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Aquaculture Sludge as Co-Substrate for Sustainable Olive Mill Solid Waste Pre-Treatment by *Anthracophyllum discolor*

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Abstract: Olive mill solid waste (OMSW) is an agro-industrial waste that has a high content of recalcitrant lignocellulose, which can adversely affect the environment. This study aimed to evaluate the phenol and lignin removal and the enzyme activity involved in the biological pre-treatment of OMSW supplemented with aquaculture sludge (AS) as an external nitrogen source by *Anthracophyllum discolor*. The highest lignin removal and enzymatic activity performance was obtained in the mixture of OMSW and AS prepared at a C/N ratio 45. In these conditions, the pre-treatment could remove 66% of lignin and 68% of phenols in the solid phase and 56% of phenols in the liquid phase and the maximum activity of laccase, manganese peroxidase and manganese independent peroxidase were of 10, 289 and 75 U L^{-1} in 25, 30, and 15 days of pre-treatment, respectively. These results propose that the addition of AS as a co-substrate for adjusting the C/N ratio allows a 41 and 141% increase in lignin removal and manganese peroxidase activity respectively, enabling the treatment of both OMSW and AS wastes and the possible recovery of an enzymatic extract of biotechnological interest.

Keywords: olive mill solid waste; white-rot fungi; aquaculture sludge; extract enzymatic; biorefinery

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1. Introduction

Resource recovery from agro-industrial wastes is considered a sustainable solution for waste management as it reduces the environmental impact and cost of waste and provides an alternative means of resource supply [1–3]. As a lignocellulosic waste generated from the two-stage olive oil production, olive mill solid waste (OMSW) can have adverse environmental impacts such as phytotoxicity in soils, toxicity to aquatic life, and offensive odors [4,5]. Moreover, the high organic matter content of OMSW, like lignin (13 to 15%) [6] and polyphenols (4.3 to 5.9 g L^{-1}) [5], makes it possible to obtain value-added by-products through the biorefining process, e.g., the biogas and biofertilizers from the anaerobic digestion of its organic matter [2,7], and the bioactive polyphenols of interest to the food, cosmetic and pharmaceutical industries [7,8].

Commonly, the OMSWs lignocellulosic waste requires a physicochemical pre-treatment to enhance the recovery of polyphenols, the detoxification of OMSW, and the generation of by-products such as biogas and biofertilizers [5,6,8–10]. Nevertheless, such a physicochemical pre-treatment involves high energy and environmental costs [2,11]. As an alternative solution, the biological pre-treatment of OMSW using white-rot fungi (WRF) is considered to be more environmentally and economically sustainable, which can generate enzymatic extracts as value-added by-products of high biotechnological interest due to their versatility in degrading many pollutants and xenobiotic compounds; for example, lignocellulosic compounds, antibiotics, dyes, and polycyclic aromatic compounds [12–16].

The ability of WRF to remove lignin and polyphenols is due to the fact that fungal mycelia can penetrate lignocellulosic materials and release polyphenols, which in turn can induce the secondary metabolism of WRF by secreting nonspecific extracellular oxidases, such as lignin peroxidase (LiP), laccase (Lac), manganese peroxidase (MnP) and manganese-independent peroxidase (MniP) [1,17–19]. Therefore, the enzymatic process resulting from the biological pre-treatment of OMSW has the potential to reduce the phenolic content of OMSW and recover ligninolytic enzymes [20]. Obtaining ligninolytic enzymes from OMSW depends on enzyme-induced variables such as correct adjustment of the C/N ratio [10]. Due to the low nitrogen content between 0.6 to 1.87%, OMSW has a high C/N ratio [21,22], which was considered to be an ideal condition for inducing the high enzymatic activity of *Anthracophyllum discolor* (*A. discolor*), a type of WRF [23], although the relevant mechanism remains unclear. However, this statement is controversial and is not accepted as a general rule for all WRFs [10,24,25], because the adjustment of the C/N ratio is found closely related to the specific process of biological pre-treatment. For instance, Zerva et al. [10] achieved an increase in the ligninolytic activity of *Pleurotus citrinopileatus* for the pre-treatment of liquid waste from olive oil production using a WRF when they attempted to replace inorganic nitrogen sources with nitrogen-enriched corn steep liquor.

In this sense, another waste with nitrogen-enriched and highly polluting characteristics is Aquaculture sludge (AS) consisting of uneaten feed, fish feces, other metabolites, and abundant biodegradable organic matters such as proteins and lipids [26,27]. It is estimated that only 20–30% of the nitrogen is assimilated by fish [28], and the nitrogen content of AS varies between 4.5 to 10% [28,29]. Thus, AS can be an ideal exogenous nitrogen source to regulate essential nutrients and improve enzymatic productivity in the OMSW pre-treatment.

As a research gap, the use of AS as a nitrogen source to modulate C/N ratios has not been studied when pre-treating OMSW with WRFs. This study aims to evaluate the effect of AS as a co-substrate in the pre-treatment of OMSW with *A. discolor*, improving its enzymatic activity for a possible recovery of an enzymatic extract and allow the pre-treatment of both OMSW and AS wastes. The study proposes a novel alternative for the valorization of two wastes that are not currently used and integrated as co-substrates in biorefinery processes.

2. Materials and Methods

2.1. Olive Mill Solid Waste and Aquaculture Sludge

The two-phase OMSW in this study was collected from the extra virgin olive oil producer "Olivares de Quepú", Talca, Chile. The processed olives belonged to the Arbequina variety. The AS was provided by "Hendrix Genetic", Curarrehue, Chile. AS is produced during the aquaculture stage of spawning and small-scale reproduction.

Both the raw wastes before pre-treatment were stored at −20 °C. The OMSW and AS characterization parameters include: pH, chemical oxygen demand (COD), total solids (TS), volatile solids (VS), total nitrogen (TN), total organic carbon (TOC) and were all measured according to standard methods [30]. Elemental analysis was performed for total carbon and nitrogen content using Perkin Elmer EA2400 Series II instrument to determine the C/N ratios of OMSW and AS. The centrifugation allowed the separation of a solid and liquid phase in the biological pre-treatment of OMSW and AS where total soluble phenols (TSP) were quantified using the Folin–Ciocalteu method, expressed as mg gallic

acid (GA) L^{-1} [8]. The solid phase extraction was performed using 5 g of sample in 10 mL of methanol/water (80:20 *v/v*). The mixture was stirred on a vortex for 30 s and heated to 70 °C for 1 h. The samples were centrifuged at 5000 rpm for 10 min and filtered by a 0.22 µm pore size PVDF membrane (Merck). The liquid phase was centrifuged and filtered under the same conditions. Additionally, the lignin content of the biological pre-treatment was measured using the Klason lignin method (TAPPI T 222 om-02) [31].

2.2. Fungal Strain and Inoculum Preparation

The fungi strain *Anthracophyllum discolor* that was used for this study was collected from the culture collection of the Center of Excellence in Biotechnological Research Applied to the Environment (CIBAMA) at the Universidad de La Frontera, Chile.

A. discolor was isolated from decaying wood in the rainforests of southern Chile. The culture was cultivated in Petri dishes (94 × 16 mm) at 25 °C for 7 days in a medium consisting of 39 g L^{-1} Potato Dextrose Agar (PDA) sterilized at 121 °C for 20 min [20].

Six plugs (6 mm diameter) of *A. discolor* active mycelia from seven days cultures on PDA medium were inoculated in 100 mL of modified Kirk's medium in a 1 L Erlenmeyer flask containing: glucose (10 g L^{-1}), peptone (2 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), $CaCl_2$ (0.1 g L^{-1}) and mineral salts solution (10 mL L^{-1}) of KH_2PO_4 (2 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.1 g L^{-1}), $MnSO_4{\cdot}5H_2O$ (50 mg L^{-1}), NaCl (10 mg L^{-1}), $FeSO_4{\cdot}7H_2O$ (1 mg L^{-1}), $CoCl_2{\cdot}6H_2O$ (1 mg L^{-1}), $ZnSO_4{\cdot}7H_2O$ (1 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.1 mg L^{-1}), $AlK(SO_4)_2$ (0.1 mg L^{-1}), H_3BO_3 (0.1 mg L^{-1}), and $NaMoO_4{\cdot}2H_2O$ (0.1 mg L^{-1}). The culture medium was adjusted to pH 5.5 [17]. The growth was carried out for an additional 7 days under static conditions at 25 °C. The mycelia were separated from the liquid medium using filtration and homogenized with 200 mL of sterile distilled water in a blender to obtain the *A. discolor* inoculum [32].

2.3. Assessment of Aquaculture Sludge Effect

2.3.1. Pre-Treatment Olive Mill Solid Waste and Aquaculture Sludge at Different C/N Ratio

Pre-treatments ware performed under sterile and static conditions in 250 mL Erlenmeyer flasks covered with hydrophobic cotton. OMSW and AS were sterilized at 121 °C for 20 min. The flasks were inoculated by adding 0.5 mL blended inoculum per dry gram of OMSW and incubated at 25 °C for 30 days of pre-treatment [32]. All assays were carried out at the same time and the control corresponded to OMSW pre-treatment with *A. discolor*.

Five different C/N ratios were selected for the pre-treatments (Table 1). The C/N ratios of 58 and 7 correspond to the pre-treatment of OMSW and AS, respectively (Table 1). The C/N ratios of 45, 32 and 19 correspond to different configurations of OMSW and AS mixtures. The pre-treatments are composed of a fixed volume of AS or water (75 mL) and the necessary amount of OMSW on a dry basis to adjust the selected C/N ratio.

Table 1. Mixture conditions for the pre-treatment of Olive mill solid waste and Aquaculture sludge.

Pre-Treatment (C/N Ratio/Substrate)	Dry Basis Mass OMSW (g)	Volume AS (mL)	Volume H_2O (mL)	Pre-Treatment TS (g L^{-1})
C/N-58 OMSW	5.2	0	75	69
C/N-45 OMSW/AS	12.2	75	0	171
C/N-32 OMSW/AS	5.0	75	0	75
C/N-19 OMSW/AS	1.8	75	0	32
C/N-7 AS	0	75	0	8

The TSP content in the solid and liquid phase was measured at the beginning and at the end of each pre-treatment. Lignin content was measured at the two best TSP yields and the activity of LiP, Lac, MnP and MniP was monitored every 5 days.

2.3.2. MnP Activity Assessment in Pre-Treatment at the Same TS Concentration

It has been reported that increasing the concentration of solids during fungi pre-treatment may promote fungi enzymatic activity [33,34]. To study the effect of TS concentration on the pre-treatment, C/N mixtures of 45, 32 and 19 were prepared with three different TS concentration, i.e., 171, 75 and 32 g TS L^{-1} (Table 2). Controls were prepared for each pre-treatment by replacing the volume of added AS with water, and OMSW maintaining the TS concentration (Table 2).

Table 2. Mixture conditions for pre-treatment of Olive mill solid waste and Aquaculture sludge at the same TS concentration.

Pre-Treatment (C/N Ratio/Substrate)	Dry Basis Mass OMSW (g)	Volume AS (mL)	Volume H_2O (mL)	Pre-Treatment TS (g L^{-1})
C/N-45 OMSW/AS	12.2	75	0	171
C/N-58 OMSW [a]	12.8	0	75	171
C/N-32 OMSW/AS	5.0	75	0	75
C/N-58 OMSW [b]	5.6	0	75	75
C/N-19 OMSW/AS	1.8	75	0	32
C/N-58 OMSW [c]	2.4	0	75	32

([a, b, c]) control pre-treatment without AS; ([a]) 171, ([b]) 75 and ([c]) 32 g L^{-1}.

The pre-treatments were carried out under the same operating conditions as in Section 2.3.1.

Due to its biotechnological interest, only MnP was evaluated. The enzymatic activity of MnP produced with *A. discolor* was evaluated at three control times: 15, 25 and 30 days of pre-treatment.

2.4. Enzyme Activity Assessment

Enzyme activity was measured in the liquid phase using a UV-VIS spectrophotometer (Thermo Scientific GenesysTM 10S) at 30 °C, after centrifugation of each biological pre-treatment at 5000 rpm for 10 min [17,35]. LiP activity was monitored every 10 seconds for 2 minutes at 310 nm (Molar extinction coefficient used 9300 M cm^{-1} [23]. Lac, MnP and MniP activities ware monitored every 10 s for 1 minute at 468 nm (The molar extinction coefficient was 49,600 M cm^{-1} [35].

LiP activity was determined by the oxidation of veratryl alcohol to veratraldehyde. The reaction mixture contained 710 µL disodium tartrate dihydrate (0.1 mM, pH 3.0), 200 µL veratryl alcohol (2 mM) and 50 µL sample. The reaction was initiated by adding 100 µL H_2O_2 (10 mM). Lac activity was determined by the oxidation of 2,6-dimethoxyphenol (DMP). The reaction mixture contained 200 µL sodium malonate (pH 4.5, 250 mM), 50 µL 2,6- DMP, 50 µL sample, and 550 µL distilled water. MnP activity was monitored by the oxidation of 2,6- DMP. The reaction mixture contained 200 µL of sodium malonate (pH 4.5, 250 mM), 50 µL of 2,6-DMP (20 mM), 50 µL of $MnSO_4$-H_2O (20 mM), 550 µL of distilled water and 50 µL of sample. The reaction was initiated by adding 100 µL of H_2O_2 (4 mM). MniP activity was determined in a reaction mixture containing 200 µL sodium malonate (pH 4.5, 250 mM), 50 µL 2,6-DMP (20 mM), 100 µL EDTA (20 mM), 500 µL distilled water and 50 µL sample. The reaction was initiated by adding 100 µL of H_2O_2 (4 mM). MnP and MniP were corrected for laccase activity [35]. The unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of oxidized product per minute, expressed as U L^{-1} [12].

An analysis of variance (ANOVA) was performed to determine if the values obtained for enzyme activity of the different pre-treatments were significantly different. In all cases, a significance level (α) of 0.05 was considered. All pre-treatments were carried out in triplicate.

3. Results and Discussion

3.1. Characterization of Olive Mill Solid Waste and Aquaculture Sludge

Table 3 details the results obtained from the OMSW and AS characterization, obtaining 0.84% nitrogen and a C/N ratio of 58 for OMSW. The nitrogen content was within the reported range, which varies from 0.56 to 1%, and the C/N ratio was higher than that reported for OMSW, which varies from 36.56 and 52 [2,21]. The low nitrogen content may limit the enzymatic activity of *A. discolor*, hindering the growth and differential regulation of ligninolytic gene expression [10]. The TSP content was 1.49 g GA per kg^{-1} OMSW, which is lower than 9.33 g of polyphenols per Kg^{-1} of OMSW reported by Al-Mallahi et al. [6]. The lignin content of OMSW reported by Miranda et al. [31] was 31.2%, which is close to the 33.4% lignin content obtained in this study. Additionally, the OMSW contained 61.5% water and 38.5% total solids, which makes it highly viscous, limiting the liquid phase used to recover ligninolytic enzymes. In this sense, Benavides et al. [20] used water to recover ligninolytic enzymes and concluded that it is necessary to use other liquid sources to recover enzymes in the pre-treatment of OMSW with *A. discolor*. Thus, the addition of AS should have high nitrogen content and high water content to generate a liquid phase for recovery of enzyme extracts.

Table 3. Olive mill solid waste and Aquaculture sludge characterization.

Substrate	C/N % *w*/*w*	TS % *w*/*w*	VS % *w*/*w*	TOC % *w*/*w*	COD mg L^{-1}	TN mg Kg^{-1}	pH	TSP g GA Kg^{-1}	Lignin % *w*/*w*
OMSW	48.4/0.84	38.5	34.5	29.2	87,867	282.9	5.10	1.49	33.4
AS	53.4/7.92	0.8	0.7	200 mg L^{-1}	15,491	897.5 mg L^{-1}	5.11	nd	nd

OMSW: Olive mill solid waste; AS: Aquaculture sludge; C/N: carbon/nitrogen ratio; TS: total solids; VS: volatile solids; TOC: total organic carbon; COD: chemical oxygen demand; TN: total nitrogen; TSP: total soluble phenols; nd: not determined.

The nitrogen content of AS was 7.92%, which was higher than the equivalent 4.5% nitrogen content reported by Khiari et al. [29]. The nitrogen content of AS was more than 9.4 times that of OMSW, making AS exhibit good C/N ratio regulation performance in the pre-treatment of OMSW with *A. discolor*. Furthermore, the C/N ratio for AS was quantified at approximately 7. Tortella et al. [23] reported that *A. discolor* shows better performance in limited nitrogen media. However, few previous studies have indicated an optimal C/N ratio for *A. discolor*. Instead, the reports focused only on specifying low nitrogen levels to enhance the enzymatic activity of *A. discolor*, rather than a clear minimum or maximum nitrogen content. Additionally, AS has a high water content (99.2%) and a low total solids content (0.8%). Such conditions allow for the creation of a liquid phase in the OMSW pre-treatment, which would facilitate the recovery of ligninolytic enzymes without adding external water to the process. In addition, OMSW and AS presented a pH on the order of 5.1 units, which provides a suitable environment for the enzymatic activity produced with *A. discolor*, as reported by Bustamante et al. [35], who obtained the highest enzymatic activity at pH 5.5.

3.2. Assessment of Aquaculture Sludge Effect

3.2.1. Assessment of Total Soluble Phenol and Lignin Removal

Figure 1a shows the TSP concentrations in the solid and liquid phase of each pretreatment at days 0 and 30. The solid phase experienced a decreasing removal efficiency of TSP as the C/N ratio decreased, showing a TSP reduction of 81, 68, 62, and 27% for 58, 45, 32 and 19 C/N ratio, respectively. The same effect occurred in the liquid phase of the pretreatments, where removals of 80, 56, 43, and 35% of TSP were recorded for 58, 45, 32 and 19 C/N ratio, respectively. Figure 1b shows the lignin removal of the two best TSP removals in the solid phase (C/N 58 and 45), showing a 25% reduction for the pre-treatment without AS addition (C/N 58) and a 66% reduction for the pre-treatment with AS addition (C/N 45). These results indicate that the addition of AS as a co-substrate to the

pre-treatment of OMSW with *A. discolor* could improve lignin removal, but not phenol reduction. However, the decrease in TSP removal efficiency would be explained by the high concentration of phenols in the medium, a product of lignin depolymerization that can produce phenolic derivatives [36]. Therefore, in future work, we would recommend studying the evolution of the phenols profile during the pre-treatment, in order to understand the effect of lignin degradation on TSP removal.

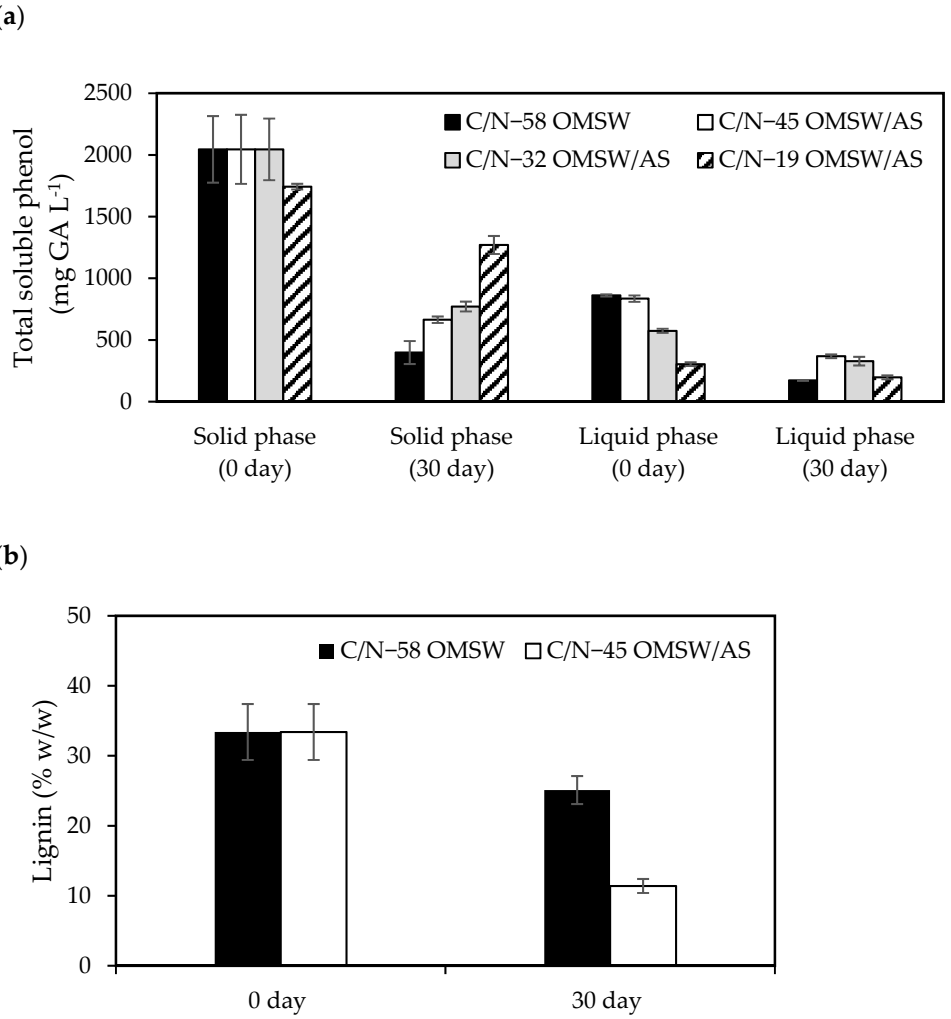


Figure 1. Total soluble phenols in the solid and liquid phase (**a**) of the pre-treatments at initial time and 30 days and lignin removal (**b**) at the same times.

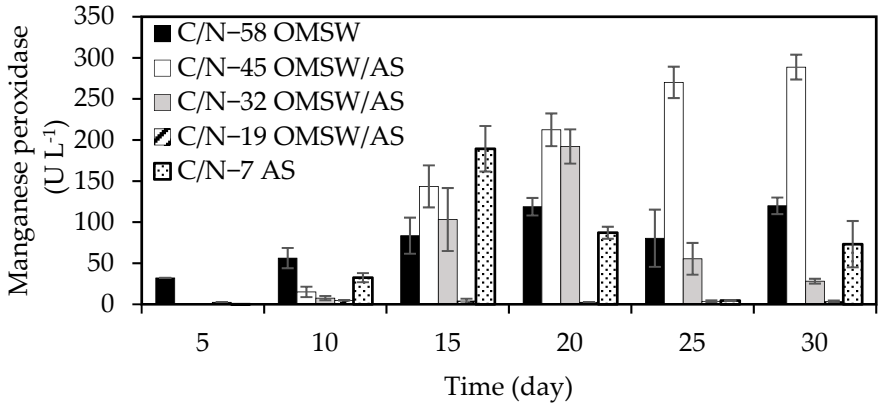
The results obtained from TSP removal are close to those reported by Benavides et al. [20], who with *A. discolor* achieved 90% TSP removal in OMSW. Other WRF species such as *Coriolopsis rigida*, *Pycnoporus cinnabarinus*, *Pleurotus citrinopileatus*, *Irpex lacteus*, *Phanerochaete chrysosporium*, *Lentinellus castoreus*, *Phlebia radiata* and *Hericium erinaceus* have reported removals between 47 to 95% TSP in solid and liquid olive wastes [10,14,18,22,37]. Therefore, pre-treatment with *A. discolor* is viable and registers good results in TSP and lignin removal.

3.2.2. Enzyme Activity Assessment

Enzymatic activities of MnP (Figure 2a) and MniP (Figure 2b) were higher than those for Lac and LiP among all the pre-treatment scenarios (Figure S1). LiP activity in the pre-treatments did not exceed 3.3 U L^{-1}. The low LiP activity may be due to the high production of MnP, since it has been reported that *A. discolor* is characterized by

high MnP production [12] and the presence of MnP can reduce the oxygen stress of the cells and inhibit LiP production [19]. Moreover, Lac activity did not exceed 10 U L^{-1} in all pre-treatments, which is consistent with reports in the literature that Lac activity of *A. discolor* can vary between 3.9 to 36.8 U L^{-1} [20,23,35]. Figure 2a,b shows that MnP and MniP activity with *A. discolor* was strongly influenced by the C/N ratio as a C/N ratio of 32 reached a maximum production of 192 and 17 U L^{-1} for MnP and MniP, respectively, and a C/N ratio of 19 reached a maximum production of just 5 U L^{-1} and undetected for MnP and MniP, respectively. The highest MnP and MniP activity was measured at a C/N ratio of 45, reaching a maximum activity in 30 and 15 days of 289 and 75 U L^{-1}, respectively. The C/N ratio of 58, without AS addition, needed 30 days to reach a MnP and MniP activity of 120 and 21 U L^{-1}, respectively. These results indicate that the adjustment of the C/N ratio, adding AS as co-substrate, is a key factor for improving the enzymatic activity of *A. discolor* and, at the same time, improving the phenolic compound removal. In particular, when the C/N ratio was 45, the production of MnP and MniP improved by 141% and 250% compared with the control, respectively. Reports have indicated that MnP exhibits a higher redox potential than laccase [19]. Therefore, the increased MnP activity could be explained by higher lignin removal at the same C/N ratio of 45 (Figure 1b).

(**a**)



(**b**)

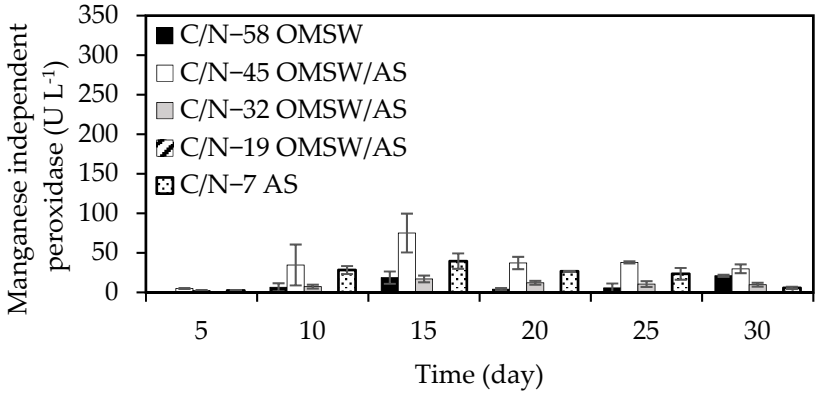


Figure 2. Time evolution of enzyme activity; manganese peroxidase (**a**) and manganese independent peroxidase (**b**), in the pre-treatment of OMSW and AS with *A. discolor* at different C/N ratios.

Thus, despite the low production of LiP and Lac, the production of MnP and MniP with A. discolor was sufficient to degrade a high percentage of phenolic compounds and lignin from OMSW (Figure 1a,b). On the other hand, the high production of MnP and MniP allows the recovery of these enzymes as value-added products for other biotechnological applications, such as enzyme immobilization in nanocomposites [15,16], lignin removal from other lignocellulosic residues [1,19,38], or bioremediation [12,23,39]. In addition, *A. discolor* is a native species isolated from southern Chile [23]. These characteristics would allow the development of local OMSW pre-treatments and enzyme recovery without introducing foreign species [20].

The TS concentration can also impact the time when the MnP achieves maximum. For the TS concentrations of 8 (C/N-7 AS), 32 (C/N-19 OMSW/AS), 69 (C/N-58 OMSW), 75 (C/N-32 OMSW/AS), and 171 g L^{-1} (C/N-45 OMSW/AS), the MnP activity peak times were 15, 10, 20, 20, 30 days, respectively (Figure 2a). Within this study scope, MnP activity required more time to reach maximum as the TS concentration increased. There is a need to balance the pre-treatment performance and required time in a future study.

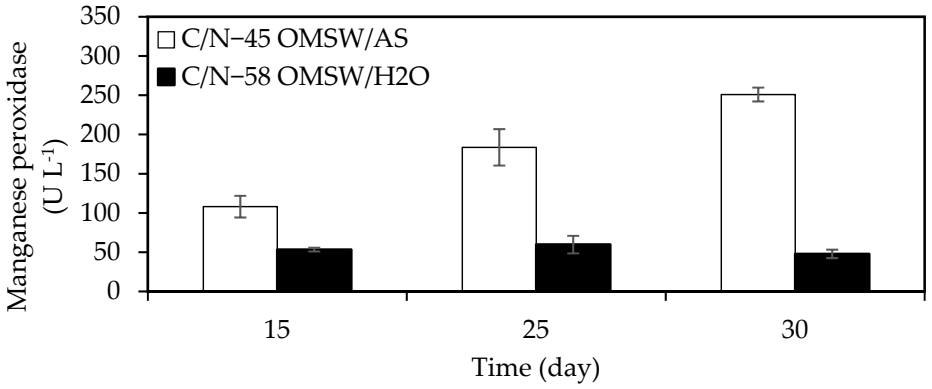
These results indicate that the adjustment of the C/N ratio by adding AS as co-substrate is a key factor in improving the enzymatic activity of *A. discolor* and at the same time improving the phenolic compound removal (Table S1).

3.2.3. Assessment of MnP Activity at Same TS Concentration

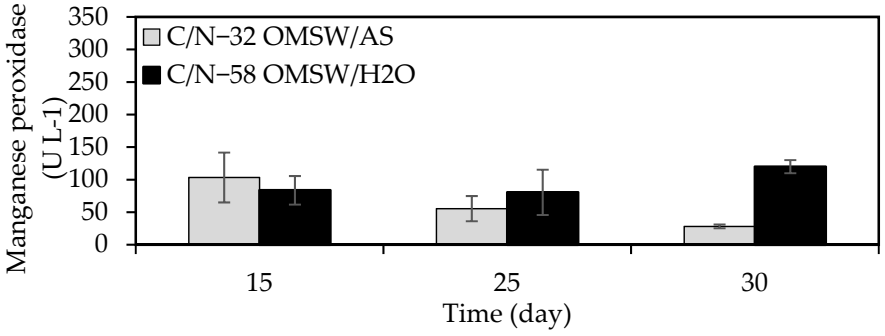
Figure 3 shows the AS supplementation effect on OMSW pre-treatment with *A. discolor*, particularly on MnP activity for three TS concentrations of 171 (Figure 3a), 75 (Figure 3b) and 32 g L^{-1} (Figure 3c). It has been reported that increasing TS concentration in WRF-pretreated OMSW could cause enhancement of MnP activity [33,34] and is not the effect of C/N ratio. Nevertheless, Figure 3a shows that with a constant TS concentration of 171 g L^{-1} and varying the C/N ratio with the addition of AS, it is possible to obtain a maximum MnP activity in *A. discolor* of 250 U L^{-1} using a C/N ratio of 45, with AS addition, and only 60 U L^{-1} using a C/N ratio of 58, without AS addition. In contrast, decreasing the TS concentration to 71 g L^{-1}, the MnP activity using a C/N ratio of 58, without AS addition, is similar to a C/N ratio of 32, with AS addition, reaching 100 U L^{-1} and 120 U L^{-1}, respectively (Figure 3b). Figure 3c shows 230 U L^{-1} of MnP activity using a TS concentration of 32 g L^{-1} at C/N of 19, with AS addition, while without AS addition the MnP activity was only 9 U L^{-1}. These results indicate a considerable decrease in MnP activity with respect to the pre-treatments using 171 g L^{-1} with AS addition. Demonstrating that the addition of AS in the pre-treatment of OMSW with *A. discolor*, using a constant TS concentration of 171 g L^{-1} and a C/N ratio of 45 or a TS concentration of 32 g L^{-1} at C/N 19, it is possible to obtain similar MnP activities. The use of AS as co-substrate with adequate C/N ratio and TS concentration has a positive effect on MnP activity with *A. discolor*. To optimize OMSW pre-treatment with *A. discolor* the interaction of C/N ratio and TS concentration should be evaluated.

It is worth noting that most studies have been conducted primarily on the liquid waste from olive oil production, or olive mill wastewater (OMWW) [10,14,21,40], and rarely on the olive mill solid waste (OMSW) [18,33]. In this regard, Lourenço et al. [33] reached a maximum MnP activity of 139 U L^{-1} for *Trametes versicolor* in OMSW, which is half of that obtained in this study, while Zerva et al. [10] reached an MnP activity of 304 U L^{-1} for *Pleurotus citrinopileatus* LGAM 28,684 in OMWW with external nitrogen added, which is close to the result of this study. Therefore, the results obtained for *A. discolor* in this study are comparable with those for the application of liquid OMWW and superior to those for the application of solid OMSW. The addition of AS as a co-substrate could be a viable alternative to improve the enzymatic activity of *A. discolor* in the OMSW pre-treatment, resulting in an integrated biorefinery process for both wastes.

(**a**)



(**b**)



(**c**)

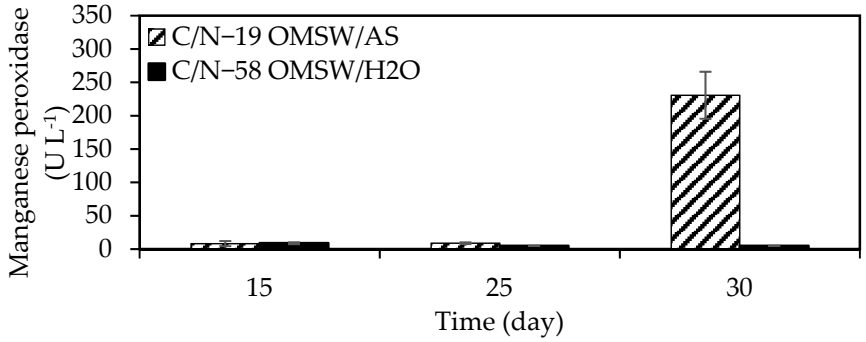


Figure 3. Comparison of manganese peroxidase activity at three control points (15, 25 and 30 days) for the OMSW/AS and OMSW/water mixtures at same concentrations; 171 (**a**), 75 (**b**) and 32 g TS L^{-1} (**c**).

Finally, the results of this study show that the adjustment of the C/N ratio and the concentration of TS are relevant parameters to improve the enzymatic activity of *A. discolor*. The enhanced enzymatic activity in this study yielded enzymes with added value such as MnP and also improved lignin removal from OMSW, evidencing that the application of AS as an external source of nitrogen has a positive effect. This research presents an opportunity to improve the sustainable development of olive oil production, incorporating another type

of waste into the OMSW management, with the possibility of obtaining value-added value products during the pre-treatment (Table S2).

4. Conclusions

Using AS as co-substrate to adjust the C/N ratio and TS concentration in the pre-treatment of OMSW with *A. discolor* had a positive effect on the improvement of MnP and MniP activity by 141 and 250%, respectively, compared to the control. The enhanced enzymatic activity improved lignin removal by up to 66%. In a biorefinery approach, the present research shows that, additional to the potential to produce both biogas and fertilizers from OMSW, the pre-treatment could also allow the recovery of MnP-enriched enzymatic extracts. This value-added product can be used in a variety of biotechnological applications. Despite the results, there is a need to understand the synergic and individual effects of the enzymes in biocatalytic processes, as well as to evaluate new alternatives to avoid sterilization, and thus make the biorefining process more efficient.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030724/s1, Figure S1: Enzymatic activities of Lac (a) and LiP (b) among all the pre-treatment; Table S1: Enzymatic Activity; Table S2: MnP activity.

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References

1. Bentil, J.A. Biocatalytic Potential of Basidiomycetes: Relevance, Challenges and Research Interventions in Industrial Processes. *Sci. Afr.* **2021**, *11*, e00717. [CrossRef]
2. Elalami, D.; Monlau, F.; Carrere, H.; Abdelouahdi, K.; Oukarroum, A.; Zeroual, Y.; Barakat, A. Effect of Coupling Alkaline Pretreatment and Sewage Sludge Co-Digestion on Methane Production and Fertilizer Potential of Digestate. *Sci. Total Environ.* **2020**, *743*, 140670. [CrossRef] [PubMed]
3. Espadas-Aldana, G.; Vialle, C.; Belaud, J.P.; Vaca-Garcia, C.; Sablayrolles, C. Analysis and Trends for Life Cycle Assessment of Olive Oil Production. *Sustain. Prod. Consum.* **2019**, *19*, 216–230. [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. Messineo, A.; Maniscalco, M.P.; Volpe, R. Biomethane Recovery from Olive Mill Residues through Anaerobic Digestion: A Review of the State of the Art Technology. *Sci. Total Environ.* **2020**, *703*, 135508. [CrossRef]
6. Al-Mallahi, J.; Furuichi, T.; Ishii, K. Appropriate Conditions for Applying NaOH-Pretreated Two-Phase Olive Milling Waste for Codigestion with Food Waste to Enhance Biogas Production. *Waste Manag.* **2016**, *48*, 430–439. [CrossRef]
7. Negro, M.J.; Manzanares, P.; Ruiz, E.; Castro, E.; Ballesteros, M. *The Biorefinery Concept for the Industrial Valorization of Residues from Olive Oil Industry*; Elsevier Inc.: Amsterdam, The Netherlands, 2017; ISBN 9780128053140.
8. Serrano, A.; Fermoso, F.G.; Rodríguez-Gutierrez, G.; Fernandez-Bolaños, J.; Borja, R. Biomethanization of Olive Mill Solid Waste after Phenols Recovery through Low-Temperature Thermal Pre-Treatment. *Waste Manag.* **2017**, *61*, 229–235. [CrossRef]

9. Caroca, E.; Serrano, A.; Borja, R.; Jiménez, A.; Carvajal, A.; Braga, A.F.M.; Rodriguez-Gutierrez, G.; Fermoso, F.G. Influence of Phenols and Furans Released during Thermal Pretreatment of Olive Mill Solid Waste on Its Anaerobic Digestion. *Waste Manag.* **2021**, *120*, 202–208. [CrossRef]
10. Zerva, A.; Zervakis, G.I.; Christakopoulos, P.; Topakas, E. Degradation of Olive Mill Wastewater by the Induced Extracellular Ligninolytic Enzymes of Two Wood-Rot Fungi. *J. Environ. Manag.* **2017**, *203*, 791–798. [CrossRef]
11. Behera, S.; Arora, R.; Nandhagopal, N.; Kumar, S. Importance of Chemical Pretreatment for Bioconversion of Lignocellulosic Biomass. *Renew. Sustain. Energy Rev.* **2014**, *36*, 91–106. [CrossRef]
12. 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]
13. Aydin, S. Enhanced Biodegradation of Antibiotic Combinations via the Sequential Treatment of the Sludge Resulting from Pharmaceutical Wastewater Treatment Using White-Rot Fungi Trametes Versicolor and Bjerkandera Adusta. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6491–6499. [CrossRef] [PubMed]
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] [PubMed]
15. Siddeeg, S.M.; Tahoon, M.A.; Mnif, W.; Ben Rebah, F. Iron Oxide/Chitosan Magnetic Nanocomposite Immobilized Manganese Peroxidase For. *J. Clean. Prod.* **2020**, *243*, 118634.
16. Tortella, G.R.; Diez, M.C.; Durán, N. Fungal Diversity and Use in Decomposition of Environmental Pollutants. *Crit. Rev. Microbiol.* **2005**, *31*, 197–212. [CrossRef]
17. Acevedo, F.; Pizzul, L.; Castillo, M.D.P.; Rubilar, O.; Lienqueo, M.E.; Tortella, G.; Diez, M.C. A Practical Culture Technique for Enhanced Production of Manganese Peroxidase by Anthracophyllum Discolor Sp4. Brazilian Arch. *Biol. Technol.* **2011**, *54*, 1175–1186. [CrossRef]
18. Aranda, E.; Sampedro, I.; Ocampo, J.A.; García-Romera, I. Phenolic Removal of Olive-Mill Dry Residues by Laccase Activity of White-Rot Fungi and Its Impact on Tomato Plant Growth. *Int. Biodeterior. Biodegrad.* **2006**, *58*, 176–179. [CrossRef]
19. Datta, R.; Kelkar, A.; Baraniya, D.; Molaei, A.; Moulick, A. Enzymatic Degradation of Lignin in Soil: A Review Sustainability Enzymatic Degradation of Lignin in Soil: A Review. *Sustainability* **2017**, *9*, 1163. [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*, 1587. [CrossRef]
21. Kaouachi, A.; Amane, M.; Jaafari, S. El Biovalorisation of Two-Phase Olive Mill Waste by Composting Process. *Int. J. Sci. Res. ISSN* **2017**, 2319–7064. [CrossRef]
22. Sampedro, I.; Marinari, S.; D'Annibale, A.; Grego, S.; Ocampo, J.A.; García-Romera, I. Organic Matter Evolution and Partial Detoxification in Two-Phase Olive Mill Waste Colonized by White-Rot Fungi. *Int. Biodeterior. Biodegrad.* **2007**, *60*, 116–125. [CrossRef]
23. 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]
24. Levin, L.; Melignani, E.; Ramos, A.M. Effect of Nitrogen Sources and Vitamins on Ligninolytic Enzyme Production by Some White-Rot Fungi. Dye Decolorization by Selected Culture Filtrates. *Bioresour. Technol.* **2010**, *101*, 4554–4563. [CrossRef] [PubMed]
25. Kaal, E.E.J.; Field, J.A.; Joyce, T.W. Increasing Ligninolytic Enzyme Activities in Several White-Rot Basidiomycetes by Nitrogen-Sufficient Media. *Bioresour. Technol.* **1995**, *53*, 133–139. [CrossRef]
26. Jasmin, M.Y.; Syukri, F.; Kamarudin, M.S.; Karim, M. Potential of Bioremediation in Treating Aquaculture Sludge: Review Article. *Aquaculture* **2020**, *519*, 734905. [CrossRef]
27. Wu, Y.; Song, K. Process Performance of Anaerobic Co-Digestion of Waste Activated Sludge and Aquaculture Sludge. *Aquac. Eng.* **2020**, *90*, 102090. [CrossRef]
28. Yogev, U.; Gross, A. Reducing Environmental Impact of Recirculating Aquaculture Systems by Introducing a Novel Microaerophilic Assimilation Reactor: Modeling and Proof of Concept. *J. Clean. Prod.* **2019**, *226*, 1042–1050. [CrossRef]
29. Khiari, Z.; Kaluthota, S.; Savidov, N. Aerobic Bioconversion of Aquaculture Solid Waste into Liquid Fertilizer: Effects of Bioprocess Parameters on Kinetics of Nitrogen Mineralization. *Aquaculture* **2019**, *500*, 492–499. [CrossRef]
30. Federation, W.E.; Association, A. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2005; p. 21.
31. Miranda, I.; Simões, R.; Medeiros, B.; Nampoothiri, K.M.; Sukumaran, R.K.; Rajan, D.; Pereira, H.; Ferreira-Dias, S. Valorization of Lignocellulosic Residues from the Olive Oil Industry by Production of Lignin, Glucose and Functional Sugars. *Bioresour. Technol.* **2019**, *292*, 121936. [CrossRef]
32. Martínez-Patiño, J.C.; Lu-Chau, T.A.; Gullón, B.; Ruiz, E.; Romero, I.; Castro, E.; Lema, J.M. Application of a Combined Fungal and Diluted Acid Pretreatment on Olive Tree Biomass. *Ind. Crops Prod.* **2018**, *121*, 10–17. [CrossRef]
33. Lourenço, L.A.; Debiasi, M.; Magina, A.; Benathar, L.; Tavares, B.; Maria, S.; Guelli, A.; De Souza, U.; García, M. Biosurfactant Production by Trametes Versicolor Grown on Two-Phase Olive Mill Waste in Solid State Fermentation. *Environ. Technol.* **2017**, *39*, 3066–3076. [CrossRef]

34. Schalchli, H.; Hormazábal, E.; Rubilar, O.; Briceño, G.; Mutis, A.; Zocolo, G.J.; Diez, M.C. Production of Ligninolytic Enzymes and Some Diffusible Antifungal Compounds by White-rot Fungi Using Potato Solid Wastes as the Sole Nutrient Source. *J. Appl. Microbiol.* **2017**, *123*, 886–895. [CrossRef] [PubMed]
35. Bustamante, M.; González, M.E.; Cartes, A.; Diez, M.C. Effect of Soya Lecithin on the Enzymatic System of the White-Rot Fungi Anthracophyllum Discolor. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 189–197. [CrossRef] [PubMed]
36. Chen, C.; Jin, D.; Ouyang, X.; Zhao, L.; Qiu, X.; Wang, F. Effect of Structural Characteristics on the Depolymerization of Lignin into Phenolic Monomers. *Fuel* **2018**, *223*, 366–372. [CrossRef]
37. Koutrotsios, G.; Larou, E.; Mountzouris, K.C.; Zervakis, G.I. Detoxification of Olive Mill Wastewater and Bioconversion of Olive Crop Residues into High-Value-Added Biomass by the Choice Edible Mushroom Hericium Erinaceus. *Appl. Biochem. Biotechnol.* **2016**, *180*, 195–209. [CrossRef] [PubMed]
38. Kumar, A.; Chandra, R. Ligninolytic Enzymes and Its Mechanisms for Degradation of Lignocellulosic Waste in Environment. *Heliyon* **2020**, *6*, e03170. [CrossRef]
39. Acevedo, F.; Pizzul, L.; Castillo, M.D.P.; Cuevas, R.; Diez, M.C. Degradation of Polycyclic Aromatic Hydrocarbons by the Chilean White-Rot Fungus Anthracophyllum Discolor. *J. Hazard. Mater.* **2011**, *185*, 212–219. [CrossRef]
40. Fenice, M.; Federici, F.; Annibale, A.D. Submerged and Solid-State Production of Laccase and Mn-Peroxidase by *Panus tigrinus* on Olive Mill Wastewater-Based Media. *J. Biotechnol.* **2003**, *100*, 77–85. [CrossRef] [PubMed]