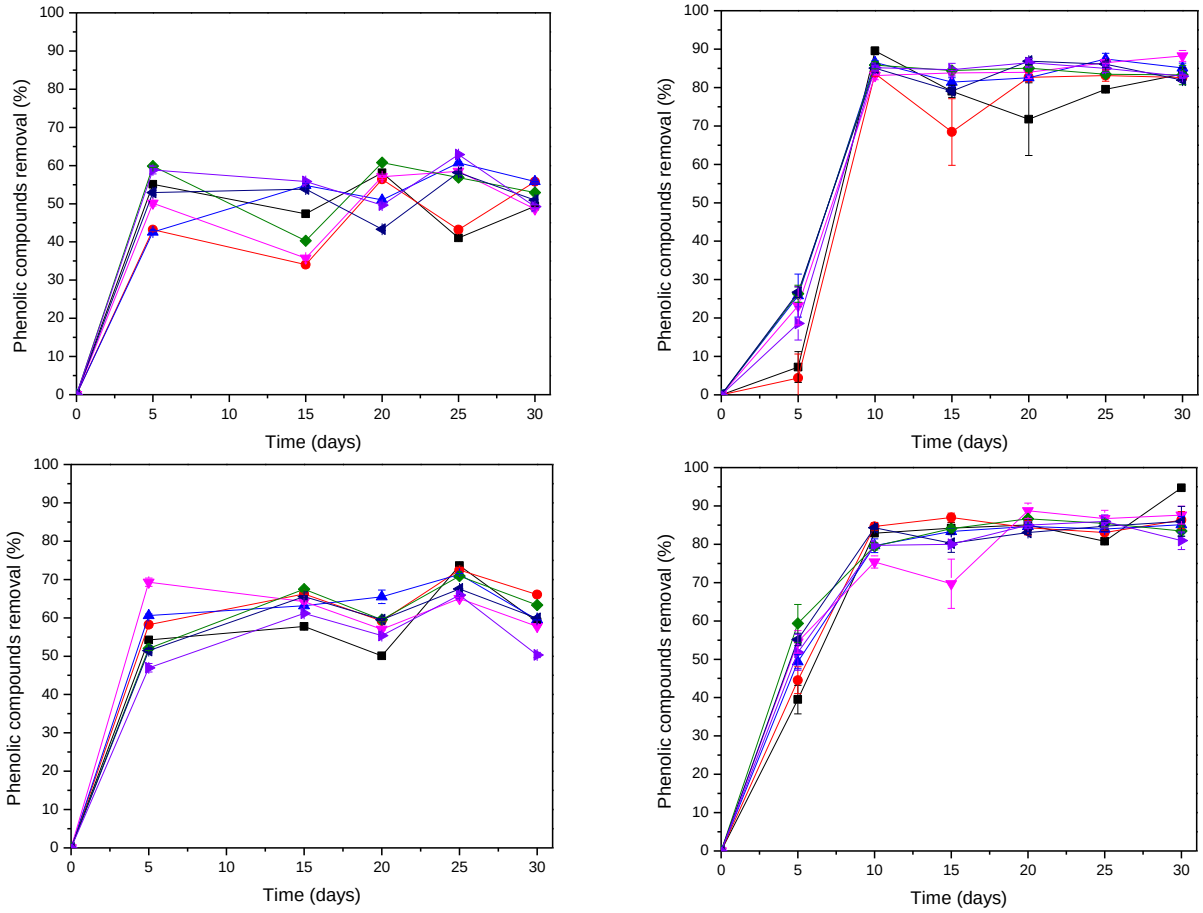

Figure 7. Phenol removal as a function of culture time using OMSW as substrate supplemented with metals for both strains studied: a) Removal in OMSW and b) Removal in leachate by *A. discolor;* c) Removal in OMSW and d) Removal in leachate by *S. hirsutum*

Table 3 shows the individual phenolic compound concentrations of the raw OMSW and at the OMSW treated with *A. discolor* and *S. hirsutum* and their leachates at day 20 when 2.5 mg Kg^{-1} of Cu and 17.6 mg Kg^{-1} of Mn were added. As can be seen, the final concentration of the

measured individual phenolic compounds indicated that most of them were widely metabolized during the process (Table 3), consistent with the high total phenol removal efficiency described in Figure 4. Despite most of the phenolic compounds being effectively degraded, some specific compounds were accumulated compared to the initial concentration during the fungal treatments. Specifically, in OMSW treated by *S. hirsutum* and its leachate, gallic acid hexoside, protocatechuic acid hexoside, and 3-hydroxytyrosol were detected (Table 3). In OMSW treated by *A. discolor* and its leachate, several compounds were accumulated concerning the raw OMSW, highlighting the accumulation of gallic acid hexoside, protocatechuic acid hexoside, 3-hydroxytyrosol, 3,4-dimethoxybenzyl alcohol, and 2,6-dimethoxyphenol (Table 3). The accumulation of these compounds would be related to the breakdown of the lignocellulosic fibers of the OMSW, entailing the release of the phenolic compounds integrated into these fibers (Luo *et al*., 2021). Likewise, some specific phenolic compounds can accumulate because they are intermediates of the incomplete degradation of other phenolic compounds (Serrano *et al*., 2017). The presence of a higher concentration of specific phenolic compounds in the OMSW treated by *A. discolor* and its leachate is due to the lower Lac activity observed with this strain compared to *S. hirsutum* (Figures 2a and 4a), which did not fully degrade these phenolic compounds.

Table 3. Individual phenolic compounds concentration (mg L^{-1}) in OMSW and its leachate obtained from the SSF assays at day 20.

Phenolic compounds	**OMSW**	**Leachate from OMSW pretreated by *A. discolor***	**Leachate from OMSW pretreated by *S. hirsutum***	**OMSW pretreated by *A. discolor***	**OMSW pretreated by *S. hirsutum***
4-Hydroxybenzoic acid	0.476 ± 0.028	ND	ND	ND	ND
Gallic acid hexoside	0.752 ± 0.062	1.016±0.042	0.805±0.013	ND	ND
3,4-dihydroxyphenylglycol	4.589 ± 0.389	ND	ND	ND	ND
Protocatechuic acid hexoside	ND	4.229±0.279	3.357±0.158	ND	4.257±1.608
3-hydroxytyrosol	2.142 ± 0.006	1.931±0.100	2.123±0.053	ND	ND
p-tyrosol	0.161 ± 0.002	ND	ND	ND	ND
Vanillic acid	0.324 ± 0.003	ND	ND	ND	ND
3,4-dimethoxybenzyl alcohol	6.348 ± 0.097	1.371±2.374	ND	6.758±1.925	ND
Vanillin	11.012 ± 0.205	ND	ND	ND	ND
2,6-dimethoxyphenol	0.425 ± 0.001	16.761±1.222	ND	5.424±1.473	ND
Hydroxycinnamic acids and derivatives					
Coutaric acid	ND	0.759±0.056	0.793±0.037	Traces	Traces
Chlorogenic acid	1.271 ± 0.017	ND	ND	ND	ND
p-Coumaric acid	Traces	ND	ND	ND	ND
Trans-ferulic acid	0.105 ± 0.003	ND	ND	ND	ND
Quercetin 3-rutinoside	0.378 ± 0.020	ND	ND	ND	ND
Quercetin 3-glucoside	0.623 ± 0.012	ND	ND	ND	ND
Myricetin	0.233 ± 0.037	ND	ND	ND	ND
Quercetin	2.631 ± 0.015	ND	ND	ND	ND
5-Hydroxymethylfurfural	0.354 ± 0.003	1.901±0.140	1.835±0.106	Traces	0.227±0.030

ND: not detected

4.4. Conclusions

Both fungal strains treated the OMSW under SSF conditions. Lac activity improvement was more related to Cu addition, whereas Mn addition improved MnP activity. The positive effect of Mn on MnP may have released phenols from the OMSW, which explains the positive relationship between Mn and Lac.

The use of Cu and Mn allows the enhancement of specific enzymes, which opens an opportunity to design specific supplements according to the fungus application.

In this study, there was a limited impact of the added metals on lignin degradation, with a high phenol removal being observed in every case.

CRediT authorship contribution statement

V. Benavides: Methodology, Formal analysis, Writing - Original Draft. A. Serrano: Conceptualization, Writing - Original Draft. F. Pinto-Ibieta: Writing - Review and Editing. O. Rubilar: Writing - Review and Editing, Resources, Funding acquisition. G. Ciudad: Writing - Review and Editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER V

Efficacy of *Anthracophyllum discolor* and *Stereum hirsutum* to recover valuable compounds from Olive Mill Solid Waste and mitigating phytotoxic effects

Manuscript in preparation

Efficacy of *Anthracophyllum discolor* and *Stereum hirsutum* to recover valuable compounds from Olive Mill Solid Waste and mitigating phytotoxic effects

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Abstract

The circular economy concept aims changing the paradigm in waste management, transitioning from spending resources on waste treatment to considering waste as a potential source of benefits. These strategies can be vital for sectors with competitiveness problems, such as the olive oil sector. In this sector, olive mill solid waste (OMSW) is a substrate with enormous potential due to its high content of phenolic compounds and/or volatile fatty acids (VFA). This study aimed to assess the efficacy of two fungi: *Anthracophyllum discolor* and *Stereum hirsutum* to recover valuable compounds from OMSW, such as enzymatic extracts, volatile fatty acids (VFA), and/or phenolic compounds, under the stimulation of extracellular enzymes production in solid-state fermentation conditions during 30 days and simultaneously determine its phytotoxic effects on tomato plants.

The laccase activity of *A. discolor* (15-day) was improved in 88.7% and *S. hirsutum* (20-day) was improved in 32.3% with Cu-Mn addition. The Cu-Mn addition also resulted in an increment of 97.2% in MnP activity by *S. hirsutum*. Regarding the phenols release from OMSW into the liquid fraction, *S. hirsutum* exhibited a higher release capacity compared to *A. discolor*, achieving a concentration up to 6001 ± 236 mg gallic acid eq L^{-1}. This release of phenolic compounds also resulted in a reduction of these compounds in the obtained solid fraction, where *A. discolor* produced a 40.8% of reduction of the phenols, while *S. hirsutum* reached 20%, respect to the raw OMSW. Again, *S. hirsutum* was able to generate the higher concentration of VFA (1627±325 mg L^{-1}), mainly isobutyric acid and acetic acid. Using OMSW treated, the solid fraction showed a slightly lower phytotoxicity than the raw OMSW for both strains. However, it was not enough to avoid severe phytotoxicity effects in the germination of *Solanum lycopersicum* at OMSW doses higher than 10% (w/w).

5.1. Introduction

The olive mill solid waste (OMSW) is a substrate generated during the extraction of olive oil in two phase processes. The management of this substrate currently represents a challenge for the olive oil sector due to the enormous volume generated, i.e., 4 tons of OMSW per ton of olive oil (Enaime *et al*., 2024; Lenzuni *et al*., 2024). Furthermore, incorrect management of OMSW generates harmful environmental effects, such as coloration of natural waters, toxicity to aquatic life, degradation of soil quality by inhibiting plant germination and growth, phytotoxicity, and the generation of nuisance odors (Genethliou *et al*., 2020; Sounni *et al*., 2021). Developing an effective treatment for OMSW to avoid these undesirable environmental impacts is complex due to a relatively high moisture combined with a high lignocellulosic fiber content, i.e. 20-35% cellulose, 33-38% lignin and 16-45% of hemicellulose (Abu Tayeh *et al*., 2020; Benavides *et al*., 2022a).

The implementation of the circular economy concept in the olive oil sector would allow changing the consideration of OMSW from waste to be treated to a possible source of benefits. For example, the OMSW is a rich source of valuable phenolic compounds, such as tyrosol or hydroxytyrosol (Abu Tayeh *et al*., 2020; Serrano *et al*., 2017b), or volatile fatty acids (VFA), which can be used as building-blocks molecules for diverse bioprocesses (Jiménez-Páez *et al*., 2023). Phenolic compounds have important industrial interest due to their antioxidant and/or antimicrobial properties (Fernández-Prior *et al*., 2020). However, its presence in untreated OMSW is a problem since it has been related to its phytotoxic potential (Aranda *et al*., 2007; Reina *et al*., 2013b). Thus, the recovery of these compounds would mean a double benefit since,

in addition to obtaining economically interesting compounds, their presence in the OMSW would be reduced, thus reducing their phytotoxicity.

The recovery of added-value compounds from the OMSW can be challenging because they are part of the cellular structures of the OMSW, e.g. linked to lignocellulosic fibers. Therefore, it is necessary to release the compounds into an aqueous phase to favor their extraction and recovery (Rubio-Senent *et al*., 2013; Serrano *et al*., 2017b). However, the molecular structure of lignocellulosic fibers is composed of a strong association between the lignin, cellulose and hemicellulose, making necessary a delignify process, i.e., pretreatment to break this structure and to release the cellulose and hemicellulose (Hendriks & Zeeman, 2009a; Taherzadeh & Karimi, 2008). For that, many different treatments have been applied, including physical, chemical, and/or thermal treatments, among others (Hendriks & Zeeman, 2009b). However, some of these treatments could not be suitable from an economic or technical point of view, requiring large inputs of energy (Serrano *et al*., 2021) or due to the generation of undesirable compounds such as furfural or 5-hydroxymethylfurfural (Serrano *et al*., 2017b). These compounds are among the most toxic compounds for fermenters in lignocellulosic hydrolysates (Becerra *et al*., 2022). Thus, the presence of them can hinder any subsequent bioprocess, limiting the available management options for the stabilization of the OMSW.

To resolve the above-mentioned problems, biological treatments have been proposed as an alternative of less energy demand and low investment (Bilal & Iqbal, 2020). Among them, the use of white root fungi (WRF) has been reported as a feasible alternative to delignify lignocellulosic substrates by the action of extracellular enzymes such as laccase (Lac), manganese peroxidase (MnP) and/or manganese-independent peroxidase (MniP) (Ntougias *et al*., 2015; Shirkavand *et al*., 2017; Suryadi *et al*., 2022). Furthermore, the production of these

enzymes can be also another valuable product in the form of enzymatic extracts with many different industrial applications such as medicine, bioremediation, agricultural applications and/or industrial processes (El-Gendi *et al*., 2022). Among the different species of fungi capable of degrading lignocellulosic fibers, the use of strains isolated from the region where they will be subsequently applied can be advantageous, since they should be adapted to the existing environmental conditions and, in addition, the release of foreign species into the environment is avoided. Recently, *Anthracophyllum discolor* and *Stereum hirsutum*, WRF isolated from temperate forests in southern Chile, have proven capable of partially delignify and remove phenols from OMSW (Benavides *et al*., 2022b). These authors reported lignin and phenols removals above 94% and 80% were achieved, respectively, for both fungi strains. However, the enzymatic activity of *A. discolor* and *S. hirsutum* was not very high in comparison with other WRF, being necessary to implement different strategies to improve it.

Some strategies to improve the enzymatic activity include the addition of organic and inorganic inducers in the culture medium (Gutierrez *et al*., 1999; Ellegaard *et al*., 2014), and/or the use of specific co-substrates like lignocellulosic biomass (Atilano-Camino *et al*., 2020; Majul *et al*., 2020; Mishra *et al*., 2017). Inducers such as copper (Cu), manganese (Mn), iron (Fe), including others, plays an essential role for improving the activities of ligninolytic enzymes secretion by WRF (Baldrian & Gabriel, 2003; Hermosilla *et al*., 2017). Cu plays a wide variety of functions in nature that mostly involve electron transfer, O_2 binding, activation, and reduction, NO_2^- and N_2O reduction, and substrate activation (Solomon *et al*., 2014). In WRF, Cu plays an important role in the lignin degradation process serving as a cofactor in the laccase catalytic center (Akpinar & Ozturk Urek, 2020; Baldrian, 2003). On the other hand, Mn plays a key role in the WRF transcriptional phase as well as in the activation of the peroxidase enzyme and directly

participates in the process of lignin degradation through the reaction cycle of Mn-dependent peroxidase (MnP) (Baldrian, 2003). The use of lignocellulosic biomasses as growth supporting for various fungal strains have shown an enhancement in the growth, colonization, and enzymatic activity, allowing an improvement in the efficiency for lignin removal and, thus, improving the transformation of cellulose and hemicellulose into fermentable sugars (Mishra *et al*., 2017). Both strategies have been shown to be effective for enhancing the enzymatic activity of *A. discolor* and *S. hirsutum*. For example, an increment in Lac activity of up to 220% at dosing 7.3 mg Mn/Kg and 2.5 mg Cu/Kg was observed in the solid-state fermentation of OMSW with *A. discolor* respect to a control with no metal addition (Chapter 4). Although there is information available regarding the characteristics, capabilities for to grow and degrade lignocellulosic residues and xenobiotic compounds and activity of *A. discolor* and *S. hirsutum*, the applicability of these fungal strains for the valorization of the OMSW has not been explored yet. Furthermore, although some works have proposed the use of fungi and/or fungal enzymes as pre-treatments combined with bioprocesses such as anaerobic digestion (Kainthola *et al*., 2021), whereas studies that propose it as a unitary valorization process are less common (Chai *et al*., 2022).

Therefore, this work aimed to evaluate the application of *A. discolor* and *S. hirsutum* to OMSW for the recovery of valuable compounds such as enzymatic extracts, VFA, and/or phenolic compounds, as well as, at the same time, reducing the phytotoxicity of the OMSW. The ligninolytic enzymatic activity, identification and quantification of phenols and VFA, and phytotoxicity were monitored under solid-state fermentation conditions with the addition of 0.5 mM of Cu and 2.41 mM of Mn for 30 days for each strain.

5.2. Materials and methods

5.2.1 Microorganisms

The fungal strains *A. discolor* and *S. hirsutum* were used, both isolated from decayed wood in the temperate forests of southern Chile belonging to the Culture Collection of the Environmental Nanobiotechnology Laboratory at the Universidad de La Frontera, Chile (Tortella *et al*., 2008). The fungi were stored at 4 °C in Petri dishes with Malt Extract Agar medium (MEA) until their use. The culture medium was prepared using 40 g of MEA in 1 L of distilled water and sterilized in an autoclave for 20 min at 121 °C. Then, the strains were activated in MEA medium using one disk of 6 mm as the initial inoculum and incubated at 28 °C for 7 days for the assays.

5.2.2 Olive Mill Solid Waste (OMSW)

OMSW was obtained from "Oleícola El Tejar" located in Marchena (Seville), Spain. OMSW was preserved under freezing conditions (-18 °C) until its characterization and use. The main characteristics of OMSW were as follows: pH = 4.6 ± 0.1; total solids (TS) = 252,604 ± 3000 mg L^{-1}; volatile solids (VS) = 236,847 ± 3120 mg L^{-1}; mineral solids (MS) = 15,756 ± 1 mg L^{-1}; moisture = 75 %w/w; chemical oxygen demand (COD) = 334 ± 31 g O_2 Kg^{-1}; total phenols = 6135 ± 131 mg gallic acid eq L^{-1}; VFA = 35.13 mg L^{-1}; copper = 242.7 ± 0.3 ppb; manganese = 282.6 ± 0.3 ppb; 3.4-dihydroxyphenylglycol = 295.8 ± 5.8 mg L^{-1}; 3-hydroxytyrosol = 2749.65 ± 65.00 mg L^{-1}; p-tyrosol = 798.6 ± 11.0 mg L^{-1}.

5.2.3 Experimental set-up and design

For all assays, OMSW was directly used, without applying any pretreatment such as grinding or sieving. For the inoculum, the fungal strains were initially pre-cultured in wheat grains-based media (18 g wheat and 30 mL distilled water per flask) based on the procedure described by Reina *et al*., (2017) with some modifications. The flasks with wheat-based media after sterilization in an autoclave were inoculated with 9 mL from the mixture of 4 fungal MEA plates homogenized in 80 mL sterile water (55% v/w) and incubated in static conditions at 28 °C for 7 days. Then, the flasks were mixed with 18 g of sterile OMSW, and 2 mL of sterile water supplemented with 0.5 mM of Cu and 2.41 mM of Mn, added as $CuSO_4$ and $MnSO_4$, respectively. The concentrations of both metals were selected in accordance with the optimal values previously described for *A. discolor* and *S. hirsutum* in Chapter 4. Flasks with wheat-based medium and incubated with *A. discolor* and *S. hirsutum* without the addition of OMSW were used as controls. The cultures were incubated in static conditions at 28 °C for 30 days. The flasks containing both media were heat-inactivated by autoclaving after inoculation with the corresponding fungal mycelium and were used as biotic controls. Three flasks were sacrificed per fungal strain at each sampling time (0, 5, 10, 15, 20, 25 and 30 days) for their characterization in order to monitor the processes.

5.2.4 Preparation of extracts

At each sampling point, an liquid extract from flasks with fermented OMSW was obtained using 100 mL of distilled water. This mixture was placed in a rotary shaker for 2 h. Then, the mixtures were centrifuged, filtered, and the obtained extracts were used for measuring enzyme activities, pH, soluble phenols, metals, and VFA described below. In the residual solid

phase, total phenols content was also determined. All the determinations were carried out in triplicate, and the data reported are mean values with standard deviation of triplicates.

5.2.5 Phytotoxicity tests

Phytotoxicity tests of the liquid extract and residual solid phase obtained at the end of each experiment were carried out to analyze their effect on *Solanum lycopersicum* germination. The tests were performed according to Zucconi (1981) and Pinho *et al*., (2017), with some modifications. Before the phytotoxicity tests, *S. lycopersicum* seeds were hydrated with tap water to imbibe the seeds and to eliminate the non-viable seeds. After, ten seeds were placed in a Petri dish of 9 cm diameter lined with filter paper containing 2 mL of liquid undiluted extract and at different dilutions with distilled water (90, 75, 50, 25, and 10% v:v). To test with the residual solid phase, ten seeds were placed in a Petri dish lined with filter paper containing 10 g of residue and variably doses adjustment with vermiculite (90, 75, 50, 25, and 10% w:w). In both cases, the Petri dishes were incubated to 25 °C in the dark for 72 h. Controls, with distilled water and vermiculite and without OMSW, were also included in the tests. The germination index (Zucconi, 1981) was calculated according to the following equations (eq. 1-3):

$$GI(\%) = \frac{RSG(\%)xRRG(\%)}{100} \qquad \text{(eq. 1)}$$

$$RSG(\%) = \frac{G}{G_0 x 100} \qquad \text{(eq. 2)}$$

$$RRG(\%) = \frac{L}{L_0 x 100} \qquad \text{(eq. 3)}$$

where RSG is relative seed germination, RRG is relative radicle growth, G is the number of germinated seeds with the sludge extract, G_0 is the number of germinated seeds into the control dish, L is the length of the radicle in the seeds germinated with the sludge extract and L_0 is the length of the radicle in the seeds germinated into the control dish.

5.2.6 Analytical methods

Enzymatic activity was periodically monitored every five days for 30 days. The enzyme activity of Lac (EC.1.10.3.2), MnP (EC.1.11.1), and MniP was determined through the 2,6-dimethoxyphenol assay, where the reaction mixture contained 200 µL of 50 mM of sodium malonate (pH 4.5), 50 µL of 20 mM 2,6- dimethoxyphenol, 50 µL of 20 mM $MnSO_4 \cdot H_2O$, and 50 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, and the absorbance of the colored product was measured at 468 nm using a UV-vis spectrophotometer (Thermo Scientific EvolutionTM 60S) at 30 °C and corrected for the Lac activity (Wariishi *et al*., 1992). One Unit (U) of MnP activity was defined as the amount of enzyme transforming 1 µmol 2,6- dimethoxyphenol per minute at pH 4.5 and 30 °C (Wariishi *et al*., 1992). Total and soluble phenols were quantified by spectrophotometry with a calibration curve of gallic acid through the Folin-Ciocalteu method (Singleton *et al*., 1999). In the solid residue, an extraction with a methanol/water solution (80:20) was prepared and the values were expressed in mg gallic acid eq 100 g^{-1} of OMSW. Soluble phenols (mg L^{-1}) were quantified in samples filtrated by Whatman Nº 1 filter paper (pore size 11 µm). In order to determine the phenolic profile, a liquid-liquid extraction using ethyl acetate was conducted on crude extract samples derived OMSW treated by WRF at day 30, according to De Marco *et al*., (2007). The crude extracts were acidified to pH 2 using HCl and subsequently subjected to hexane washes to eliminate the lipid fraction. Specifically, 10 mL of crude extract were combined with 15 mL of hexane, vigorously shaken,

and centrifuged at 2400 ×*g* for 5 minutes. This process was repeated twice to ensure thorough washing. Following the lipid removal, extraction of phenolic compounds was performed using ethyl acetate. The previously washed crude extracts were mixed with 10 mL of ethyl acetate, vigorously shaken, and centrifuged at 2400 ×*g* for 5 minutes. This extraction procedure was repeated four times to enhance efficiency. Subsequently, the ethyl acetate solvent was evaporated under vacuum, leaving behind a dry residue. This extract was then dissolved in 3 mL of methanol, forming a solution suitable for the characterization, quantification, and fractionation of phenolic compounds. Individual phenols were quantified by reversed phase high-performance liquid chromatography (HPLC-DAD) using an Agilent 1200 series instrument (Agilent Technologies, USA) (the wavelength used for quantification was 280 nm). The chromatographic separation was achieved on a Synergi 4u Fusion-RP (250 mm x 4.6 mm i.d.) reversed-phase column, 5 µm particle size (Phenomenex, Macclesfield, UK); isocratic elution at a flow rate of 0.5 mL min^{-1} was used. The mobile phase A was Milli-Q water acidified with 0.01% trichloroacetic acid and mobile phase B was acetonitrile utilizing the following gradient over a total run time of 55 min: 95% A initially, 75% A in 30 min, 50% A in 45 min, 0% A in 47 min, 75% A in 50 min, and 95% A in 52 min until completion of the run. Quantification was carried out by peaks in function of the compounds, with reference to calibrations made using external pure standards. At the end of the experimental time, Volatile Fatty Acids (VFA) were determined in triplicate using a gas chromatograph Shimadzu GC-2025 (Shimadzu Corporation, Japan) equipped with a Stabilwax-DA column (Crossbond Carbowax polyethylene glycol, RESTEK) of 0.25 mm i.d. x 30 m and a flame ionization detector (FID) at 250 °C. The oven temperature gradually increases from 100 to 170 °C at 5 °C/min. Nitrogen (30 mL min^{-1}), hydrogen (40 mL min^{-1}), and air (400 mL min^{-1}) were used as carrier gas at a flow rate of 40 mL min^{-1} at 504 kPa.

5.3. Results and discussion

5.3.1 Ligninolytic enzyme activity by A. discolor and *S. hirsutum*

The Lac activity of *A. discolor* with Cu-Mn addition reached 987.2 ± 26.6 U L^{-1} at day 15, improving 88.7% the lac activity produced by the control experiment control (111.3 ± 28.9 U L^{-1}) (Figures 1a-b). After day 20, the Lac activity tend to decrease over time at the different evaluated conditions for *A. discolor*. MnP activity for *A. discolor* with Cu-Mn addition reached values up to a maximum of 2089.7 ± 153.9 U L^{-1}, showing an increase of 49.7% with respect to the maximum obtained in the control (1051.3 ± 34.6 U L^{-1}). In opposite, *A. discolor* secreted low amounts of MniP (<250 U L^{-1}) with respect to Lac and MnP, both in the treatment without metals and in the treatment with Cu-Mn addition (Figure 1a-b).

Figure 1c-d show the Lac, MnP, and MniP activity variation over experimental time by *S. hirsutum* without metals (control) and with Cu-Mn addition. The higher Lac activity was obtained at day 20 both for the control and Cu-Mn addition, reaching values of 966.4 ±7.5 U L^{-1} and 654.3 ±76.8 U L^{-1}, respectively. The addition of Cu and Mn also resulted in a high increment of the MnP activity, which increased 97.2% respect to the control up to a maximum concentration of 2008.5 ± 204 U L^{-1} (Figure 1c-d). However, after day 25, the enzymatic activity showed a gradual decreasing over time in all treatments.

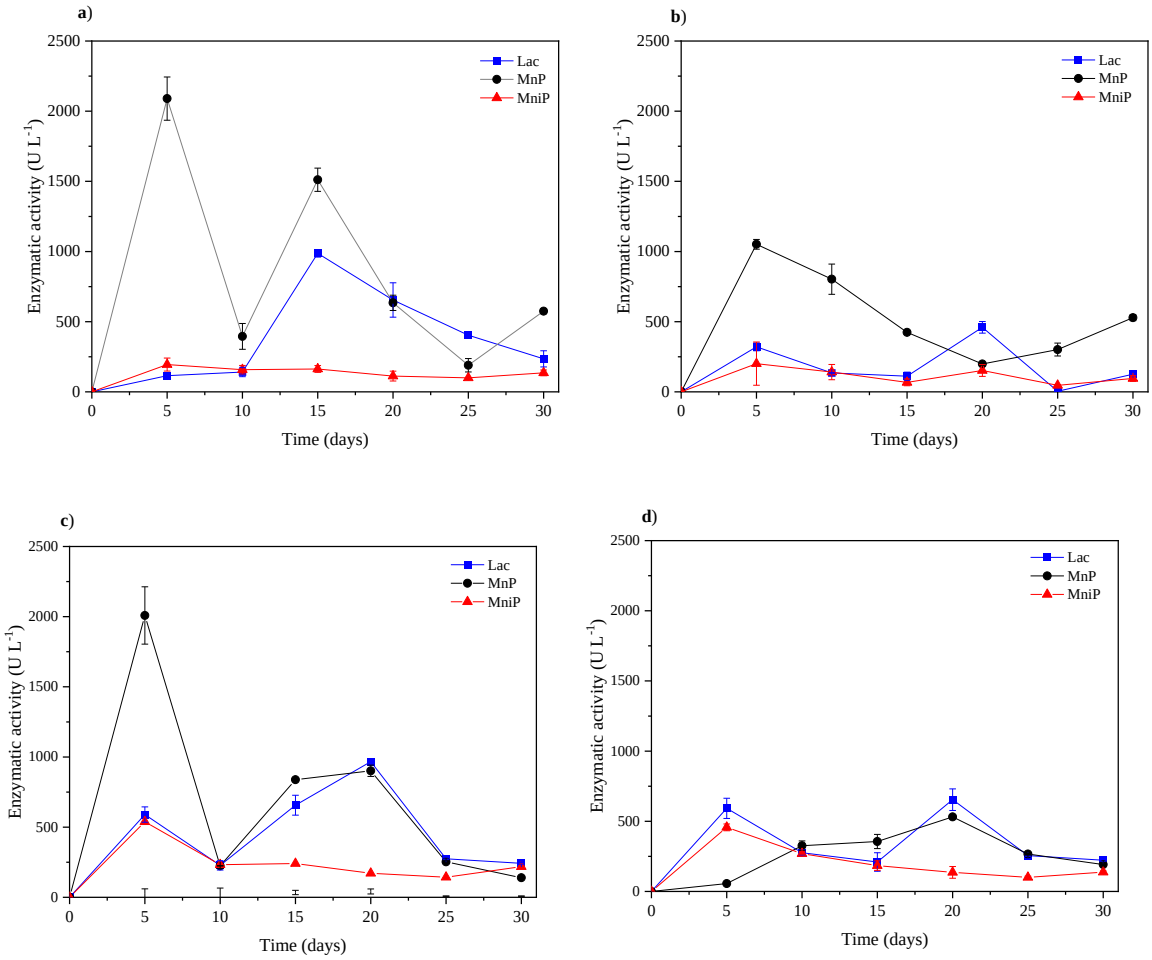

Figure 1. Enzymatic activity of Laccase (Lac) Manganese peroxidase (MnP) and Manganese independent peroxidase (MniP) behaviour by *Anthracophyllum discolor* (a) *Anthracophyllum discolor* with Cu-Mn addition (b) *Stereum hirsutum* (c); *Stereum hirsutum* with Cu-Mn addition (d) in SSF at 30°C during experimental time. Error bars indicate standard error of the mean (n = 3).

For both fungal strains, operation times longer than 15-20 days would not be recommended to obtain an enzyme extract with a high concentration of enzymes (Figure 1). On the opposite, the strategy of Cu-Mn addition clearly showed a positive effect on the obtained enzymatic activity, in line with the previous findings described in Chapter 4 for the same fungal

strains in the fermentation of OMSW. The improvement of the enzymatic activity can be explained by the inductive effect of Cu-Mn addition. Concretely, Cu effect could be associated to that copper has an important role in the regulation of peroxidase activity, causing induction at an appropriate dosed concentration (Slavens *et al*., 2019). For example, Liu *et al*., (2020) reported an improvement in the Lac activity by *Ganoderma lucidum* in a 61.3% by adding 0.19 mM Cu. Likewise, the MnP activity increase is possible because this enzyme is a heme-containing enzyme, which consists of Mn-binding sites, and catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Kannaiyan *et al*., 2015). In addition, Mn^{2+} plays a key role in the transcriptional phase and in the activation of the peroxidase enzyme (Baldrian, 2003; Slavens *et al*., 2019), which may explain the obtained results. Additionally, to the individual effect of the dosed metals, interactions between Mn and Cu metal ions can also play an essential role in the fungal response. Similar results were described in Chapter 4, under similar experimental conditions to the present research, where the higher improvements of the enzymatic activities were achieved at Cu-Mn co-addition for the different enzymatic activities in comparison to the individual metal additions.

5.3.2 Phenolic compounds release from the OMSW to a liquid fraction

The obtained results showed that the present fungal treatment allowed the release and recovery the phenolic compounds contained in the OMSW in a generated liquid fraction and, at the same time, a reduction of the phenolic content in the OMSW (Figure 2a-b). As it can be seen in Figure 2a and 2b, *S. hirsutum* was able to release a higher concentration of phenolic compounds to the liquid fraction in comparison with *A. discolor*, reaching maximum concentrations of 6001 ± 236 mg gallic acid eq L^{-1} and 3886 ± 186 mg gallic acid eq L^{-1}, respectively. For *A. discolor*, the effect of the Cu-Mn addition showed a little improvement

respect to the control (Figure 2a). Contrary, the Cu-Mn addition with *S. hirsutum* increased the maximum reached concentration of phenolic compounds around 20% (Figure 2b). This positive effect could be related to the greater enzymatic activity, especially of Lac and MnP, previously described for *S. hirsutum* as a consequence of the Cu-Mn addition (Figure 1c-d). It is worth to mention that the use of *S. hirsutum* would be preferable respect to *A. discolor* for the obtaining of phenolic compounds in the liquid phase because, as it is shown in Figure 2a, *A. discolor* strongly reduced the concentration of phenolic compounds at increasing the operation time, even reaching values close to zero.

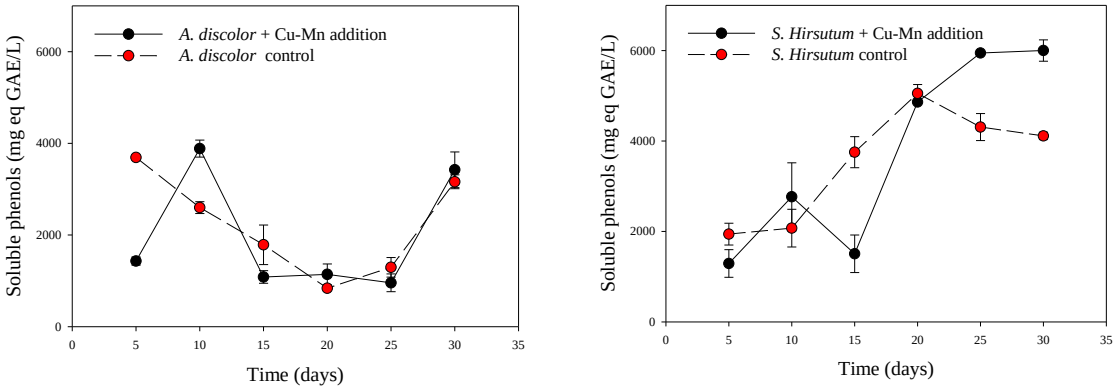

Figure 2. Variation of the soluble phenolic compounds concentration in the liquid fraction of OMSW obtained with *Anthracophyllum discolor* (a) and *Stereum hirsutum* (b) with Cu-Mn addition and without metals (control) in SSF at 30°C during experimental time. Error bars indicate standard error of the mean (n = 3).

The obtained concentrations of phenolic compounds in the liquid fraction were higher than the obtained by other physic-chemical alternatives reported in literature. For example, Fattoum *et al*., (2023) described that, at the most effective conditions, liquid-liquid extraction methods using ethyl acetate and chloroform/methanol (1:1) only reached concentrations of soluble phenolic compounds of up to 1.17 g gallic acid eq L^{-1} of OMSW and 1.07 g gallic acid eq

L^{-1} of OMSW, respectively. Likewise, the obtained phenolic compound concentration in the present research was also higher than the reported by Serrano *et al*., (2017) by implementing a steam-explosion thermal treatment for OMSW, i.e. 1834 ± 126 mg gallic acid eq L^{-1} of OMSW. However, this unexpected difference, since the steam-explosion is a highly severe treatment capable of breaking-down even the lignin structures, could be due to the water added during the steam-explosion, which diluted the concentration of phenols, instead of only a lower release of the phenols contained in the OMSW.

The concentrations of individual phenolic compounds in the liquid fraction from treatments with *A. discolor* and *S. hirsutum* after 30 days are outlined in Table 1. It is evident from the results that the final concentrations of the measured phenolic compounds indicate these were metabolized during the treatment process. Notably, certain specific compounds exhibited accumulation compared to their initial concentrations during the fungal treatments. For instance, in the liquid fraction from *S. hirsutum* without Cu-Mn addition, the presence of 4-methylcatechol and 4-ethylphenol were detected (Table 1). This accumulation can be attributed to the degradation of lignocellulosic fibers of OMSW, resulting in the release of phenolic compounds integrated into these fibers (Luo *et al*., 2021). Additionally, specific phenolic compounds may accumulate as intermediates of the incomplete degradation of others (Serrano *et al*., 2017b). Conversely, in the liquid phase treated by *A. discolor*, it is inferred that phenols were metabolized (Table 1).

Table 1. Individual phenolic compounds concentration (mg L^{-1}) in liquid fraction of OMSW at day 30.

Phenolic compounds	Liquid fraction without treatment	Treatment by *A. discolor*	Treatment by *A. discolor* and Cu-Mn	Treatment by *S. hirsutum*	Treatment by *S. hirsutum* and Cu-Mn
2-4 Hydroxiphenylethanol	25.48±	ND	ND	ND	ND
4-methylcatechol	ND	ND	ND	29.09±	ND
4-ethylphenol	ND	ND	ND	24.58±	ND
Vanillic acid	19.60±	ND	ND	ND	19.53±
p-Coumaric acid	0.43±	ND	ND	ND	ND

ND: not detected

The action of *A. discolor* allowed, in parallel to the release of phenolic compounds to the liquid fraction, a substantial reduction of the phenolic compounds in the solid fraction of the OMSW, which decreased from 18.1 ± 0.1 g gallic acid eq 100 g^{-1} OMSW at 0-day up to a minimal value of 10.7 ± 0.3 g gallic acid eq 100g^{-1} OMSW at 30-day (Figure 3a). As it can be also seen, the Cu-Mn addition showed almost no impact on the phenolic removal with *A. discolor,* reaching phenol removal efficiencies around 45% respect to the raw OMSW. These phenol removal efficiencies are in the range of the values reported in literature for other fungal strains applied to OMSW. For example, Sampedro *et al*., (2009) found that, in OMSW pretreated by *Coriolopsis rigida (*current *Funalia floccosa)*, the phenolic removal efficiency was about 36% after 14 days of incubation. Similarly, Reina *et al*. (2013a) described an efficiency in phenol compounds removal between 60 to 80% for *Auricularia auricula-judae*, *Bjerkandera adusta*, and *Coprinellus radians* after 28 days of OMSW pretreatment. On the contrary, *S. hirsutum* showed a very low phenol removal efficiency in both conditions, i.e. Cu-Mn addition, and control (Figure 3b). Concretely, the phenol removal efficiencies were only of 5% and 20% for Cu-Mn addition

and control, respectively. This low removal efficiencies of *S. hirsutum* would be related with the release of new phenolic compounds from complex lignocellulosic structures during the OMSW degradation as reported by Benavides *et al*., (2022); Serrano *et al*., (2017b). Therefore, the selection of the fungal strain seems to be a crucial factor that must be adjusted to the objective of the process, being more recommended *S. hirsutum* for generating an enriched phenolic compound extract and *A. discolor* if the objective is reducing the phenolic content of the OMSW.

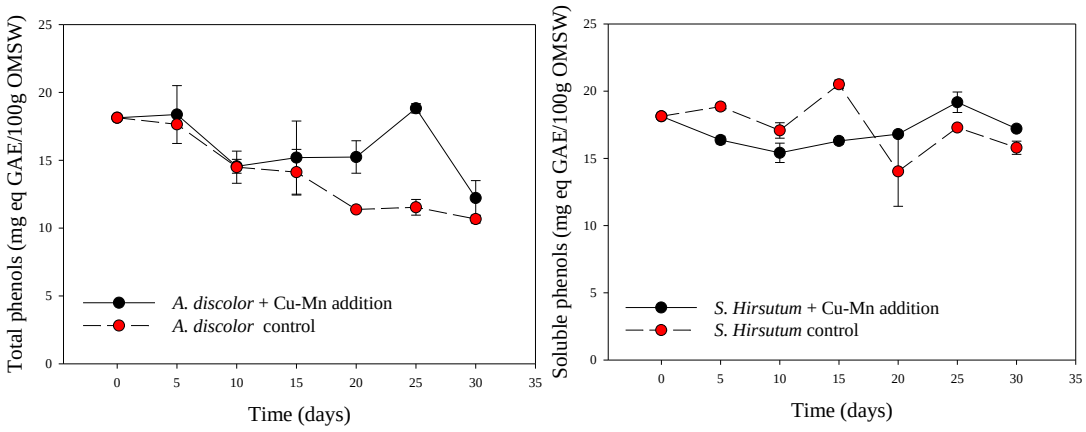

Figure 3. Variation of the total phenolic compounds concentration in the solid phase of the OMSW obtained with *Anthracophyllum discolor* (a) and *Stereum hirsutum* (b) with Cu-Mn addition and without metals (control) in SSF at 30°C during experimental time. Error bars indicate standard error of the mean (n = 3).

5. 3.3 Volatile fatty acids (VFAs) composition

The profiles of VFAs in the liquid fraction of the OMSW pretreatment by *A. discolor* and *S. hirsutum* with Cu-Mn addition and the controls are shown in Figure 4a-d. *S. hirsutum* was able to generate a higher concentration of VFA and with a more diverse VFA profile than *A. discolor.* It is worth to note that the Cu-Mn addition did not imply a marked difference respect to the control for both fungal strains, except for *A. discolor* at 25-day when an anomalous concentration

of isovaleric acid was detected (Figure 4a). The maximum sum of individual VFA were 1627±325 mg L^{-1} for *S. hirsutum* at 25-day without Cu-Mn addition and 1227±245 mg L^{-1} for *A. discolor* at 25-day with Cu-Mn addition. The effectiveness of these fungal strain for accumulating VFA was much higher than the reported by Serrano *et al*., (2017a), which described that the steam-explosion treatment of OMSW was not able to generate any relevant concentration of acetic acid. By contrast, the OMSW has been described as a feasible biomass for VFA production by anaerobic fermentation. For example, Jiménez-Páez *et al*., (2023) reported the accumulation of up to 2625 ± 375 mg L^{-1} of VFA during the batch anaerobic fermentation of OMSW at controlled pH 9. However, these same authors justify this high effectiveness by the combined action of both the anaerobic microorganisms and the chemical action of the hydroxides from the NaOH used to control the pH.

The VFAs profile obtained with *S. hirsutum* shown that at 30-day were present four VFAs, where the highest amounts corresponded to isobutyric acid (748 ± 154 – 776 ± 168 mg L^{-1}) followed by acetic acid (457 ± 78 – 478 ± 85 mg L^{-1}). This concentration of acetic acid was around 2-fold higher with respect to the concentration obtained with *A. discolor*, which was the almost unique VFA determined with this fungal strain (Figure 4a-b). *S. hirsutum* also was able to accumulate a relevant concentration of valuable isovaleric acid after 10-days of operation, i.e. (217 ± 59 – 223 ± 26 mg L^{-1}) (Figure 4c-d). The production of isobutyric or isovaleric acids can be very interesting because are widely used in the food industry as preservatives and flavor enhancers (Bhatia & Yang, 2017). It is worth to note that higher production yields have been reported for other fungi such as *Clostridium butyricum*, i.e., concentrations between 12.25 to 20 g L^{-1} (He *et al*., 2005; Zigova, 1999). However, the difference would be explained by the use of synthetic sugars (sucrose and glucose) as substrate in such research, being difficult to compare

with the results obtained from a substrate as complex as the OMSW. Thus, in line with the results obtained for the phenolic compounds, *S. hirsutum* was more interesting for VFA accumulation than *A. discolor* in both total VFA concentration and the obtained VFA profile.

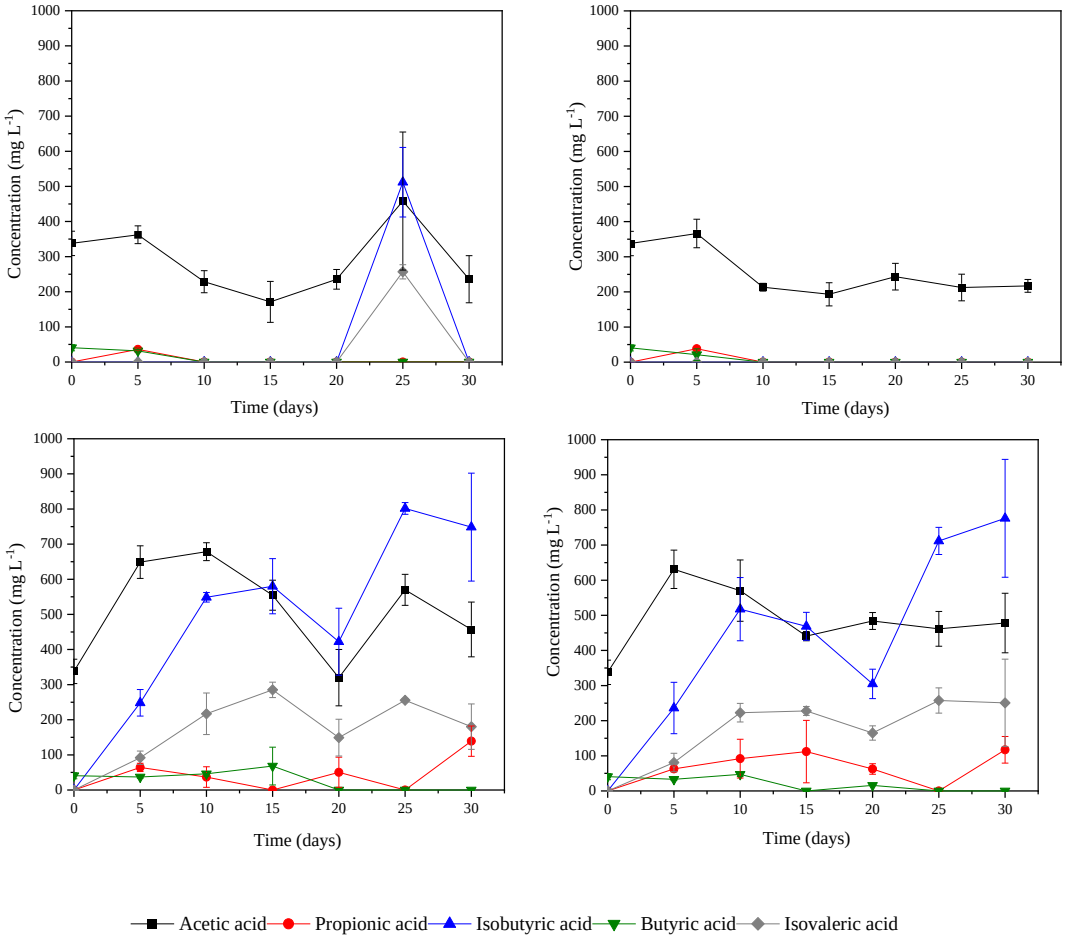

Figure 4. Volatile fatty acid variation derived from the liquid extract of OMSW, subject to treatment by *Anthracophyllum discolor* with Cu-Mn supplementation (a), *Anthracophyllum discolor* (b), *Stereum hirsutum* with Cu-Mn supplementation (c), and *Stereum hirsutum* (d) in SSF at 30°C during experimental time. Error bars indicate standard error of the mean (n = 3).

5.3.4 Effect of OMSW on *S. lycopersicum* seeds germination.

The results showed that raw OMSW negatively affected *S. lycopersicum* seeds germination after 72 hours of incubation. The greatest negative effect occurred when the solid phase was used as the substrate, reaching a germination index of below 20% at 10% of OMSW and an almost total germination inhibition at higher OMSW doses (Figure 5a). By contrast, the germination index in the liquid extract presented germination indexes above 70% at dosed up to 75% of liquid extract, rapidly decreasing at higher dosed up to a minimal 10.94± 1.8% of seed germination at a 100% dose (Figure 5b).

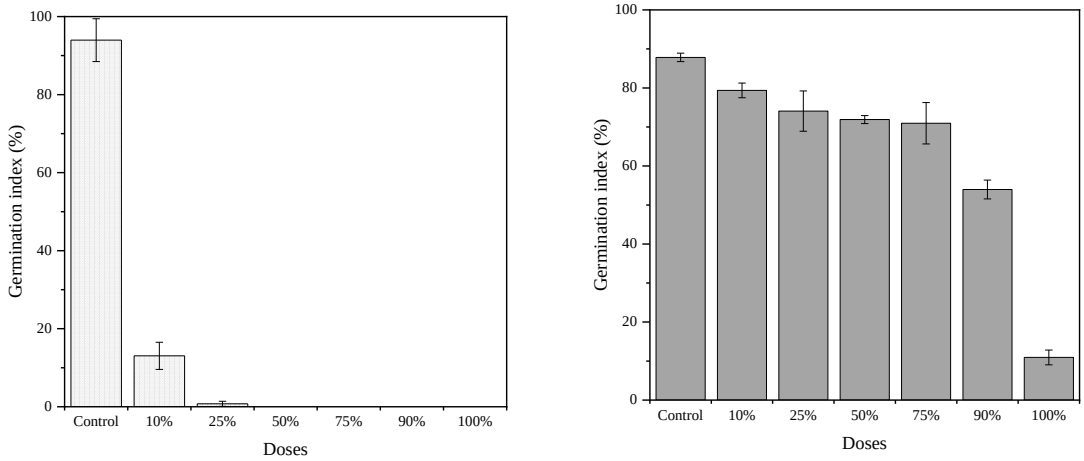

Figure 5. Effect of different doses of solid fraction and liquid extract of OMSW raw on *Solanum lycopersicum* seeds germination. Solid fraction, control with vermiculite (a), and liquid extract, control with distilled water (b). Error bars indicate standard error of the mean (n = 3).

When the assays were carried out using OMSW treated, the solid fraction of the OMSW showed a slightly lower phytotoxicity than the raw OMSW for both *A. discolor* and *S. hirsutum* (Figure 6c-d and Figure 7c-d). Concretely, a dose 10% of OMSW allowed germination indexes of around 35% for *A. discolor* and around 40-50% for *S. hirsutum*. Cu-Mn addition did not result in a relevant enhancement of the phytotoxicity decrease respect to the control for both fungal

strains. However, higher doses of OMSW also resulted in an almost total germination inhibition, such as with the raw OMSW. This reduction in the phytotoxicity respect to the raw OMSW would be related to the decrease in the phenolic compounds concentration previously described, since these compounds has been reported to be closely related to the phytotoxicity (Capasso *et al*., 1992; Pinho *et al*., 2017). In the opposite, the action of *A. discolor* and *S. hirsutum* resulted in an increment of the phytotoxicity of the liquid extract respect to the raw OMSW (Figure 6a-b and Figure 7a-b). Concretely, *A. discolor* presented germination indexes between 20-40% with Cu-Mn addition and 30-60% in the control, for doses between 10% to 75% (Figure 6a-b). *S. hirsutum* presented an even higher phytotoxicity than *A. discolor*, with an almost total inhibition of the germination of *S. lycopersicum* seeds at concentrations higher than 25% (Figure 7a-b). That would be a consequence of the accumulation of the released compounds, both phenolic compounds and VFA, in these liquid fractions since both are potential contributors to the phytotoxicity of the fraction (Kong *et al*., 2022; Pinho *et al*., 2017). Thus, the higher phytotoxicity of *S. hirsutum* would be explained by the higher concentration of these compounds in the obtained liquid fractions.

Therefore, due to the partial detoxification of OMSW, fungal treatment with *A. discolor* and *S. hirsutum* could be not enough to be considered as an integral management method for this biomass, being necessary in this case a further stabilization method such as composting (Aviani *et al*., 2010) or the use of other fungal strains with higher dephenolization capacity such as *Phlebia sp*, which can reduce the OMSW phytotoxicity up to 80% Sampedro *et al*., (2009).

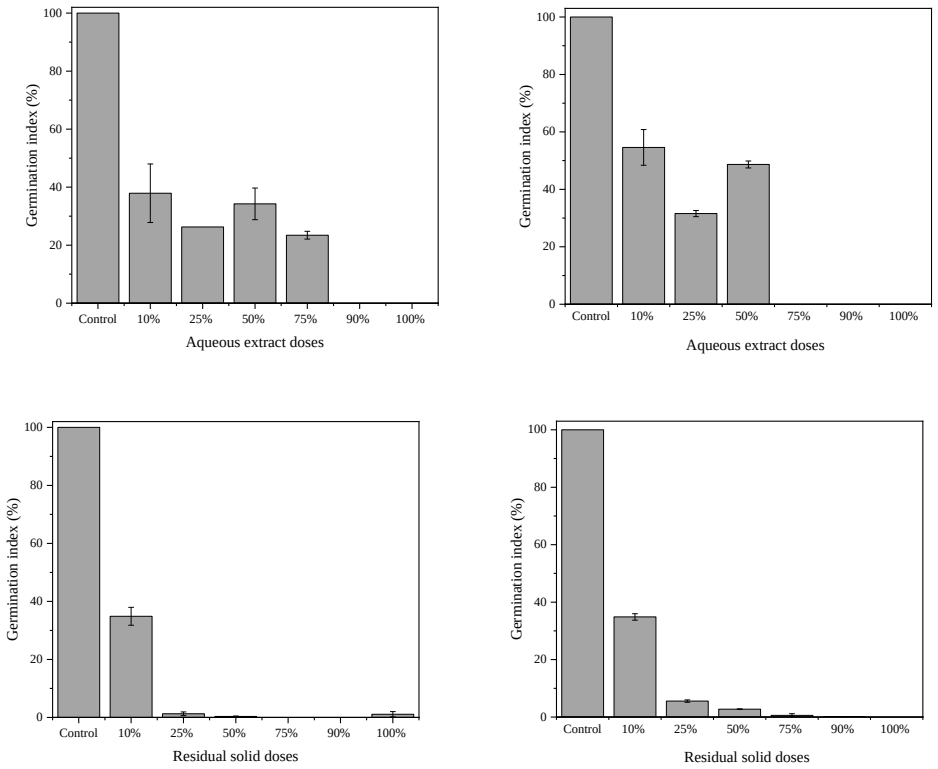

Figure 6. Effect of different doses of solid fraction and liquid extract of OMSW treated by *Anthracophyllum discolor* with and without Cu-Mn added in SSF at 30°C obtained at final treatment (30-day) on *Solanum lycopersicum* seeds germination. Liquid extract doses from treatment with Cu-Mn added (a), Liquid extract doses from treatment without Cu-Mn added (b). Solid fraction doses from treatment with Cu-Mn added (a), Solid fraction doses from treatment without Cu-Mn added (b). Error bars indicate standard error of the mean (n = 3).

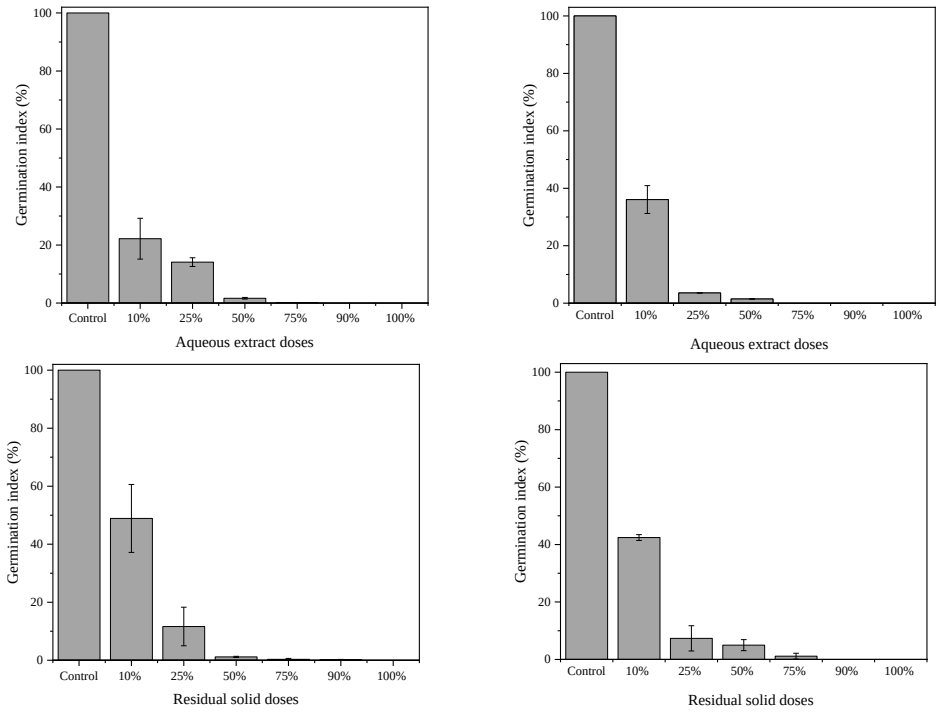

Figure 7. Effect of different doses of solid fraction and liquid extract of OMSW treated by *Stereum hirsutum* with and without Cu-Mn added in SSF at 30°C obtained at final treatment (30-day) on *Solanum lycopersicum* seeds germination. Liquid extract doses from treatment with Cu-Mn added (a), Liquid extract doses from treatment without Cu-Mn added (b). Solid fraction doses from treatment with Cu-Mn added (a), Solid fraction doses from treatment without Cu-Mn added (b). Error bars indicate standard error of the mean (n = 3).

5.4 Conclusions

The results showed that *S. hirsutum* can be an interesting fungal strain for the valorization of OMSW, allowing the generation of a liquid fraction enriched in both phenolic compounds and VFAs. The release of phenolic compounds by *S. hirsutum* was clearly favored by the proposed Cu-Mn addition. In opposite, *A. discolor* did not resulted in relevant accumulation of phenols

neither VFAs, despite of a similar enzymatic activity at both fungal strains. Despite a reduction of the phytotoxicity in comparison to the raw OMW, the treated OMSW by both fungal strains still showed severe phytotoxic effects at doses of OMSW higher than 10%. Therefore, despite the valuable interest of the proposed fungal treatment as valorization process, it should be still necessary a further stabilization method for a complete management of the OMSW.

CRediT authorship contribution statement

V. Benavides: Methodology, Formal analysis, Writing - Original Draft. F. Pinto-Ibieta: Writing - Review and Editing. E. Aranda: Writing - Review and Editing, Resources. V. Ramos: Methodology. M. Rao: Writing - Review and Editing, Resources. G. Ciudad: Writing - Review and Editing, Resources A. Serrano: Conceptualization, Writing - Original Draft, Writing - Review and Editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER VI
General discussion and conclusions

6.1 General discussion

As mentioned in the previous chapters, the intricate molecular structure of lignocellulosic biomasses, predominantly due to lignin strong bonding with cellulose and hemicellulose, imposes significant limitations on their processing and utilization (Ning *et al*., 2021). Nevertheless, this biomass presents immense potential as feedstock for biorefineries, offering opportunities for obtaining value-added products that have been explored using different thermal, physic-chemical, or biological pre-treatments (Bilal & Iqbal, 2020; van Kuijk *et al*., 2016; Wan & Li, 2012).

Taking advantage of the enzymatic machinery of fungi, composed primarily of laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP), several alternatives to physicochemical treatments have been proposed (Chaudhary *et al*., 2023; Khalil, 2023). Fungal treatments offer several key advantages, notably the absence of toxic organic solvents (Sheldon & Woodley, 2018), minimal energy consumption requirements (El-Gendi *et al*., 2022), and the ease of obtaining crude enzymatic extracts during treatment for subsequent applications or valorization (Bilal & Iqbal, 2020; Corbu *et al*., 2023). Particularly, the direct use of WRF has been proposed as an alternative to the application of pure enzymes to delignify lignocellulosic residues, avoiding the long production times of enzyme extracts and the processes of purification and stabilization of enzymes (Mori *et al*., 2021; Shirkavand *et al*., 2017; Suryadi *et al*., 2022). Enhancing the application of fungal treatments to biodegradation processes and improving the resulting enzymatic extracts remains a challenge. Various strategies can be employed to address this challenge, including optimizing nutrient balance through careful substrate selection (Atilano-Camino *et al*., 2020; Henske *et al*., 2018), incorporating metals as

inducers or cofactors (Baldrian, 2003; Mishra *et al*., 2017), and selecting appropriate fungal strains (Tortella *et al*., 2005).

During this Ph. D. thesis, a biodegradation and detoxification process for olive mill solid waste (OMSW) by WRF using inducers was deeply studied. The proposal aimed to go a further step on conventional procedures for the treatment of OMSW and based on the experimental proposal, the acquired knowledge of the Ph. D. student and also the expertise of several collaborators, achieve a low energy demand and effective process, including its possible valorization in valuable products.

As outlined in **Chapter II**, the literature review underscores the biotechnological potential of basidiomycete fungi in enzyme production when supplemented with metals, thereby potentially augmenting biodegradation processes and the generation of biomass-derived products. This result is attributed to the influence of metal-fungi interactions on enzymatic activity. Furthermore, the impact of metal supplementation is contingent upon the type of substrate employed, with distinctions observed between synthetic models and lignocellulosic substrates. Metals present an intriguing avenue for enhancing the enzymatic activity of ligninolytic fungi, particularly within the phylum Basidiomycota, operating through diverse, interconnected mechanisms. In addition, determining an optimal metal addition range proves challenging, given the multitude of influencing factors such as fungal strain variability and substrate composition. Notably, enzymatic activity thrives when utilizing substrates rich in lignocellulosic fibers, suggesting a less pronounced impact of metal supplementation compared to synthetic substrates. This observation may stem from inherent copper concentrations within the substrate, mitigating the need for external metal inputs. Consequently, precise quantification of metal concentrations

within the substrate is imperative for accurately assessing bioavailable metal levels for fungal assimilation. Nonetheless, synergistic effects emerge between metal supplementation and the utilization of lignocellulosic substrates, underscoring their combined potential for enhancing enzymatic activity.

In the initial experimental phase of this study, the efficacy of *A. discolor* and *S. hirsutum* as biological pretreatments for OMSW was investigated, as detailed in **Chapter III**. Encouragingly, both white rot fungi demonstrated the ability to thrive and secrete lignocellulosic enzymes directly onto OMSW substrates without the requirement for supplementary nutrients, external carbon sources, or agitation systems. Notably, by day 10, *A. discolor* showcased its peak Manganese Peroxidase (MnP) production, reaching an impressive 176.7 U L^{-1}. These findings align with previous research by Schalchli *et al*., (2017), who observed maximal enzymatic activity from *A. discolor* cultivated on potato peel waste after 15 days. This underscores the potential of *A. discolor* as a robust ligninolytic enzyme producer, offering insights into its applicability for sustainable bioprocessing of lignocellulosic substrates such as OMSW. Hermosilla *et al*., (2017b) reported findings akin to ours, utilizing wheat straw inoculated with *Ganoderma lobatum*. However, noteworthy differences in the timeframes for achieving optimal results were observed, with their study requiring 40 days for similar results. This variance in incubation duration may be attributed to variations in substrate composition. Specifically, our study demonstrated a remarkable reduction in incubation time to attain peak enzyme activity values, indicative of OMSW's unique ability to induce ligninolytic enzyme production despite its lower lignocellulosic content (approximately 33.6%) compared to other substrates such as wheat straw, which typically contains 35–45% cellulose, 20–30% hemicellulose, and around 15% lignin (del Río *et al*., 2012). It is worth noting that the enhanced production of ligninolytic enzymes

observed in our study may be attributed to the presence of phenolic compounds, including ferulic, caffeic, coumaric, and chlorogenic acids (González *et al*., 2019; Torres-Mancera *et al*., 2011). The efficiency of phenolic compound removal achieved by both strains, amounting to 80%, coupled with the significant relative degradation of lignin in OMSW (94.1% by *A. discolor* and 94.4% by *S. hirsutum*) after 24 days of incubation, underscores the efficacy of our approach. Our findings surpass previous pretreatment methods proposed for OMSW detoxification, including thermal pretreatment with vapor injection (achieving 69.5% phenolic compound removal) (Serrano *et al*., 2017b) and steam explosion pretreatment (yielding 58.3% phenolic compound removal) (Serrano *et al*., 2017c). Moreover, our pretreatment strategy, requiring fewer stages and energy inputs compared to existing methodologies, offers a promising avenue for OMSW valorization under mild conditions (lower temperature (24 °C), no additional nutrient supplementation or agitation systems), thus facilitating biomass valorization without imposing a significant energy demand.

The influence of heavy metals on extracellular lignocellulolytic enzymes, both transcriptionally and functionally during the breakdown of lignocellulosic compounds and xenobiotics by WRF and their enzymatic extracts, has been well-documented (Baldrian, 2003). Consequently, metal supplementation emerges as a crucial factor in biological treatment, impacting the efficacy of WRF in degrading lignocellulosic biomass. Metals such as Cu and Mn are indispensable for the survival of all organisms, yet their availability may be restricted for certain fungi depending on their environmental conditions. Hence, supplementation with these metals may be necessary at times to meet the physiological requirements of the fungi and enhance their lignocellulose breakdown capabilities (Baldrian & Gabriel, 2003; Vrsanska & Langer, 2016). Previous literature suggests that appropriate dosages of Mn and Cu can stimulate

the production of Manganese Peroxidase (MnP) and laccase enzymes **(Chapter II)**. Therefore, **Chapter IV** described the impact of Cu and Mn supplementation on OMSW inoculated with *A. discolor* and *S. hirsutum*, focusing on ligninolytic enzyme activity, lignin degradation, and phenolic compound removal. In this section, we demonstrated the positive effects of Cu and Mn addition resulting in improved lignin degradation and phenolic compound removal. These findings underscore the importance of considering metal supplementation strategies in the design and optimization of biological treatments for lignocellulosic biomass degradation. In addition, the results revealed optimal metal dosages enhanced enzyme activity, varying efficacy depending on the fungal strain. A notable positive effect, attributed to Mn supplementation, was also observed, directly correlating with improved manganese peroxidase (MnP) and Laccase activity. The observed positive effects of Mn-Cu supplementation were related to those reported by Kannaiyan *et al*. (2015), who explained a cross-regulation phenomenon by Mn, which can act as an inducer of Lac activity. The highest levels of lignin degradation were associated with the dosage and the fungal strain. Specifically, *S. hirsutum* supplemented with 2.5 mg Kg^{-1} of Cu and 7.3 mg Kg^{-1} of Mn demonstrated the most favorable conditions (41.5 ± 3.2% at 20-day). This positive effect can directly result from improving the MnP activity described in section 3.3.2. Accordingly, Sari *et al*. (2016) reported that enhancing the MnP activity favors the oxidizing of Mn^{2+} to Mn^{3+} by the MnP. The highly reactive Mn^{3+} can combine with organic chelating compounds and acts as a low-molecular-weight redox mediator targeting the oxidation of the phenolic components of lignin and, thus, enhancing the lignin degradation (Kannaiyan *et al*., 2015). *S. hirsutum* also exhibited 65-75% phenol removal efficiencies in OMSW over a 25-day period with Cu-Mn supplementation. The higher values in the leachate reached 80-95% after 30 days. The phenol removal efficiencies achieved in this section were close to those reported by other authors, such as Reina *et al*. (2013), who described an efficiency in phenol compound removal from OMSW of

90% by *Auricularia auricula-judae*, 80% by *Bjerkandera adusta* and 100% by *Coprinellus radians*, all at 14-day under SSF. Additionally, the final concentration of the individual phenolic compounds indicated that most of them were widely metabolized during the process. However, some specific compounds were accumulated compared to the initial concentration during the fungal treatments. Specifically, in OMSW treated by *S. hirsutum* and its leachate, gallic acid hexoside, protocatechuic acid hexoside, and 3-hydroxytyrosol were detected. In OMSW treated by *A. discolor* and its leachate, the accumulation of gallic acid hexoside, protocatechuic acid hexoside, 3-hydroxytyrosol, 3,4-dimethoxybenzyl alcohol, and 2,6-dimethoxyphenol were detected. The accumulation of these compounds would be related to the breakdown of the lignocellulosic fibers of the OMSW, entailing the release of the phenolic compounds integrated into these fibers (Luo *et al*., 2021). Likewise, some specific phenolic compounds can accumulate because they are intermediates of the incomplete degradation of other phenolic compounds (Serrano *et al*., 2017b). Accordingly, the WRF and optimal metal dosages represent a promising strategy for future mycoremediation processes.

Once the efficacy of the fungi to grow on OMSW without supplemental nutrient sources was demonstrated, and the optimal doses of Cu and Mn were found to stimulate enzymatic activity, leading to enhanced lignin breakdown and phenol elimination from OMSW **(Chapters III and IV)**, the application of *A. discolor* and *S. hirsutum* into OMSW for the recovery of valuable compounds such as enzymatic extracts, VFA, and/or phenolic compounds, as well as, at the same time, reducing the phytotoxicity of the OMSW was monitored under solid-state fermentation conditions with the addition of 0.5 mM of Cu and 2.41 mM of Mn for 30 days for each strain. **(Chapter V)**. The results found demonstrated that operation times exceeding 15-20 days are not recommended for obtaining enzyme extracts with high enzyme concentrations for

both fungal strains. Conversely, the Cu-Mn addition strategy demonstrated a clear positive effect on enzymatic activity, consistent with previous findings described in **Chapter V** for the same fungal strains during OMSW fermentation. The enhancement of enzymatic activity can be attributed to the inductive effect of Cu-Mn addition. Specifically, the presence of copper is known to regulate peroxidase activity, inducing activity at appropriate dosages (Slavens *et al*., 2019). For instance, Liu *et al*., (2020) reported a 61.3% improvement in Lac activity by *G. lucidum* with the addition of 0.19 mM Cu. Similarly, the increase in MnP activity can be attributed to its heme-containing nature, featuring Mn-binding sites and catalyzing Mn^{2+} oxidation to Mn^{3+} in the presence of H_2O_2 (Kannaiyan *et al*., 2015). Additionally, Mn^{2+} plays a crucial role in the transcriptional phase and activation of peroxidase enzymes (Baldrian, 2003; Slavens *et al*., 2019), which may account for the observed results. Furthermore, interactions between Mn and Cu ions may play a significant role in fungal responses. Similar results were reported in **Chapter IV** under comparable experimental conditions, highlighting the superior enhancements in enzymatic activities achieved with Cu-Mn co-addition compared to individual metal additions. *S. hirsutum* demonstrated a higher capacity to release phenolic compounds into the liquid fraction compared to *A. discolor*, with maximum concentrations of 6001 ± 236 mg gallic acid eq L^{-1} and 3886 ± 186 mg gallic acid eq L^{-1}, respectively. While the Cu-Mn addition exhibited marginal improvement in phenolic compound release for *A. discolor*, it notably enhanced the maximum concentration of phenolic compounds by approximately 20% for *S. hirsutum*. This enhancement may be attributed to the increased enzymatic activity, particularly of Lac and MnP, observed in *S. hirsutum* following Cu-Mn addition. Moreover, the decline in phenolic compound concentration observed in A. discolor over time suggests that *S. hirsutum* may be preferable for phenolic compound extraction from the liquid phase. These findings

underscore the potential of *S. hirsutum* over *A. discolor* in phenolic compound extraction, particularly in conjunction with Cu-Mn supplementation.

Finally, this study considered the circular economy concept applied in waste management, transitioning from spending resources on waste treatment to considering waste as a potential source of benefits. In the olive oil sector, OMSW is a substrate with enormous potential due to its high content of phenolic compounds and/or volatile fatty acids (VFA). Therefore, the efficacy of *A. discolor* and *S. hirsutum* to recover valuable compounds from OMSW, such as enzymatic extracts, volatile fatty acids (VFA), and/or phenolic compounds, under the stimulation of extracellular enzymes production in solid-state fermentation conditions during 30 days and simultaneously determine its phytotoxic effects on tomato plants were described in **Chapter VI**. The results demonstrated that extended operation times exceeding 15-20 days are not recommended for obtaining enzyme extracts with high enzyme concentrations for both fungal strains. Conversely, the strategy of Cu-Mn addition showed a clear positive effect on enzymatic activity, consistent with previous findings detailed in **Chapter V** for the same fungal strains during OMSW fermentation. The enhancement of enzymatic activity can be attributed to the inductive effect of Cu-Mn addition. Specifically, copper is known to regulate peroxidase activity, inducing activity at appropriate dosages (Slavens *et al*., 2019). For instance, Liu *et al*., (2020) reported a 61.3% improvement in Lac activity by *G. lucidum* with the addition of 0.19 mM Cu. Similarly, the increase in MnP activity is plausible due to its heme-containing nature, featuring Mn-binding sites and catalyzing Mn^{2+} oxidation to Mn^{3+} in the presence of H_2O_2 (Kannaiyan *et al*., 2015). Additionally, Mn^{2+} plays a crucial role in the transcriptional phase and activation of peroxidase enzymes (Baldrian, 2003; Slavens *et al*., 2019), further explaining the observed results. Furthermore, interactions between Mn and Cu metal ions may also play an essential role

in fungal response. Similar results were described in **Chapter V** under comparable experimental conditions, highlighting superior enhancements in enzymatic activities achieved with Cu-Mn co-addition compared to individual metal additions. These findings underscore the importance of considering metal supplementation strategies in optimizing enzymatic activity for lignocellulosic biomass degradation. The concentrations of phenolic compounds extracted from the liquid fraction in this study surpassed those achieved by other physicochemical methods reported in the literature. For instance, Fattoum *et al*., (2023) reported maximum concentrations of soluble phenolic compounds of up to 1.17 g gallic acid eq L^{-1} and 1.07 g gallic acid eq L^{-1} using liquid-liquid extraction methods with ethyl acetate and chloroform/methanol (1:1), respectively. Similarly, the phenolic compound concentration obtained in our study exceeded that reported by Serrano *et al*., (2017b) using steam-explosion thermal treatment for OMSW, which reached 1834 ± 126 mg gallic acid eq L^{-1} of OMSW. However, this discrepancy may be attributed to the dilution effect caused by water addition during steam-explosion treatment, rather than a lower release of phenols from OMSW. The observed metabolism of phenolic compounds during fungal treatment underscores the dynamic nature of the biotransformation process. The accumulation of specific compounds, such as 4-methylcatechol and 4-ethylphenol, suggests complex interactions between fungal enzymes and OMSW components, leading to the release and subsequent accumulation of certain phenolic intermediates. These findings align with previous studies highlighting the intricate pathways involved in the degradation of lignocellulosic materials by white rot fungi. Moreover, the distinct metabolic profiles observed between *A. discolor* and *S. hirsutum* further emphasize the species-specific mechanisms underlying phenolic compound metabolism. Overall, these results contribute to our understanding of the potential applications of fungal treatment in phenolic compound extraction and waste valorization, paving the way for the development of sustainable waste management strategies. Also, *S. hirsutum* exhibited a higher

concentration of volatile fatty acids (VFA) with a more diverse profile compared to *A. discolor*. Interestingly, Cu-Mn addition did not significantly affect VFA concentrations for either fungal strain, except for an anomalous concentration of isovaleric acid detected in *A. discolor* at day 25. The maximum sum of individual VFAs reached 1627±325 mg L^{-1} for *S. hirsutum* at day 25 without Cu-Mn addition, and 1227±245 mg L^{-1} for *A. discolor* at day 25 with Cu-Mn addition. These concentrations significantly surpassed those reported for steam-explosion-treated OMSW by Serrano *et al*., (2017a), highlighting the effectiveness of fungal strains in VFA accumulation. While anaerobic fermentation of OMSW has been described as a feasible method for VFA production, our results suggest that fungal treatment offers a promising alternative for VFA generation from OMSW. However, further research is needed to elucidate the underlying mechanisms and optimize VFA production using fungal treatment methods. The VFAs profile obtained with *S. hirsutum* at day 30 revealed the presence of four VFAs, with the highest amounts of isobutyric acid (748 ± 154 – 776 ± 168 mg L^{-1}) followed by acetic acid (457 ± 78 – 478 ± 85 mg L^{-1}). In contrast, *A. discolor* predominantly produced acetic acid, albeit at a concentration around half of that obtained with *S. hirsutum*. Additionally, *S. hirsutum* accumulated a significant concentration of isovaleric acid after 10 days of operation (217 ± 59 – 223 ± 26 mg L^{-1}). These findings are noteworthy as isobutyric and isovaleric acids are commonly used in the food industry as preservatives and flavor enhancers. While higher production yields have been reported for other fungi, such as *Clostridium butyricum*, using synthetic sugars as substrates, the complex nature of OMSW makes direct comparisons challenging. Overall, *S. hirsutum* demonstrated greater potential for VFA accumulation compared to *A. discolor*, both in terms of total VFA concentration and the obtained VFA profile. The differential VFA profiles observed between *S. hirsutum,* and *A. discolor* underscore the species-specific metabolic capabilities of white rot fungi in VFA production from complex substrates like OMSW. The

substantial accumulation of isobutyric and isovaleric acids by *S. hirsutum* highlights its potential to produce valuable VFA compounds with applications in the food industry. Although higher production yields have been reported for certain bacteria using synthetic sugars, the feasibility of using OMSW as a substrate for VFA production underscores the importance of exploring fungal treatment methods in waste valorization efforts. Further research is warranted to optimize VFA production using fungal treatment strategies and elucidate the underlying metabolic pathways involved in VFA synthesis.

Additionally, when OMSW treated with *A. discolor* and *S. hirsutum* was assessed, the solid fraction showed slightly lower phytotoxicity compared to raw OMSW, with germination indexes around 35% for *A. discolor* and 40-50% for *S. hirsutum* at a 10% dose. However, Cu-Mn addition did not notably decrease phytotoxicity for either fungal strain. Higher OMSW doses resulted in almost total germination inhibition, like raw OMSW, suggesting that reduced phytotoxicity is linked to decreased phenolic compound concentrations. Conversely, the liquid extract's phytotoxicity increased compared to raw OMSW, particularly with *S. hirsutum*, which exhibited nearly complete germination inhibition at concentrations above 25%. This heightened phytotoxicity may be attributed to the accumulation of released compounds, including phenolic compounds and volatile fatty acids, in the liquid fractions. The greater phytotoxicity observed with S. *hirsutum* likely stems from higher concentrations of these compounds in its liquid fractions. The differential phytotoxic effects observed between solid and liquid fractions treated with *A. discolor* and *S. hirsutum* underscore the complex interplay between fungal treatment, substrate composition, and phytotoxic compound release. While solid fractions showed reduced phytotoxicity compared to raw OMSW, likely due to decreased phenolic compound concentrations, liquid fractions exhibited increased phytotoxicity, attributed to the accumulation

of released compounds. These findings highlight the importance of considering both solid and liquid fractions in assessing the overall impact of fungal treatment on phytotoxicity. Furthermore, the varying phytotoxic effects observed between fungal strains underscore the need for further research to elucidate the underlying mechanisms and optimize fungal treatment strategies for minimizing phytotoxicity while maximizing waste valorization potential.

6.2 Conclusions

1. *A. discolor* and *S. hirsutum,* two fungi isolated from rainforest at southern of Chile, were able to grow under static conditions using OMSW as the sole substrate without supplementing other nutrients, reaching more than 80% of total phenol removal, with clear evidence of lignin degradation after 24 days. These results highlighted the potential of WRF for the future design of an in-situ treatment for the valorization of OMSW as an alternative in the current management of OMSW and the associated limitations of smaller farms, avoiding complex systems or transportation.

2. The use of Cu and Mn allows the enhancement of specific enzymes, which opens an opportunity to design specific supplements according to the fungus application. As expected, laccase activity improvement was more related to Cu addition, whereas Mn addition improved MnP activity. On the other hand, there was a limited impact of the added metals on lignin degradation, but with a high phenol removal being observed in every case evaluated, that may have released with the positive effect of Mn on MnP and the positive relationship between Mn and Lac.

3. *A. discolor* and *S. hirsutum* in a culture medium based on wheat grains and supplemented with optimal doses of Cu and Mn, biodegrade OMSW under static conditions by increasing ligninolytic enzyme activity. Specifically, the effect of Cu-Mn supplementation was associated with the strain, with a distinct impact on the removal of soluble and total phenolic compounds, showing a degradation selectivity related to the physiology of phenol-degrading by the strains studied.

4. Residual solid fraction from OMSW treated by strains showed a phytotoxic effect more marked on *Solanum lycopersicum*. In contrast, residual liquid fraction from treatments with *A. discolor* slowed down the germination but did not inhibit germination until 75% of the concentration of this fraction, leaving open a possibility to study the effect on other aspects important for the plant.

5. The findings indicate that *S. hirsutum* holds promise as a fungal strain for the valorization of OMSW, resulting in the production of a liquid fraction enriched in phenolic compounds and VFAs. The addition of Cu-Mn notably facilitated the release of phenolic compounds by *S. hirsutum*. In contrast, A. discolor did not demonstrate significant accumulation of phenols or VFAs, despite exhibiting similar enzymatic activity to *S. hirsutum*. Although the treated OMSW showed reduced phytotoxicity compared to raw OMSW, severe phytotoxic effects were still observed at doses exceeding 10%. Hence, while fungal treatment presents an appealing valorization process for OMSW, additional stabilization methods are warranted for comprehensive OMSW management.

6.3 Future perspectives

This study suggests the feasibility of devising an in-situ pretreatment method for OMSW valorization, circumventing the need for complex systems or transportation. Therefore, future research under non-sterile conditions is needed to evaluate the competition of WRF with other microorganisms present in the OMSW.

Depending on the OMSW treatment design, it will be important to consider minimizing water use to optimize the process. In that sense, the evaluation of the process in the solid substrate obtained from the three-phases olive oil extraction process would be an interesting challenge, facing a substrate with less water but, also, higher relative abundance of recalcitrant compounds to be degraded because most of the easily biodegradable compounds are previously displaced during the olive extraction process to a liquid phase.

Despite the use of promising fungi for OMSW treatment, as well as metals addition as inducers, the generation of VFA still remains below the desirable values. At the same time the generated treated solid fraction still present a high seeds inhibitory effect. Thus, further research on new strategies for improving the fungal activity will be necessary to enhance the process, reducing the waste toxicity and to broaden the possible agronomic applications of the residual fractions from OMSW.

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Appendix

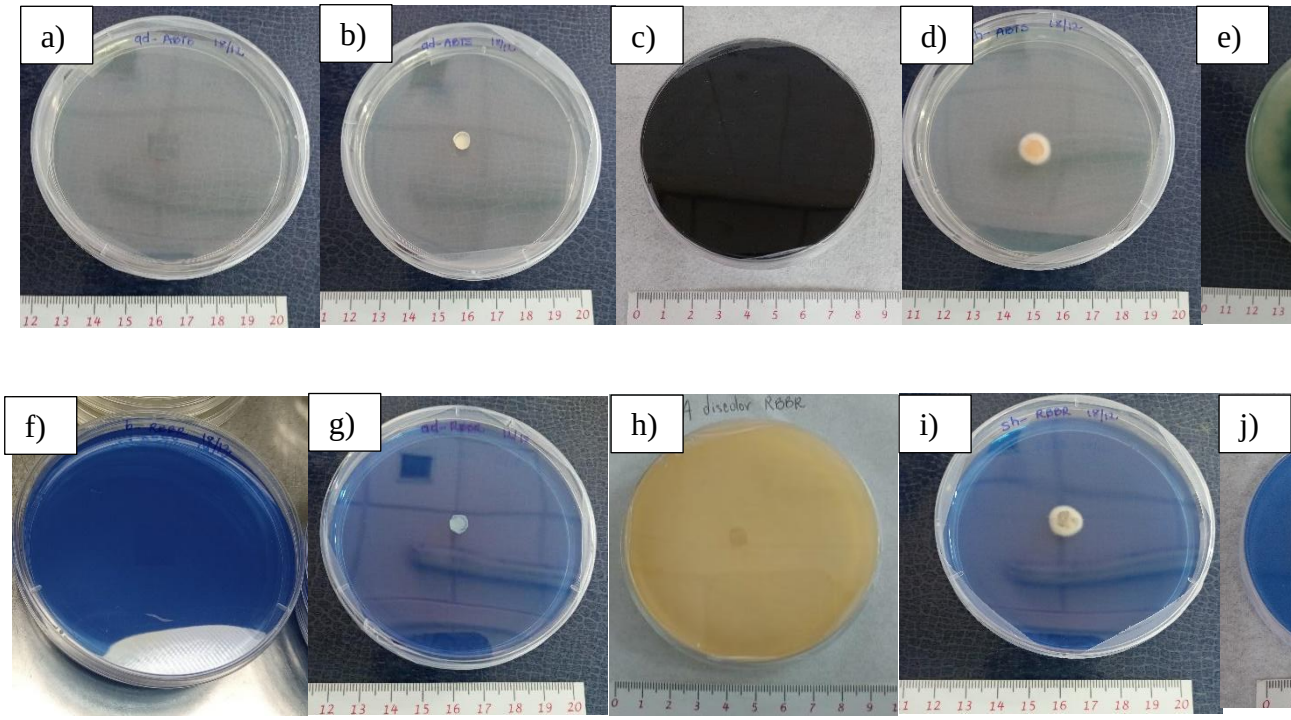

Figure S1. Qualitative detection of lignocellulosic enzymes. (a) control with ABTS and PDA, (
PDA with ABTS day 1, (c) *A. discolor* in PDA with ABTS day 14, (d) *S. hirsutum* in PDA with AB
hirsutum in PDA with ABTS day 14, (f) control with RBBR and PDA, (g) *A. discolor* in PDA with RB
discolor in PDA with RBBR day 14, (i) *S. hirsutum* in PDA with RBBR day 1, (j) *S. hirsutum* in PDA
14.