agronomy

MDPI

Article

Fungal Pretreatment of Alperujo for Bioproduct Recovery and Detoxification: Comparison of Two White Rot Fungi †

Viviana Benavides [1,2,3], Gustavo Ciudad [1,4,‡], Fernanda Pinto-Ibieta [5,*], Elisabet Aranda [2,6], Victor Ramos-Muñoz [7], Maria A. Rao [8] and Antonio Serrano [2,6,‡]

1 Departamento de Ingeniería Química, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco 4780000, Chile; d.benavidesarteaga@uandresbello.edu (V.B.); gustavo.ciudad@ufrontera.cl (G.C.)
2 Instituto Universitario de Investigación del Agua, Universidad de Granada, 18071 Granada, Spain; earanda@ugr.es (E.A.); antonio.serrano@ugr.es (A.S.)
3 Escuela de Ciencias Ambientales y Sustentabilidad, Facultad de Ciencias de la Vida, Universidad Andres Bello, Santiago 7550196, Chile
4 Centro de Excelencia en Investigación Biotecnológica Aplicada al Ambiente (CIBAMA), Universidad de La Frontera, Temuco 4780000, Chile
5 Departamento de Procesos Industriales, Facultad de Ingeniería, Universidad Católica de Temuco, Casilla 15-D, Temuco 4810399, Chile
6 Departamento de Microbiología, Facultad de Farmacia, Campus Universitario de Cartuja s/n, Universidad de Granada, 18012 Granada, Spain
7 Instituto de la Grasa, Spanish National Research Council (CSIC), Campus Universitario Pablo de Olavide, Ed. 46, Ctra. de Utrera, km. 1, 41013 Seville, Spain; vmramos@ig.csic.es
8 Department of Agricultural Sciences, University of Naples, Federico II, Via Università 100, 80055 Portici, Italy; mariarao@unina.it
* Correspondence: fpinto@uct.cl; Tel.: +56-45-2325556
† This research has been previously published as part of the following PhD thesis: Benavides Arteaga, Viviana. Development of an Olive Mill Solid Waste Treatment Using White-Rot Fungi with Cu and Mn as Inducers/Cofactors for Improving Its Biodegradability. Ph.D. Thesis, Universidad de La Frontera, Temuco, Chile & Universidad de Granada, Granada, Spain, 2024.
‡ These authors contributed equally to this work.

check for updates

Academic Editors: Caterina Morcia and Francesca Valerio

Received: 29 May 2025
Revised: 25 July 2025
Accepted: 25 July 2025
Published: 31 July 2025

Citation: Benavides, V.; Ciudad, G.; Pinto-Ibieta, F.; Aranda, E.; Ramos-Muñoz, V.; Rao, M.A.; Serrano, A. Fungal Pretreatment of Alperujo for Bioproduct Recovery and Detoxification: Comparison of Two White Rot Fungi. *Agronomy* **2025**, *15*, 1851. https://doi.org/10.3390/agronomy15081851

Abstract

Alperujo, a solid by-product from the two-phase olive oil extraction process, poses significant environmental challenges due to its high organic load, phytotoxicity, and phenolic content. At the same time, it represents a promising feedstock for recovering value-added compounds such as phenols and volatile fatty acids (VFAs). When used as a substrate for white rot fungi (WRF), it also produces ligninolytic enzymes. This study explores the use of two native WRF, *Anthracophyllum discolor* and *Stereum hirsutum*, for the biotransformation of alperujo under solid-state fermentation conditions, with and without supplementation of copper and manganese, two cofactors known to enhance fungal enzymatic activity. *S. hirsutum* stood out for its ability to release high concentrations of phenolic compounds (up to 6001 $\pm$ 236 mg gallic acid eq L^{-1}) and VFAs (up to 1627 $\pm$ 325 mg L^{-1}) into the aqueous extract, particularly with metal supplementation. In contrast, *A. discolor* was more effective in degrading phenolic compounds within the solid matrix, achieving a 41% reduction over a 30-day period. However, its ability to accumulate phenolics and VFAs in the extract was limited. Both WRF exhibited increased enzymatic activities (particularly Laccase and Manganese Peroxidase) with the addition of Cu-Mn, highlighting the potential of the aqueous extract as a natural source of biocatalysts. Phytotoxicity assays using *Solanum lycopersicum* seeds confirmed a partial detoxification of the treated alperujo. However, none of the fungi could entirely eliminate inhibitory effects on their own, suggesting the need for complementary stabilization steps before agricultural reuse. Overall, the results indicate that *S. hirsutum*, especially when combined with metal supplementation, is better suited for valorizing alperujo through the recovery of bioactive compounds. Meanwhile, *A. discolor* may be more suitable for detoxifying the solid phase strategies. These findings support the integration of fungal pretreatment into biorefinery schemes that valorize agroindustrial residues while mitigating environmental issues.

Keywords: ligninolytic enzymes; alperujo; valorization; phenolic compound recovery; volatile fatty acids

1. Introduction

Alperujo is the principal residue from the two-phase olive oil extraction system, with an estimated generation of 4 tons per ton of olive oil produced [1,2]. Alperujo management poses significant environmental concerns due to its high organic load, dark coloration of effluents, phytotoxicity, and strong odor emissions, which can negatively impact soil, water bodies, and plant germination [3,4]. These challenges could be resolved by developing sustainable strategies for its valorization. In a circular bioeconomy, alperujo is increasingly viewed as a potential feedstock rather than a waste product, owing to its content of valuable compounds. These include volatile fatty acids (VFAs), which serve as platform chemicals for biotechnological applications [5], and phenolic compounds, such as hydroxytyrosol and tyrosol, known for their antioxidant and antimicrobial properties [6,7]. Although these phenolic compounds contribute to the phytotoxic nature of alperujo [8,9], this substrate can still present a high potential as an organic amendment, reinforcing the interest of the phenol extraction for both valorization and detoxification purposes.

Their entrapment impedes the effective recovery of these compounds within a complex lignocellulosic matrix composed of lignin, cellulose, and hemicellulose [10,11]. Conventional pretreatment methods, including thermal and chemical strategies, have been proposed to disrupt this structure. However, these often involve high energy inputs or generate fermentation-inhibiting by-products such as furfural and 5-hydroxymethylfurfural [12,13]. In this regard, biological pretreatments using white-rot fungi (WRF) present a more environmentally benign alternative. These fungi produce ligninolytic enzymes such as laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP), which can effectively degrade lignin and release bound bioactive compounds [14–16]. Beyond delignification, these enzymes represent high-value biocatalysts with applications across several industries [17]. While several WRF species have shown potential for alperujo treatment [8,18], there is growing interest in native fungi, which adapt better to local conditions and present fewer ecological risks. In this context, *Anthracophyllum discolor (A. discolor)* and *Stereum hirsutum (S. hirsutum)*, isolated from temperate forests in southern Chile [19], have demonstrated efficient delignification and phenolic removal capabilities [20,21].

Currently, treatment with WRF is no longer considered an independent valorization strategy. Still, it has evolved into a key pretreatment step within biorefinery schemes integrating complementary downstream techniques, such as anaerobic digestion (AD). Although numerous strategies have focused on enhancing enzymatic activity by adding organic or inorganic cofactors and lignocellulosic co-substrates [22–25], there is a paucity of studies evaluating how these enhancements impact the downstream recovery of value-added compounds and the sustainability of interconnected processes. For instance, the addition of metallic cofactors may restrict the subsequent agricultural application of the treated substrate [26]. At the same time, the use of nitrogen-rich co-substrates could cause ammonium accumulation, thereby inhibiting downstream anaerobic digestion [27]. Thus, within this integrated context, a broader perspective is required that considers the impacts of these strategies beyond merely optimizing enzymatic activity.

Therefore, this study aimed to reuse alperujo by biotransformation to recover bioactive compounds and reduce its phytotoxicity. To reach these aims, the biotransformation will be carried out using, independently, two native WRF, *A. discolor* and *S. hirsutum*, under solid-state fermentation in the presence or absence of copper and manganese supplementation. Ultimately, the study aims to inform the selection of fungi, considering whether the primary goal is the recovery of bioactive compounds or the reduction in phytotoxicity, thereby supporting the integration of fungal pretreatment into circular biorefinery schemes. Therefore, the work focused on two main outcomes: (i) the generation of an aqueous extract enriched in value-added compounds, namely, phenolic compounds, volatile fatty acids, and ligninolytic enzymes with potential biotechnological applications, and (ii) the partial detoxification of the residual solid phase to assess its suitability for agricultural reuse.

2. Materials and Methods

2.1. Microorganisms

The fungi *A. discolor* and *S. hirsutum* were used, both isolated from decayed wood in the temperate forests of southern Chile, and belong to the Culture Collection of the Environmental Nanobiotechnology Laboratory at the Universidad de La Frontera, Chile [28]. The fungi were maintained at 4 °C in Petri dishes containing malt extract agar medium (VWR chemicals, Radnor, PA, USA), prepared by dissolving 33.6 g L^{-1} in distilled water, adjusted to pH 5.5 before autoclaving at 121 °C for 15 min. Prior to experimental use, the fungi were pre-cultivated to ensure active growth. Reactivation was carried out by transferring a 6 mm diameter agar plug, taken from the edge of an actively growing colony, to fresh malt extract agar plates. The cultures were then incubated at 28 °C in the dark for 7 days under static conditions to allow full mycelial development [20].

2.2. Alperujo

Alperujo was obtained from "Oleícola El Tejar," located in Marchena, Seville, Spain. Alperujo was preserved under freezing conditions (−18 °C) until its characterization and use. The main characteristics of alperujo were as follows: pH = 4.6 ± 0.1; total solids (TSs) = 252.6 ± 3.0 mg L^{-1}; volatile solids (VSs) = 236.8 ± 3.1 mg L^{-1}; mineral solids (MSs) = 15.8 ± 0.1 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}; volatile fatty acids (VFAs) = 35.13 mg L^{-1}; copper = 242.7 ± 0.3 ppb; manganese = 282.6 ± 0.3 ppb [20].

2.3. Experimental Set-Up and Design

The four assessed experimental conditions were (i) *A. discolor* without Cu-Mn supplementation, (ii) *A. discolor* with Cu-Mn supplementation, (iii) *S. hirsutum* without Cu-Mn supplementation, and (iv) *S. hirsutum* with Cu-Mn supplementation. Alperujo was directly used for all assays without pretreatment, such as grinding or sieving. For the inoculum, the fungi were initially pre-cultured in a wheat grain-based medium (18 g wheat and 30 mL distilled water per flask) based on the procedure described by Reina et al. [29] with some modifications. The flasks with wheat grain-based medium, after sterilization in an autoclave, were inoculated with 9 mL of a mixture of 4 active fungal plates homogenized using an Ultra-Turrax in 80 mL of sterile water (55% v/v). The mixture was then incubated in static conditions at 28 °C for 7 days. Afterward, the flasks were mixed with 18 g of sterile alperujo and 2 mL of sterile water supplemented with 0.5 mM of Cu and 2.41 mM of Mn, added as $CuSO_4 \cdot 5H_2O$ and $MnSO_4 \cdot H_2O$, respectively. The concentrations of both metals were selected by the optimal values previously described for *A. discolor* and *S. hirsutum* in Benavides et al. [20]. Flasks containing wheat grain-based medium and incubated with *A. discolor* and *S. hirsutum* without the addition of alperujo were used as controls. The cultures were incubated in static conditions at 28 °C for 30 days. Twenty-one 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 fungus at each sampling time (0, 5, 10, 15, 20, 25, and 30 days) for their characterization to monitor the processes.

2.4. Sampling Procedure and Analytical Methods

An aqueous extract was obtained from flasks with fermented alperujo at each sampling point using 100 mL of distilled water. This mixture was placed in a rotary shaker for 2 h. Then, the mixtures were centrifuged and filtered, and the obtained extracts were used to measure enzyme activities, pH, soluble phenols, metals, and VF, as described below. In the residual solid phase, the total phenol content was also determined. All the determinations were carried out in triplicate, and the data reported are mean values with the standard deviation of the triplicate.

Enzymatic activities of laccase (Lac, EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1), and Mn-independent peroxidase (MniP) were evaluated every five days over a 30-day period using the 2,6-dimethoxyphenol assay. The reaction mixture (pH 4.5) contained sodium malonate buffer, 2,6-dimethoxyphenol, $MnSO_4 \cdot H_2O$, and sample supernatant, and was initiated with H_2O_2. Absorbance was recorded at 468 nm and corrected for Lac activity. Enzymatic activity was expressed as U, defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per minute at 30 °C [30,31] (Supplementary Material S.1). Phenolic compounds were quantified by spectrophotometry following the Folin–Ciocalteu method, using gallic acid as calibration standard. Solid samples were extracted with methanol/water (80:20 *v*/*v*) via vortexing and centrifugation (1200× *g*, 10 min), repeated four times. Liquid samples were centrifuged and microfiltered (0.22 µm), while soluble phenols were recovered from Whatman Nº1-filtered supernatants. The colorimetric assay involved the reaction of 100 µL of extract with 0.2 M Folin–Ciocalteu reagent and 0.7 M sodium carbonate. Absorbance was measured at 655 nm after 15 min incubation at ~25 °C using a Bio-Rad iMark reader (Bio-Rad Laboratories, Hercules, CA, USA). Results were expressed as mg gallic acid equivalents per liter (mg GAE L^{-1}) or per 100 g of OMSW (mg GAE/100 g) [32,33] (Supplementary Material S.2).

At the end of the experimental period, volatile fatty acids (VFAs, C2–C5) were determined in triplicate and quantified using an internal-standard method. Analyses were performed on a Shimadzu GC-2025 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a Stabilwax-DA capillary column (Crossbond acid-deactivated Carbowax polyethylene glycol, 30 m × 0.25 mm i.d., 0.25 µm film thickness; RESTEK) and a flame ionisation detector (FID) set at 250 °C. A detailed description of the analytical method, covering the calibration of acetic (HAc), propionic (HPr), isobutyric (i-HBu), butyric (HBu), isovaleric (i-HVa), and valeric (HVa) acids, is provided in the Supplementary Material S.3 [34].

2.5. Phytotoxicity Tests After Fungal Treatment

Phytotoxicity tests of the aqueous extract and residual solid phase obtained at the end of each experiment were conducted to assess their impact on *S. lycopersicum* germination. The tests were performed according to the method described by Pinho et al. [35] and Zucconi et al. [36], with some modifications. Before conducting the phytotoxicity tests, *S. lycopersicum* seeds were hydrated with tap water to imbibe them and eliminate the non-viable seeds. Afterward, ten seeds were placed in a 9 cm diameter Petri dish lined with filter paper containing 2 mL of undiluted aqueous extract, as well as 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 variable dose adjustments with vermiculite (90, 75, 50, 25, and 10% *w:w*). In both cases, the Petri dishes were incubated at 25 °C in the dark for 72 h. Controls, with distilled water and vermiculite and without alperujo, were also included in the tests. The germination index was calculated according to the following equations (Equations (1)–(3)):

$$GI(\%) = \frac{RSG(\%) \times xRRG(\%)}{100} \quad (1)$$

$$RSG(\%) = \frac{G}{(G_0 \times x100)} \quad (2)$$

$$RRG(\%) = \frac{L}{(L_0 \times x100)} \quad (3)$$

where GI is germination index, G is germination; 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.

2.6. Statistical Analysis

All analyses were carried out at least in triplicate. The results are expressed as mean values with their corresponding standard deviations from the replicates. Differences among the variables were evaluated using analysis of variance (ANOVA). When significant differences were detected ($p < 0.05$), means were compared using Tukey's Honestly Significant Difference (HSD) test at a 5% significance level to identify statistically different groups [37]. All statistical analyses were performed using InfoStat software, v. 2020.

3. Results and Discussion

3.1. Value-Added Compounds Obtained in the Aqueous Extract

3.1.1. Ligninolytic Enzyme Activity by *A. discolor* and *S. hirsutum*

Figure 1a–c depicts the temporal profiles of laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MniP) activities in aqueous extracts from *A. discolor* and *S. hirsutum*, with and without Cu-Mn supplementation. Adding Cu-Mn to *A. discolor* significantly enhanced Lac activity ($p < 0.05$, see Supplementary Material S.4), peaking at 987 ± 27 U L^{-1} on day 15, a value 88.7% higher than the 111 ± 29 U L^{-1} recorded without metal addition (Figure 1a). After day 20, a consistent decline in Lac activity was observed across all treatments. Similarly, MnP activity increased by 49.7% with metal supplementation, reaching 2090 ± 154 U L^{-1} compared to 1051 ± 35 U L^{-1} without supplementation. MniP activity remained comparatively low (<250 U L^{-1}) under all conditions.

For *S. hirsutum*, the peak Lac activity was recorded at day 20, with Cu-Mn supplementation increasing the value from 654 ± 77 U L^{-1} to 966 ± 8 U L^{-1}, indicating a 45% enhancement (Figure 1a). The maximum MnP activity also increased significantly with metal addition, reaching 2008 ± 204 U L^{-1} at day 5, compared to only 500 ± 10 U L^{-1} without supplementation ($p < 0.05$, see Supplementary Material S.4).

Overall, enzymatic activity was highest before day 20 for both fungi, suggesting this as the optimal timeframe for enzyme extraction (Figure 1). Cu-Mn supplementation consistently enhanced enzymatic output, corroborating previous findings by Benavides et al. [23], who also associated copper with increased Lac activity and manganese with elevated MnP activity in fungal treatments of alperujo. Specifically, for *S. hirsutum*, the addition of 6.1 mg Cu kg^{-1} and 7.3 mg Mn kg^{-1} resulted in MnP activity of 173 ± 5 U L^{-1}, an 863% increase over the control [20].

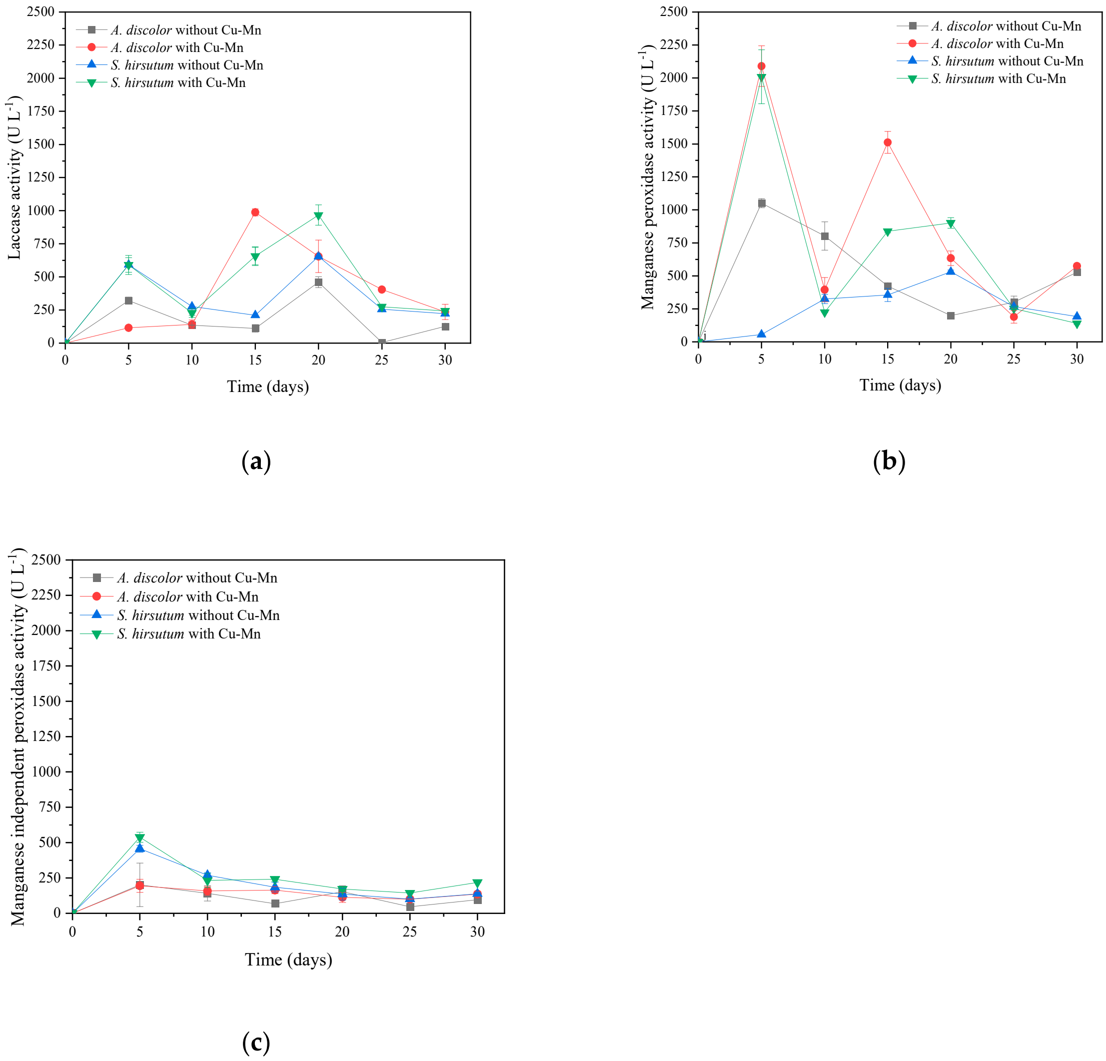


Figure 1. Enzymatic activities in aqueous extracts of *Anthracophyllum discolor* and *Stereum hirsutum* without and with Cu-Mn supplementation: Laccase activity (**a**); Manganese peroxidase activity (**b**); Manganese-independent peroxidase activity (**c**). Error bars represent the standard error of the mean (n = 3).

This observed enhancement likely stems from the inductive roles of Cu and Mn in enzymatic expression. Copper can act as a regulatory cofactor for peroxidase activity, inducing expression at optimal metal dosage [38]. Liu et al. [39] reported an improvement of 61.3% in the Lac activity of *Ganoderma lucidum* upon the addition of 0.19 mM Cu^{2+}. Likewise, the increase in MnP activity could be attributed to this enzyme being a heme-containing protein, which includes Mn-binding sites and catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 [40]. Additionally, Mn^{2+} plays a key role in both the transcriptional regulation and enzymatic activation of peroxidase enzymes [38,41]. This improvement in enzymatic activity suggests that metal supplementation can effectively transform enzymatic extracts into valuable sources for biotechnological applications, particularly in bioremediation processes.

The augmentation of enzymatic activity through metal supplementation yielded values comparable to those reported for extracts produced using synthetic culture media for bioremediation applications [42]. For instance, Sybuia et al. [43] reported the removal of acetaminophen using 40 U L^{-1} of laccase obtained from *T. versicolor*. Similarly, 200 U L^{-1} of laccase from *T. hirsutum* achieved removal efficiencies of 100% for sulfamethazine, 99% for acetaminophen, 87% for levothyroxine, and 82% for lincomycin, all at an initial concentration of 10 µg L^{-1} [44]. These findings highlight the potential of aqueous extracts from alperujo as a low-cost enzymatic source for biotechnological applications. Reina et al. [9] similarly described alperujo as an enzymatic elicitor for producing high-interest enzymes by *Auricularia auricula judae*.

3.1.2. Phenolic Compounds Release from the Alperujo to the Aqueous Extract

The fungal pretreatment promoted the solubilization of phenolic compounds into the aqueous extract, with *S. hirsutum* outperforming *A. discolor* in terms of both yield and stability of released compounds. Maximum concentrations reached 6001 ± 236 mg gallic acid eq L^{-1} for *S. hirsutum* and 3886 ± 186 mg gallic acid eq L^{-1} for *A. discolor* (Figure 2). Adding Cu-Mn had a limited effect on phenol recovery in *A. discolor*, which could be attributed to the elevated laccase activity (Figure 1a), promoting phenol oxidation and polymerization [23,45].

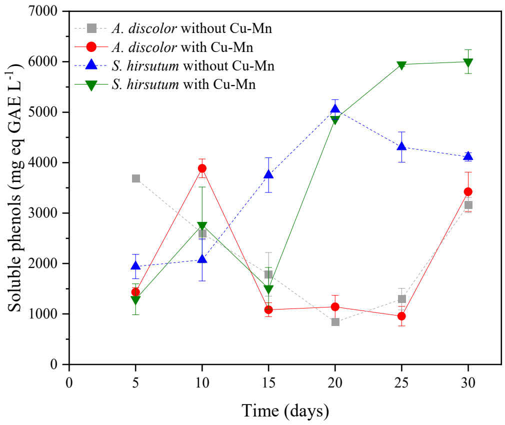


Figure 2. Variation of the soluble phenolic compounds concentration in the aqueous extract obtained with *Anthracophyllum discolor* and *Stereum hirsutum* without metals (control) and with Cu-Mn. Error bars indicate standard error of the mean (n = 3).

In contrast, Cu-Mn supplementation increased phenol accumulation by approximately 20% in *S. hirsutum* (Figure 2), likely due to the coordinated upregulation of both Lac and MnP (Figure 1a,b), which favored the liberation of phenolic compounds from lignocellulosic matrices without significant oxidative degradation.

During the experiment, *A. discolor* showed a marked decrease in phenolic content, almost depleting soluble phenols by day 30. This behavior underscores the relevance of fungus selection based on the target application. Suppose the process objective is the recovery and valorization of phenolic compounds for nutraceutical, pharmaceutical, or cosmetic applications. In that case, *S. hirsutum* is the preferred fungus due to its higher and more sustained phenol release. Conversely, if the goal is to reduce or eliminate phenolic content for environmental purposes, such as detoxifying phytotoxic residues, *A. discolor* is more suitable, as it efficiently removes phenols from the substrate, presumably through enzymatic degradation and polymerization.

Therefore, the selection of fungus should be guided by the final objective: *S. hirsutum* for value-added recovery and *A. discolor* for bioremediation and phytotoxicity mitigation. This functional differentiation aligns with previous findings, where distinct fungal species exhibited divergent capacities for either phenol solubilization or degradation, depending on their enzymatic profiles [9,20].

The concentrations of phenolic compounds obtained in the aqueous extracts were higher than those obtained by other physicochemical alternatives reported in the literature. Fattoum et al. [46] reported yields around 1170 mg gallic acid eq L^{-1} using ethyl acetate extraction, whereas Serrano et al. [11] achieved 1834 ± 126 mg gallic acid eq L^{-1} through steam-explosion pretreatment. This unexpected result could be partially attributed to dilution effects (water additions) and oxidative degradation during thermal processing, further highlighting the advantages of biological methods for phenolic extraction. Despite the high concentration of phenols obtained in this research, further analysis of phenolic profiles and bioactivities is essential to assess their industrial potential and market value.

3.1.3. Volatile Fatty Acid (VFA) Composition in the Aqueous Extract

The accumulation of VFAs in the aqueous extract was significantly more efficient with *S. hirsutum* than with *A. discolor* ($p < 0.05$, see Supplementary Materials S.4), both in total concentration and diversity of compounds (Figure 3a–d). The highest total VFA concentration was observed at day 25 for *S. hirsutum* without Cu-Mn supplementation, reaching 1627 ± 325 mg L^{-1}. In comparison, *A. discolor* achieved a maximum of 1227 ± 245 mg L^{-1} under Cu-Mn supplementation. This outcome suggests that *S. hirsutum* possesses a higher metabolic capacity to depolymerize complex lignocellulosic structures and release fermentable intermediates.

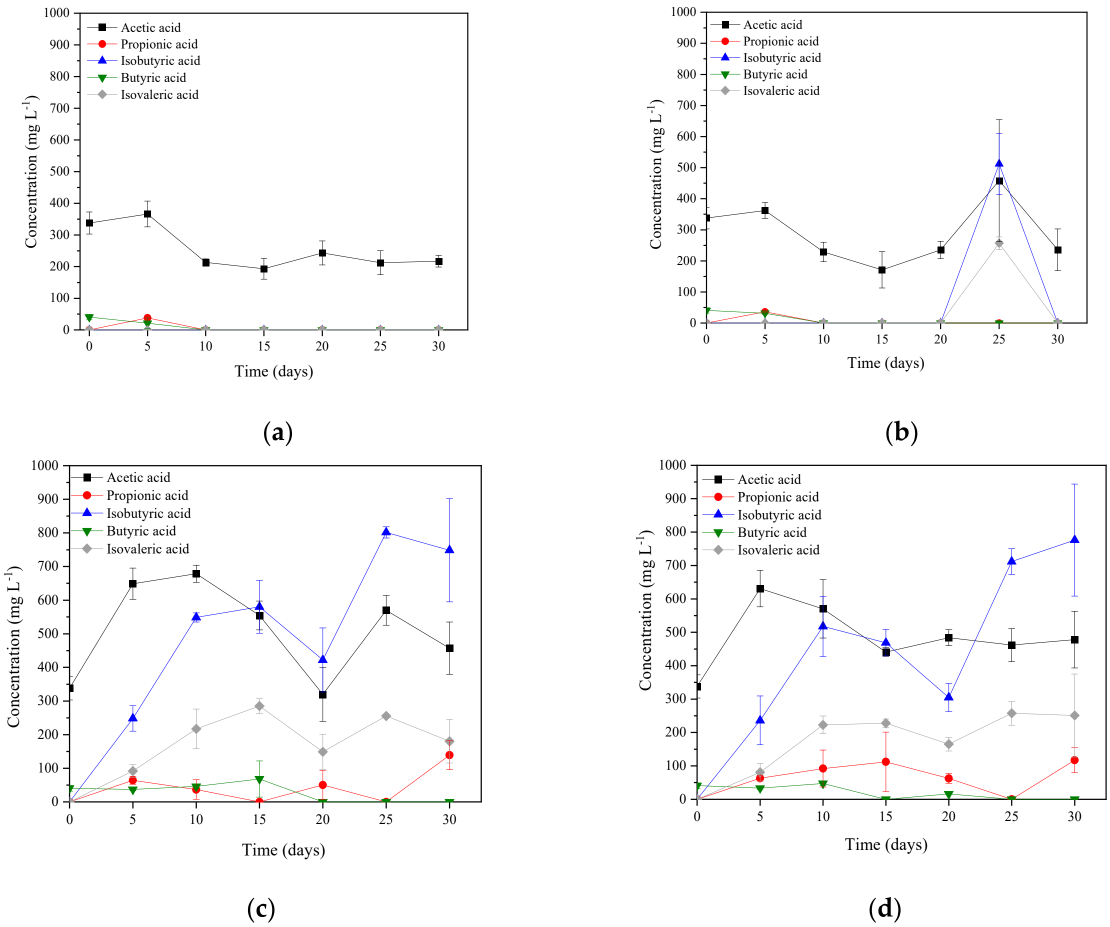


Figure 3. Volatile fatty acid variation derived from the liquid extract of alperujo, subject to treatment by *Anthracophyllum discolor* without Cu-Mn (**a**); *Anthracophyllum discolor* with Cu-Mn (**b**); *Stereum hirsutum* without Cu-Mn (**c**), and *Stereum hirsutum* without Cu-Mn (**d**) in Solid State Fermentation at 30 °C during experimental time. Error bars indicate standard error of the mean (n = 3).

The effect of Cu-Mn supplementation on VFA production was less pronounced than for phenolic compounds or enzymatic activity. Except for a peak in isovaleric acid at day 25 with *A. discolor* and Cu-Mn (Figure 3a), metal supplementation did not significantly enhance VFA yields, suggesting that VFA accumulation is governed more by substrate availability and fungal metabolic pathways than by ligninolytic enzyme induction.

The effectiveness of these fungi for accumulating VFA was higher than that reported by Serrano et al. [47], which described that the steam-explosion treatment of alperujo could not generate any relevant concentration of acetic acid. By contrast, the alperujo has been described as a feasible biomass for VFA production by anaerobic fermentation. For example, Jiménez-Páez et al. [5] reported accumulating up to 2625 ± 375 mg L^{-1} of VFA during the batch anaerobic fermentation of alperujo 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.

Figure 3c,d reveals a distinct metabolic profile for *S. hirsutum*, which produced up to four VFAs, including isobutyric, isovaleric, and acetic acids. At day 30, isobutyric acid dominated the profile with concentrations between 748 ± 154 and 776 ± 168 mg L^{-1}, followed by acetic acid (457 ± 78–478 ± 85 mg L^{-1}). These values are approximately twice as high as those achieved by *A. discolor*, which primarily generated acetic acid as the only detectable VFA (Figure 3a,b).

Notably, *S. hirsutum* began accumulating isovaleric acid as early as day 10 (217 ± 59–223 ± 26 mg L^{-1}), suggesting a faster onset of secondary metabolism. Both isobutyric and isovaleric acids are of industrial relevance due to their applications as preservatives, flavoring agents, and precursors for chemical synthesis [48]. Although the yields are lower than those obtained by pure cultures on defined substrates (e.g., 12–20 g L^{-1} by *Clostridium butyricum* using glucose or sucrose [49,50], the results are noteworthy considering the complexity and recalcitrance of alperujo as a lignocellulosic substrate.

These findings demonstrate that fungal pretreatment, particularly with *S. hirsutum*, enables partial hydrolysis and fermentation of alperujo into VFAs. In contrast to anaerobic fermentation processes, such as that reported by Jiménez-Páez et al. [5], under pH-controlled conditions, the fungal route offers a more sustainable, single-stage approach that eliminates the need for external pH regulation or synthetic nutrients.

In conclusion, producing VFAs via fungal biotransformation represents a promising strategy for converting alperujo into valuable chemical intermediates. *S. hirsutum*, owing to its broader metabolic activity and more complex VFA profile, is again highlighted as the most efficient fungus for this purpose. Optimization could include co-cultivation strategies or sequential fermentation schemes to enhance VFA recovery further.

3.2. Characterization of the Obtained Alperujo Solid Phase

3.2.1. Phenolic Compounds in the Alperujo Solid Phase

The evolution of total phenolic compounds in the solid phase during fungal treatment revealed contrasting behaviors between the two fungi (Figure 4). *A. discolor* substantially reduced the phenolic compounds in the solid phase of the alperujo, which decreased from 18.1 ± 0.1 g gallic acid eq 100 g^{-1} alperujo at 0-day up to a minimal value of 10.7 ± 0.3 g gallic acid eq 100 g^{-1} alperujo at 30-day, reaching phenol removal efficiencies around 45% (Figure 4). As can also be seen, the Cu-Mn addition showed almost no impact, demonstrating that phenol degradation by *A. discolor* is not substantially enhanced by these cofactors under the tested conditions. This behavior is consistent with the strong laccase activity exhibited by *A. discolor*, which, as previously discussed, can oxidize phenols into less soluble or polymerized forms [23].

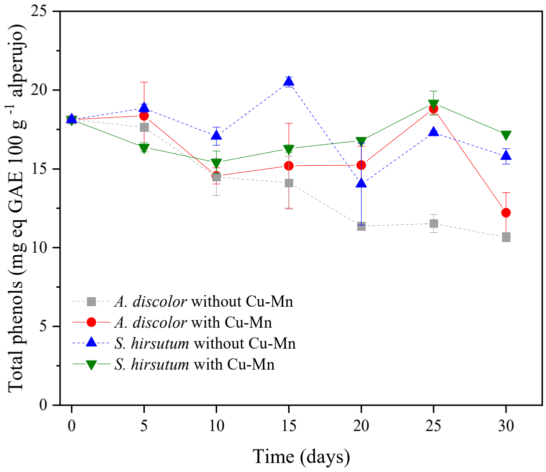


Figure 4. Variation of the total phenolic compounds concentration in the solid phase of alperujo obtained with *Anthracophyllum discolor* without Cu-Mn; *Anthracophyllum discolor* with Cu-Mn; *Stereum hirsutum* without Cu-Mn and *Stereum hirsutum* without Cu-Mn in solid state fermentation at 30 °C during experimental time. Error bars indicate standard error of the mean (n = 3).

Therefore, *A. discolor* is effective for dephenolization of the solid matrix and is a suitable candidate for bioremediation purposes where detoxification is prioritized. These phenol removal efficiencies fell within the range of values reported in the literature for other fungi applied to alperujo. Sampedro et al. [18] achieved a phenolic removal efficiency of 36% in alperujo pretreated with *Coriolopsis rigida* (current *Funalia floccosa*), after 14 days of incubation. Similarly, Reina et al. [9] described an efficiency in phenol compounds removal between 60% and 80% for *Auricularia auricula-judae*, *Bjerkandera adusta*, and *Coprinellus radians* after 28 days of alperujo pretreatment.

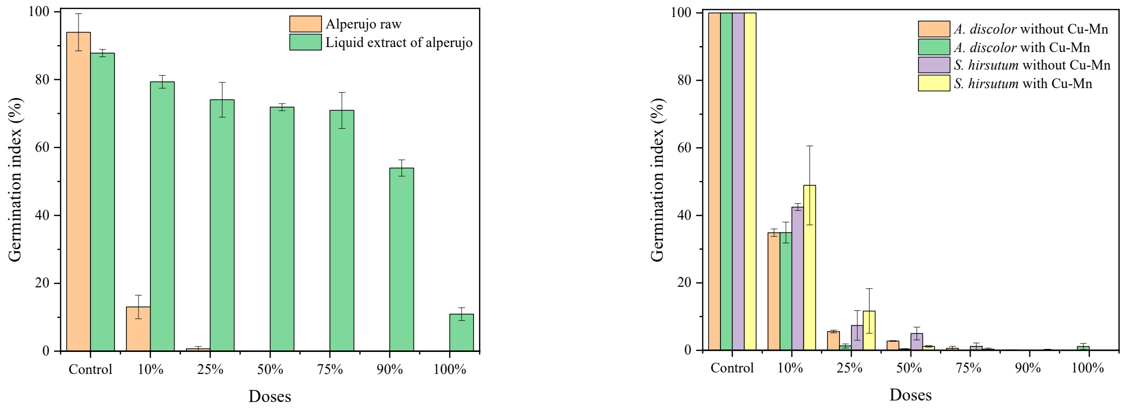
In contrast, *S. hirsutum* showed a very low phenol removal efficiency in both conditions, i.e., with and without Cu-Mn addition (Figure 4). Specifically, the phenol removal efficiencies were only 5% and 20% for Cu-Mn addition and without Cu-Mn, respectively. The low removal efficiencies of *S. hirsutum* are likely related to the release of new phenolic compounds from complex lignocellulosic structures during alperujo degradation, as reported by Benavides et al. [20] and Serrano et al. [11].

In summary, phenol removal from the solid phase is highly fungal dependent, with *A. discolor* showing a pronounced capacity for in situ degradation. At the same time, *S. hirsutum* favors phenol mobilization into the aqueous extract. This divergence again emphasizes the need to tailor fungal treatments to specific valorization or remediation targets.

3.2.2. Effect of Alperujo on *S. lycopersicum* Seed Germination

Phytotoxicity assays using *S. lycopersicum* seeds revealed that raw alperujo exhibits strong inhibitory effects, with germination index (GI) values dropping below 20 at a 10% (w/w) dose (Figure 5). This pronounced phytotoxicity is consistent with the high initial content of phenolic compounds (18.1 ± 0.1 g gallic acid eq 100 g^{-1}) and has been extensively linked to the presence of bioactive phenolics such as hydroxytyrosol and tyrosol [35,51].

Following fungal treatment, both fungi exhibited a moderate reduction in phytotoxicity in the solid phase. At the 10% application dose, GIs increased to 35% and 40–50% for *A. discolor* and *S. hirsutum*, respectively (Figure 5b). However, higher doses still caused near complete inhibition of germination, indicating that detoxification was only partial. Notably, Cu-Mn supplementation had a negligible impact on phytotoxicity mitigation, suggesting that metal-induced enzymatic enhancement does not translate directly into improved detoxification during seed germination.



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

Figure 5. Effect of alperujo on *Solanum lycopersicum* seed germination. Different doses of solid fraction and liquid extract of alperujo raw (**a**) and effect of different doses of solid fraction of alperujo treated with *Anthracophyllum discolor* without Cu-Mn; *Anthracophyllum discolor* with Cu-Mn; *Stereum hirsutum* without Cu-Mn and *Stereum hirsutum* without Cu-Mn (**b**) in solid state fermentation at 30 °C obtained at final treatment (30-day) on *Solanum lycopersicum* seed germination. Error bars indicate 406 standard error of the mean (n = 3).

The observed improvements in germination correlate with the partial depletion of phenolic content in the solid matrix, particularly in the case of *A. discolor* (Figure 4), further supporting the role of phenolics as primary phytotoxic agents. Despite *S. hirsutum* having a lower residual phenol concentration in the solid phase, its performance in the GI test was slightly better. This may be due to the qualitative composition of phenols or the generation of less toxic intermediates during fungal bioconversion.

Taken together, the results of this study suggest that valorization, rather than bioremediation, is the most promising route for alperujo management under the tested conditions.

The fungal pretreatment with *S. hirsutum*, particularly with Cu-Mn supplementation, yielded aqueous extracts enriched in value-added compounds such as phenols, VFAs, and ligninolytic enzymes, all of which have relevant applications in the food, pharmaceutical, and biotechnological sectors. In contrast, although *A. discolor* effectively reduced the phenolic content in the solid phase, the degree of detoxification achieved was insufficient to support direct agricultural reuse without further stabilization. Therefore, fungal treatment should be considered a preconditioning step within integrated biorefinery frameworks to recover bioactive molecules from agroindustrial residues.

4. Conclusions

This study demonstrates that fungal pretreatment of alperujo using *S. hirsutum* and *A. discolor* results in both the generation of an aqueous extract enriched in value-added compounds and the partial detoxification of the residual solid phase. Both fungi presented high MnP and Lac activities, reaching a significant improvement with Cu and Mn supplementation. *S. hirsutum* was significantly more effective at solubilizing phenolic compounds and VFAs into the aqueous extract than *A. discolor.* The supplementation with copper and manganese favored the accumulation of phenolic compounds in *S. hirsutum,* but not for *A. discolor.* In contrast, *A. discolor* led to a greater reduction in phenolic content in the solid phase than *S. hirsutum*, contributing to lower phytotoxicity. However, this effect was insufficient to allow direct agricultural application at doses above 10%, highlighting the need for post-treatment strategies such as composting. Despite similar enzymatic activity profiles, the differences in compound release and detoxification performance emphasize the importance of fungal selection based on the targeted application.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15081851/s1, S.1: Methodology used for enzymatic activity; S.2: Total phenol content determination; S.3: Detailed description of methodology used for Volatile Fatty Acids determination; S.4: Statistical analysis: Analysis of variance (ANOVA) ($p < 0.05$) and Tukey's Honestly Significant Difference (HSD) test at a 5%; Figure S1: Chromatogram of analyzed sample corresponding to flask with *A. discolor* without Cu-Mn at 25 days; Figure S2: Chromatogram of analyzed sample corresponding to flask with *A. discolor* supplemented with Cu-Mn at 25 days; Figure S3: Chromatogram of analyzed sample corresponding to flask with *S. hirsutum* without Cu-Mn at 25 days; Figure S4: Chromatogram of analyzed sample corresponding to flask with *S. hirsutum* supplemented with Cu-Mn at 25 days.

Author Contributions: Conceptualization, F.P.-I., E.A. and A.S.; Formal analysis, V.B.; Funding acquisition, G.C.; Methodology, V.B. and V.R.-M.; Resources, G.C., F.P.-I., E.A., M.A.R. and A.S.; Supervision, G.C., E.A. and A.S.; Writing—original draft, V.B., M.A.R. and A.S.; Writing—review and editing, G.C., F.P.-I., E.A., M.A.R., V.R.-M. and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: Fondecyt project No. 1231521, Agencia Nacional de Investigación y Desarrollo de Chile, ANID.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Acknowledgments: Viviana Benavides expresses gratitude to the Agencia Nacional de Investigación y Desarrollo (ANID), Chile, for the doctoral scholarship (21212088) that supported this research. Special thanks are extended to Jorge Castro-Rojas for their assistance with HPLC-DAD analysis. Gustavo Ciudad is grateful to the ANID for his Fondecyt Project N° 1231521. Fernanda Pinto-Ibieta thanks ANID for the project Fondecyt Iniciación No. 11241307. Antonio Serrano is grateful to the Economic Transformation, Industry, Knowledge, and Universities Department of the Andalusia Autonomous Government for his Emergia fellowship (EMERGIA20_00114).

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the study design, data collection, or analyses, manuscript writing, or the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

COD	Chemical Oxygen Demand
FID	Flame Ionization Detector
GI	Germination Index
HPLC-DAD	High-Performance Liquid Chromatography with diode-array detection
Lac	Laccase
MnP	Manganese Peroxidase
MS	Mineral Solids
RRG	Relative Radicle Growth
RSG	Relative Seed Germination
SSF	Solid State Fermentation
TS	Total Solids
VFAs	Volatile Fatty Acids
VS	Volatile Solids
WRF	White Rot Fungi

References

1. Enaime, G.; Dababat, S.; Wichern, M.; Lübken, M. Olive mill wastes: From wastes to resources. *Environ. Sci. Pollut. Res.* **2024**, *31*, 20853–20880. [CrossRef]
2. Lenzuni, M.; Converti, A.; Casazza, A.A. From laboratory- to industrial-scale plants: Future of anaerobic digestion of olive mill solid wastes. *Bioresour. Technol.* **2024**, *394*, 130317. [CrossRef] [PubMed]

3. Genethliou, C.; Kornaros, M.; Dailianis, S. Biodegradation of olive mill wastewater phenolic compounds in a thermophilic anaerobic upflow packed bed reactor and assessment of their toxicity in digester effluents. *J. Environ. Manag.* **2020**, *255*, 109882. [CrossRef]
4. Sounni, F.; Elgnaoui, Y.; El Bari, H.; Merzouki, M.; Benlemlih, M. Effect of mixture ratio and organic loading rate during anaerobic co-digestion of olive mill wastewater and agro-industrial wastes. *Biomass Convers. Biorefinery* **2021**, *13*, 1223–1229. [CrossRef]
5. Jiménez-Páez, E.; Serrano, A.; Purswani, J.; Correa-Galeote, D.; Cubero-Cardoso, J.; Fermoso, F.G. Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste. *Environ. Technol. Innov.* **2023**, *32*, 103409. [CrossRef]
6. Fernández-Prior, Á.; Bermúdez-Oria, A.; Millán-Linares, M.D.C.; Fernández-Bolaños, J.; Espejo-Calvo, J.A.; Rodríguez-Gutiérrez, G. Anti-inflammatory and antioxidant activity of hydroxytyrosol and 3,4-dihydroxyphenyglycol purified from table olive effluents. *Foods* **2021**, *10*, 227. [CrossRef] [PubMed]
7. Wang, E.; Jiang, Y.; Zhao, C. Hydroxytyrosol isolation, comparison of synthetic routes and potential biological activities. *Food Sci. Nutr.* **2024**, *12*, 6899–6912. [CrossRef] [PubMed]
8. Díaz, A.I.; Ibañez, M.; Laca, A.; Díaz, M. Biodegradation of olive mill effluent by white-rot fungi. *Appl. Sci.* **2021**, *11*, 9930. [CrossRef]
9. Reina, R.; Liers, C.; Ocampo, J.A.; García-Romera, I.; Aranda, E. Solid state fermentation of olive mill residues by wood- and dung-dwelling Agaricomycetes: Effects on peroxidase production, biomass development and phenol phytotoxicity. *Chemosphere* **2013**, *93*, 1406–1412. [CrossRef]
10. Rubio-Senent, F.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity. *LWT* **2013**, *54*, 114–124. [CrossRef]
11. Serrano, A.; Fermoso, F.G.; Alonso-Fariñas, B.; Rodríguez-Gutierrez, G.; Fernandez-Bolaños, J.; Borja, R. Phenols recovery after steam explosion of Olive Mill Solid Waste and its influence on a subsequent biomethanization process. *Bioresour. Technol.* **2017**, *243*, 169–178. [CrossRef]
12. Becerra, M.L.; Lizarazo, L.M.; Rojas, H.A.; Prieto, G.A.; Martinez, J.J. Biotransformation of 5-hydroxymethylfurfural and furfural with bacteria of bacillus genus. *Biocatal. Agric. Biotechnol.* **2022**, *39*, 102281. [CrossRef]
13. Hendriks, A.T.W.M.; Zeeman, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **2009**, *100*, 10–18. [CrossRef]
14. Ntougias, S.; Baldrian, P.; Ehaliotis, C.; Nerud, F.; Merhautová, V.; Zervakis, G.I. Olive mill wastewater biodegradation potential of white-rot fungi—Mode of action of fungal culture extracts and effects of ligninolytic enzymes. *Bioresour. Technol.* **2015**, *189*, 121–130. [CrossRef]
15. Shirkavand, E.; Baroutian, S.; Gapes, D.J.; Young, B.R. Pretreatment of radiata pine using two white rot fungal strains *Stereum hirsutum* and *Trametes versicolor*. *Energy Convers. Manag.* **2017**, *142*, 13–19. [CrossRef]
16. Suryadi, H.; Judono, J.J.; Putri, M.R.; Eclessia, A.D.; Ulhaq, J.M.; Agustina, D.N.; Sumiati, T. Biodelignification of lignocellulose using ligninolytic enzymes from white-rot fungi. *Heliyon* **2022**, *8*, e08865. [CrossRef]
17. El-Gendi, H.; Saleh, A.K.; Badierah, R.; Redwan, E.M.; El-Maradny, Y.A.; El-Fakharany, E.M. A Comprehensive Insight into Fungal Enzymes: Structure, Classification, and Their Role in Mankind's Challenges. *J. Fungi* **2022**, *8*, 23. [CrossRef]
18. Sampedro, I.; Cajthaml, T.; Marinari, S.; Stazi, S.R.; Grego, S.; Petruccioli, M.; Federici, F.; D'Annibale, A. Immobilized inocula of white-rot fungi accelerate both detoxification and organic matter transformation in two-phase dry olive-mill residue. *J. Agric. Food Chem.* **2009**, *57*, 5452–5460. [CrossRef]
19. Durán, N.; Cuevas, R.; Cordi, L.; Rubilar, O.; Diez, M.C. Biogenic silver nanoparticles associated with silver chloride nanoparticles (Ag@AgCl) produced by laccase from *Trametes versicolor*. *SpringerPlus* **2014**, *3*, 645. [CrossRef]
20. Benavides, V.; Pinto-Ibieta, F.; Serrano, A.; Rubilar, O.; Ciudad, G. Use of *Anthracophyllum discolor* and *Stereum hirsutum* as a Suitable Strategy for Delignification and Phenolic Removal of Olive Mill Solid Waste. *Foods* **2022**, *11*, 5438. [CrossRef]
21. Rubilar, O.; Tortella, G.; Cea, M.; Acevedo, F.; Bustamante, M.; Gianfreda, L.; Diez, M.C. Bioremediation of a Chilean Andisol contaminated with pentachlorophenol (PCP) by solid substrate cultures of white-rot fungi. *Biodegradation* **2011**, *22*, 31–41. [CrossRef] [PubMed]
22. Atilano-Camino, M.M.; Álvarez-Valencia, L.H.; García-González, A.; García-Reyes, R.B. Improving laccase production from *Trametes versicolor* using lignocellulosic residues as cosubstrates and evaluation of enzymes for blue wastewater biodegradation. *J. Environ. Manag.* **2020**, *275*, 111231. [CrossRef]
23. Benavides, V.; Serrano, A.; Pinto-Ibieta, F.; Rubilar, O.; Ciudad, G. Biodegradation of olive mill solid waste by *Anthracophyllum discolor* and *Stereum hirsutum*: Effect of copper and manganese supplementation. *Bioresour. Bioprocess.* **2025**, *12*, 18. [CrossRef]
24. Mishra, V.; Jana, A.K.; Jana, M.M.; Gupta, A. Enhancement in multiple lignolytic enzymes production for optimized lignin degradation and selectivity in fungal pretreatment of sweet sorghum bagasse. *Bioresour. Technol.* **2017**, *236*, 49–59. [CrossRef]
25. Siddeeg, S.M.; Tahoon, M.A.; Mnif, W.; Ben Rebah, F. Iron Oxide/Chitosan Magnetic Nanocomposite Immobilized Manganese Peroxidase for Decolorization of Textile Wastewater. *Processes* **2020**, *8*, 5. [CrossRef]

26. Hseu, Z.Y. Evaluating heavy metal contents in nine composts using four digestion methods. *Bioresour. Technol.* **2004**, *95*, 53–59. [CrossRef]
27. Capson-Tojo, G.; Moscoviz, R.; Astals, S.; Robles; Steyer, J.P. Unraveling the literature chaos around free ammonia inhibition in anaerobic digestion. *Renew. Sustain. Energy Rev.* **2020**, *117*, 109487. [CrossRef]
28. Tortella, G.R.; Rubilar, O.; Gianfreda, L.; Valenzuela, E.; Diez, M.C. Enzymatic characterization of Chilean native wood-rotting fungi for potential use in the bioremediation of polluted environments with chlorophenols. *World J. Microbiol. Biotechnol.* **2008**, *24*, 2805–2818. [CrossRef]
29. Reina, R.; Liers, C.; García-Romera, I.; Aranda, E. Enzymatic mechanisms and detoxification of dry olive-mill residue by *Cyclocybe aegerita*, *Mycetinis alliaceus* and *Chondrostereum purpureum*. *Int. Biodeterior. Biodegrad.* **2017**, *117*, 89. [CrossRef]
30. Wariishi, H.; Valli, K.; Gold, M.H. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: Kinetic mechanism and role of chelators. *J. Biol. Chem.* **1992**, *267*, 23688–23695. [CrossRef]
31. Parenti, A.; Muguerza, E.; Redin Iroz, A.; Omarini, A.; Conde, E.; Alfaro, M.; Castanera, R.; Santoyo, F.; Ramírez, L.; Pisabarro, A.G. Induction of laccase activity in the white rot fungus *Pleurotus ostreatus* using water polluted with wheat straw extracts. *Bioresour. Technol.* **2013**, *133*, 142–149. [CrossRef]
32. Singleton, V.L.; Orthofer, R.; Lamuela-Ravents, R.M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 1999; pp. 152–178.
33. García, A.; Rodríguez-Juan, E.; Rodríguez-Gutiérrez, G.; Rios, J.J.; Fernández-Bolaños, J. Extraction of phenolic compounds from virgin olive oil by deep eutectic solvents (DESs). *Food Chem.* **2016**, *197*, 554–561. [CrossRef]
34. Vargas-Muñoz, M.A.; Cerdà, V.; Cadavid-Rodríguez, L.S.; Palacio, E. Automated method for volatile fatty acids determination in anaerobic processes using in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction and gas chromatography with flame ionization detector. *J. Chromatogr. A* **2021**, *1643*, 462034. [CrossRef]
35. Pinho, I.A.; Lopes, D.V.; Martins, R.C.; Quina, M.J. Phytotoxicity assessment of olive mill solid wastes and the influence of phenolic compounds. *Chemosphere* **2017**, *185*, 258–267. [CrossRef]
36. Zucconi, F.; Pera, A.; Forte, M.; DeBertolli, M. Evaluating toxicity of immature compost. *Biocycle* **1981**, *22*, 54–57.
37. Di Rienzo, J.A.; Casanoves, F.; Balzarini, M.G.; Gonzalez, L.; Tablada, M.; Robledo, C.W. *InfoStat Versión 2020*; de Transferencia InfoStat, FCA; Universidad Nacional de Córdoba: Córdoba, Argentina, 2020; Available online: http://www.infostat.com.ar (accessed on 13 June 2024).
38. Slavens, S.; Marek, S.M.; Wilkins, M.R. Effects of copper, manganese, and glucose on the induction of ligninolytic enzymes produced by *Pleurotus ostreatus* during fungal pretreatment of switchgrass. *Trans. ASABE* **2019**, *62*, 1673–1681. [CrossRef]
39. Liu, S.-H.; Tsai, S.-L.; Guo, P.-Y.; Lin, C.-W. Inducing laccase activity in white rot fungi using copper ions and improving the efficiency of azo dye treatment with electricity generation using microbial fuel cells. *Chemosphere* **2020**, *243*, 125304. [CrossRef] [PubMed]
40. Kannaiyan, R.; Mahinpey, N.; Kostenko, V.; Martinuzzi, R.J. Nutrient media optimization for simultaneous enhancement of the laccase and peroxidases production by coculture of *Dichomitus squalens* and *Ceriporiopsis subvermispora*. *Biotechnol. Appl. Biochem.* **2015**, *62*, 173–185. [CrossRef] [PubMed]
41. Baldrian, P. Interactions of heavy metals with white-rot fungi. *Enzyme Microb. Technol.* **2003**, *32*, 78–91. [CrossRef]
42. Flórez-Restrepo, M.A.; López-Legarda, X.; Segura-Sánchez, F. Bioremediation of emerging pharmaceutical pollutants acetaminophen and ibuprofen by white-rot fungi—A review. *Sci. Total Environ.* **2025**, *977*, 179379. [CrossRef]
43. Sybuia, P.A.; Contato, A.G.; de Araújo, C.A.V.; Zanzarin, D.M.; Maciel, G.M.; Pilau, E.J.; Peralta, R.M.; de Souza, C.G.M. Application of the white-rot fungus *Trametes* sp. (C3) laccase in the removal of acetaminophen from aqueous solutions. *J. Water Process Eng.* **2024**, *57*, 104677. [CrossRef]
44. Alokpa, K.; Lafortune, F.; Cabana, H. Application of laccase and hydrolases for trace organic contaminants removal from contaminated water. *Environ. Adv.* **2022**, *8*, 100243. [CrossRef]
45. Zhang, P.; Xu, J.; Zhang, X.; Hou, C.; Wu, D. Catalytic removal of emerging contaminant and phenolic compounds by laccase: Transformation mechanisms in aquiatic environments-polymerization or degradation? *Sep. Purif. Technol.* **2024**, *355*, 129544. [CrossRef]
46. Fattoum, H.; Cherif, A.O.; Trabelsi, S.; Ben Messaouda, M. Identification of Phenolic Compounds Extracted from OMW Using LC-MS. *J. Oleo Sci.* **2023**, *72*, 1113–1123. [CrossRef] [PubMed]
47. Serrano, A.; Fermoso, F.G.; Alonso-Fariñas, B.; Rodríguez-Gutierrez, G.; Fernandez-Bolaños, J.; Borja, R. Olive mill solid waste biorefinery: High-temperature thermal pre-treatment for phenol recovery and biomethanization. *J. Clean. Prod.* **2017**, *148*, 314–323. [CrossRef]
48. Bhatia, S.K.; Yang, Y.H. Microbial production of volatile fatty acids: Current status and future perspectives. *Rev. Environ. Sci. Biotechnol.* **2017**, *16*, 327–345. [CrossRef]
49. He, G.Q.; Kong, Q.; Chen, Q.H.; Ruan, H. Batch and fed-batch production of butyric acid by *Clostridium butyricum* ZJUCB. *J. Zhejiang Univ. Sci. B* **2005**, *6*, 1076–1080. [CrossRef]

50. Zigova, J. Butyric acid production by. *Biotechnol. Bioeng.* **1999**, *34*, 835–843.
51. Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry* **1992**, *31*, 4125–4128. [CrossRef]