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Article

Toward the Fungi Whole Cell as a Suitable Strategy for Delignification and Phenolic Removal of Olive Mill Solid Waste

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Abstract: White-rot fungi (WRF) have specific enzymes to degrade lignocellulosic and phenolic compounds. Therefore, their direct application could be an alternative to biodegrade complex lignocellulosic biomass such as olive mill solid waste (OMSW). The aim of this study was to evaluate the capacity of *A. discolor* and *S. hirsutum* to grow in OMSW as the sole substrate under static conditions and evaluate the phenolic removal compounds and lignin degradation. The lignolytic enzyme activity was determined, as was the phenolic compound removal. At the same time, lignin degradation and structural changes were evaluated by confocal laser scanning microscopy (CLSM) and scanning electron microscope (SEM), respectively. Both strains were able to grow using OMSW as the sole substrate without adding other nutrients, oxygen and/or agitation. The higher ligninolytic enzyme activity was found at day 8, and the highest phenolic removal (more than 80% with both strains) was reached after 24 days of incubation. The CLSM analysis confirmed lignin degradation through the drop in lignin fluorescence from 3967 for untreated OMSW to 235 and 221 RFU after 24 days of treatment by *A. discolor* and *S. hirsutum* respectively. The results indicate that both WRF could be suitable candidates to design an in-situ pretreatment step of OMSW, as long as in future research the WRFs have the same performance in non-sterile conditions.

Keywords: olive mill solid waste (OMSW); white-rot fungi; laccase; manganese-independent peroxidase; manganese peroxidase; *Anthracophyllum discolor; Stereum hirsutum; whole cell*

1. Introduction

Every year, close to 1000 Gt of lignocellulosic biomass is generated worldwide, including wheat straw, sugarcane bagasse, corn stalks, and others [1, 2]. This huge volume of biomass can be used as suitable feedstocks in biorefineries to obtain added-value products derived from the polysaccharide fraction, including carbohydrate-enriched ruminant feed, chemical compounds, and biofuels [3, 4, 5, 6]. However, the molecular structure of lignin, as well as its strong association with cellulose and hemicellulose, strongly limit the acquisition of these added-value products [7, 8]. To resolve this challenge, enzymatic pretreatments have been proposed to enhance the use of lignocellulosic biomass as feedstock in subsequent bioprocesses [9] (Bilal and Iqbal, 2020). Some authors have reported the direct use of ligninolytic enzymes for the reduction of the lignin content in different

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lignocellulosic wastes [10, 11, 12]. 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 [13, 14, 15]. WRF can produce extracellular oxidative enzymes such as laccase (Lac), manganese peroxidase (MnP) and manganese-independent peroxidase (MniP), which have been reported to be involved in the degradation of lignocellulosic fibers [16, 17]. 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 [18, 19, 15].

Olive mill solid waste (OMSW) is a solid by-product generated during olive oil extraction, the incorrect management of which has several deleterious environmental effects like 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 [20,21]. OMSW is composed of olive husk, olive pulp and olive vegetation water, resulting in a substrate rich in lignocellulosic fibers and polyphenols [22,23]. Polyphenols in OMSW create a high antimicrobial activity that can hamper the valorization of this biomass through microbe-mediated processes [24]. 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* [17] and *Phlebia* sp. [25], respectively. More recently, *Pleurotus citrinopileatus* and *Irpex lacteus* appeared as potent degraders of olive mill wastewater, while simultaneously producing biotechnologically relevant enzymes [26]. These co-generated enzymes could be used to degrade other lignocellulosic waste or to bioremediate soil contaminated with pentachlorophenol [27, 26, 28]. Therefore, the application of WRF to OMSW might not only result in the phenolic detoxification of the biomass, but also in the acquisition of valuable ligninolytic enzymes. Moreover, WRF could be added directly to the lignocellulosic biomass using a whole cell concept. Therefore, the number of steps in this process is minimal and does not require any additional feedstock to produce fungi. In particular, *Anthracophyllum discolor* and *Stereum hirsutum* are WRF that have been reported to generate lignocellulosic enzymes such as MnP and LiP [29]. Therefore, both *A. discolor* and *S. hirsutum* could be suitable strains for the pretreatment of lignocellulosic waste like OMSW using the whole cell concept. However, to the best of our knowledge, the use of *A. discolor* and *S. hirsutum* culturedin OMSW as the sole substrate under static conditions has not been previously evaluated. Therefore, the aim of this study was to evaluate the whole cell capacity of *A. discolor* and *S. hirsutum* to grow in OMSW as the sole substrate under static conditions and evaluate remotion of the total phenols and lignin degradation.

2. Materials and Methods

2.1. Microorganisms

The fungal strains used were *A. discolor* Sp4 and *S. hirsutum*, both isolated from decayed wood in the rain forest of southern Chile belonging to the Culture Collection of the Environmental Nanobiotechnology Laboratory at the Universidad de La Frontera, Chile. The fungi were stored at 4ºC in Petri dishes with potato dextrose agar (PDA) medium until use. The culture medium was prepared using 39 g of PDA in 1L of distilled water and sterilized in an autoclave for 20 minutes at 121ºC. Then, the strains were activated in PDA medium using one disk of 6mm as the initial inoculum and incubated at 25ºC for 7 days for the assays.

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 characterization parameters included oxygen demand (COD), total solids (TS), volatile solids (VS), pH, total nitrogen (TN), ammonia nitrogen (N-ammonia) carried out using the standard methods [30]. Cu,

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Fe and Mn present in OMSW were measured by EPA method 6020A. NO_2- and NO_3- anions were measured by ion chromatography. 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^{-1} and mg GA $100g^{-1}$. For the determination of total phenol content in solid phases, a previous extraction was carried out using 1g 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 1200G for 10 min. The methanol layer was separated and the extraction repeated four times [31]. The ash, total extractives, lignin, hemicellulose and cellulose contents were measured by the Klason lignin method (TAPPI T 222 om-02) [32].

2.3. Qualitative detection of lignocellulolytic enzymes

Qualitative discoloration tests with Remazol Brilliant Blue R (RBBR) and staining of 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) [33]. The tests were performed in triplicate in Petri dishes, using uninoculated plates as controls. One agar disk (6mm diameter) of active mycelia from a 7-day-old culture in PDA medium was inoculated in Petri dishes and then incubated at 25 °C in darkness. The green coloring 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 scale of discoloration and coloration (Table 1) proposed by Tortella *et al.et al.* (2008) [34] was used in this assay.

Table 1. RBBR discoloration and ABTS coloration scale for lignocellulolytic enzyme detection.

Scale	Halo diameter (mm)
0	No effect
+	1-22
++	22-45
+++	45-67
++++	67-90

2.4. Mycelial expansion assays

Petri dishes were inoculated with one agar disk (6mm diameter) of active mycelia from a 7-day-old culture in PDA medium and from the refrigerated OMSW sample a subsample of 1000 g (62% moisture content) was extracted, which was sterilized in an autoclave for 20 minutes at 121ºC, then distributed in Petri dishes according to the treatments (Table 2) in a proportion of 20g. The Petri dishes were incubated at 25ºC for 12 days or until the dish was covered completely by the fungi. The experiment was carried out in triplicate under a completely random design with a 3x2 factorial arrangement, where factor A corresponded to culture medium and factor B to fungal strains. The mycelial growth was evaluated every 24 hours for 12 days by photographic registration of the Petri dishes, so that the current limit of the mycelia expansion halo could be visualized. The public domain program ImageJ v.1.52a was used for image processing.

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Table 2. Treatment description for mycelial expansion assays.

Treatment	Description
T1	*A. discolor* on PDA
T2	*A. discolor* in OMSW
T3	*A. discolor* on PDA and OMSW
T4	*S. hirsutum* on PDA
T5	*S. hirsutum* in OMSW
T6	*S. hirsutum* on PDA and OMSW

PDA, potato dextrose agar; OMSW, olive mill solid waste,

2.5. Enzymatic extract production using OMSW

For this assay, 12 samples of 30 g of OMSW (49.7% moisture content) were extracted and deposited in Erlenmeyer flasks (500 mL) according to treatments defined for this assay (Table 3), which were sterilized in an autoclave for 20 minutes at 121ºC. The OMSW final weight on a wet basis in the flasks after sterilization was 28.8 g. Then, 100 mL of modified Kirk's medium (MKM) were added to the flasks. Finally, they were inoculated with five disks (6 mm diameter) of active mycelium from 7-day-old cultures plates. The flasks were incubated at 25ºC under static conditions for 24 days. Control flasks without OMSW were evaluated at the same time.

Table 3. Treatment description for enzymatic extract production using OMSW.

Treatment	Strain	Culture media
1	*S. hirsutum*	100 mL MKM
2	*A. discolor*	100 mL MKM
3	*S. hirsutum*	100 mL MKM +30g OMSW
4	*A. discolor*	100 mL MKM +30g OMSW
5	*S. hirsutum*	100 mL distilled water +30g OMSW
6	*A. discolor*	100 mL distilled water +30g OMSW

OMSW, olive mill solid waste, modified Kirk's medium (MKM)

2.6. Analytical Methods

Extract recovery and enzymatic activity evaluation were performed according to Acevedo *et al.et al.* (2011) and Hermosilla, 2017 [35, 1]. The crude extracts were collected by filtration through Whatman Nº 1 filter paper (pore size 11 µm). Enzymatic activity was periodically monitored every three 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 100µL of 4 mM H_2O_2, and the absorbance of the colored product was measured at 468 nm using an UV-Vis spectrophotometer (Thermo Scientific Evolution™ 60S) at 30ºC and corrected for the Lac activity [36]. One MnP activity unit (U) was defined as the amount of enzyme transforming 1µmol 2,6-DMP per minute at pH 4.5 and 30ºC [36].

2.7. Kinetics of the fungus growth

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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 (eq. 1). The application of the modified Gompertz equation was previously reported for growth of *Fusarium verticillioides* and *Rhizopus stolonifera* by Ochoa-Velasco *et al.* (2018) [37], and is defined as:

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

Where A is the average diameter (mm) at time t (d), A_{max} is the maximum fungal growth (mm) achieved during the stationary phase, 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).

2.8. 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. Particularly, for CLSM the samples were incubated with 3 markers in the dark for 30 minutes at room temperature. The markers used were Calcofluor at 20% v/v for cell wall staining (blue) ʎ emission/excitation 405/450 nm, Safranin O at 1% w/v for lignin staining (green) ʎ emission/excitation 546/ 590 nm and Congo Red 1% w/v for cellulose staining (red) ʎ emission/excitation 633/700 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, 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, samples were outlined using silver paint. The visualization was carried out under the following parameters: 10 KV, WD 10 mm, BSE (chemical contrast) and UVD (topography) detectors in SEM (Hitachi SU3500, Japan).

3. Results and Discussion

3.1. Olive mill solid waste characterization

According to Table 4, OMSW had a lignin, hemicellulose, and cellulose content of 33.4±4%, 45.5±8% and 35.2±3%, respectively. These values are within the ranges reported in the literature for two-phase OMSW [39]. 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% [38, 25]. The determined C/N ratio for the used OMSW was 48.4/0.84 (Table 4). In fact, the much higher concentration of carbon compared to nitrogen is a feature of OMSW which has also been defined as limiting the biological treatment of this biomass, e.g., through anaerobic digestion, with supplementation with a co-substrate being recommended [39, 40].

OMSW presented a pH value around 5.0-5.1, which is within the desirable range for enzyme activity. For example, Bustamante *et al.* (2011) [41] determined that enzyme activity for *A. discolor* in modified Kirk's medium (MKM) at 25°C incubation was enhanced at pH between 5 and 6. On the other hand, some metals from OMSW were determined, giving values for Fe (<13 mg kg^{-1}), Mn (<140 mg kg^{-1}) and Cu (8.48 mg kg^{-1}). The presence of copper and manganese is important due to their role as inducers. Jain *et al.* (2019) [42] 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 [43, 35], showing that expression of MnP in the fungal cultures is dependent on

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Mn. In addition, Papinutti and Forchiassin (2003) [44] indicated that specific activity of MnP 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^{-1}. Compared to other reports, this value is in the low range of total polyphenol content that varies between 1.5 and 12 g L^{-1} [16]. OMSW has an antioxidant capacity of 214.4 mg Trolox L-1 (Table 4). 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 4.

Table 4. Physical-chemical characteristics Olive Mill Solid Waste.

Olive Mill Solid Waste	Value	Unit
α-cellulose	35.2 ± 3	%
Lignin	33.4 ± 4	%
Hemicellulose	45.5 ± 8	%
Elemental analysis (C/N)	48.4/0.84	%
Ash	2.96	%
Total nitrogen	134.2	mg kg^{-1}
Nitrate	< 1.0	mg kg-1
Nitrite	< 0.6	mg kg-1
Total copper	8.5	mg kg-1
Total iron	< 140.0	mg kg-1
Total manganese	< 13.0	mg kg-1
Chemical oxygen demand	87.9	mg O_2 L-1
Total solids	38.5	%
Volatile solids	89.7	% of TS
pH	5.08	-
Total polyphenols	149.0	mg GA 100 g-1
Antioxidant capacity	214.4	mg Trolox L-1
Gallic acid hexoside	0.9	mg 100 g-1
Gallic acid protocatechuic acid hexoside	0.5	mg 100 g-1
Hexoside ferulic acid	0.3	mg 100 g-1
Cautaric acid	1.0	mg 100 g-1
Caftaric acid	0.9	mg 100 g-1
Catechin	1.6	mg 100 g-1
Epicatechin	1.9	mg 100 g-1
Quercetin-3-rutinoside	1.3	mg 100 g-1
Quercetin-3-hexoside	1.7	mg 100 g-1
Quercetin-3-glucuronide	2.0	mg 100 g-1
Kaempferol-3-glucoside	3.5	mg 100 g-1
Kaempferol-3-hexoside	10.2	mg 100 g-1

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), and from 22 mm and 45 mm for *S. hirsutum* (Table 5). However, both strains showed a similar activity for PDA+ABTS, resulting in a coloration diameter ranging from 67 to 90 mm (Table 5). Similar results were published by Tortella *et al.* (2008) [34] for these strains, which indicated that the fungal strains maintained their enzymatic potentials after subcultures since they were isolated. The strains were selected for further assays in OMSW because they produced the highest

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reaction intensity with all enzymatic indicators in the media tested, which indicates that they have enzymatic mechanisms to degrade lignocellulosic components of OMSW.

Table 5. Qualitative detection by RBBR decoloration and ABTS coloration at evaluation day 14.

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

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.

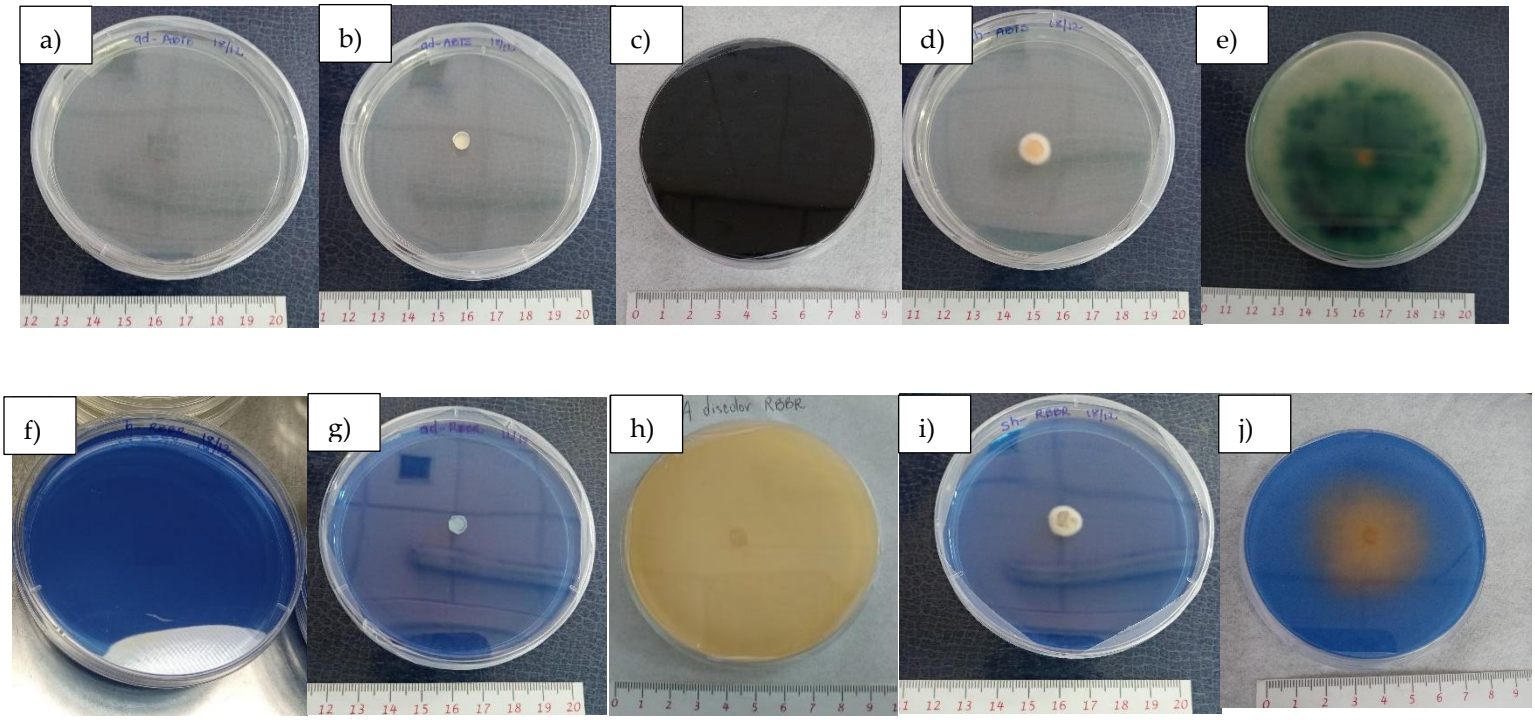

Figure 1. Qualitative detection of lignocellulosic enzymes. (a) control with ABTS and PDA, (b) *A. discolor* in PDA with ABTS day 1, (c) *A. discolor* in PDA with ABTS day 14, (d) *S. hirsutum* in PDA with ABTS day 1, (e) *S. hirsutum* in PDA with ABTS day 14, (f) control with RBBR and PDA, (g) *A. discolor*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

3.3. Mycelial expansion assays

Once the enzymatic activity of the selected WRF strains had been corroborated, the suitability of both *A. discolor* and *S. hirsutum* for degrading OMSW was evaluated. Figure 2 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 6). 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 [45]. In particular, *A. discolor* showed growth from day 6, reaching a maximum mycelial diameter of 20 mm in OMSW,

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while *S. hirsutum* left the latency state on day 2, reaching a maximum mycelial diameter of 90 mm (Figure 2). In detail, Table 6 shows the mycelial growth rate (mm d^{-1}) determined by fitting the modified Gompertz model (Table 6). 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 2b, Table 6). As expected, in both strains the highest mycelial growth rate was achieved using PDA, followed by PDA+OMSW and OMSW (Table 6), probably due to the readily biodegradable compounds provided by the PDA [45]. When the substrate was OMSW, *A. discolor* showed a mycelial growth rate of 2.5 ± 0.5 mm d^{-1}, while *S. hirsutum* reached 9.7 ± 0.7 mm d^{-1}. Although both strains were able to grow in OMSW, the higher mycelial growth rate of *S. hirsutum* respect *A. discolor* indicated a better adaptability of *S. hirsutum* to grow in this substrate.

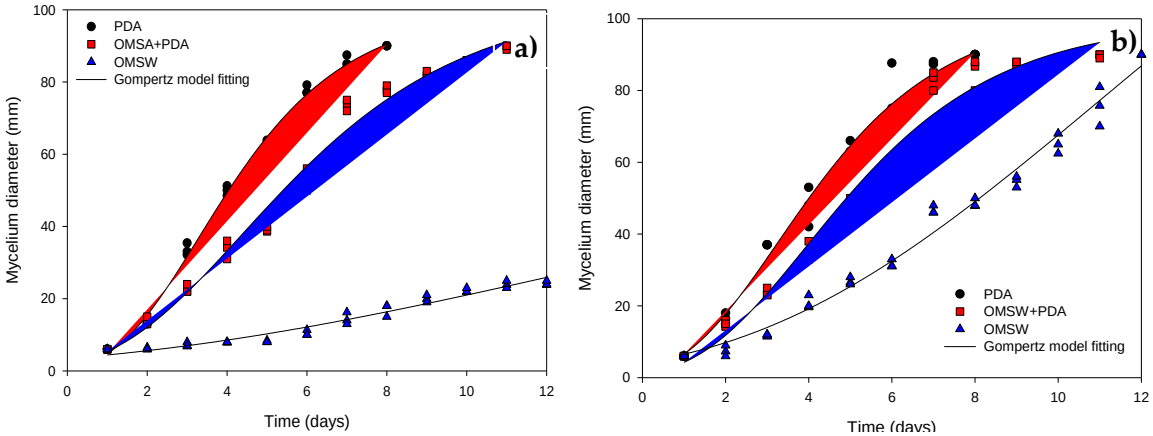

Figure 2. Evolution of fungal growth halos, equivalent diameters. a) *A. discolor*; b) *S. hirsutum.*

Table 6. Kinetics results of the modified Gompertz model applied to the fungi expansion halos studied.

Treatment	Maximum mycelial diameter (mm)	Mycelial growth rate (mm day^{-1})	Lag phase (d)	R^2
A. discolor on PDA	100 ± 2	17.9 ± 0.5	1.2 ± 0.1	0.9968
A. discolor in OMSW	78 ± 39	2.5 ± 0.5	1.6 ± 1.9	0.9585
A. discolor on PDA and OMSW	103 ± 4	12.1 ± 0.5	1.3 ± 0.1	0.9864
S. hirsutum on PDA	102 ± 5	16.8 ± 1	1.0 ± 0.2	0.9838
S. hirsutum in OMSW	212 ± 46	9.7 ± 0.7	3.0 ± 0.6	0.9857
S. hirsutum on PDA and OMSW	100 ± 3	14.3 ± 0.9	1.4 ± 0.2	0.9793

PDA, potato dextrose agar; OMSW, olive mill solid waste

3.4. Enzymatic extract production using OMSW

Figure 3 shows the 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* (Figure 3a)

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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 on day 8 (Figure 3a). By contrast, Shalchli *et al.* (2017) [27] 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 [1]. 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 4), markedly lower than the other reported substrates, e.g., wheat straw contains around 35–45% cellulose, 20–30% hemicellulose, and around 15% lignin [46]. On the other hand, *S. hirsutum* (Figure 3b) showed ligninolytic enzymes 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 3b).

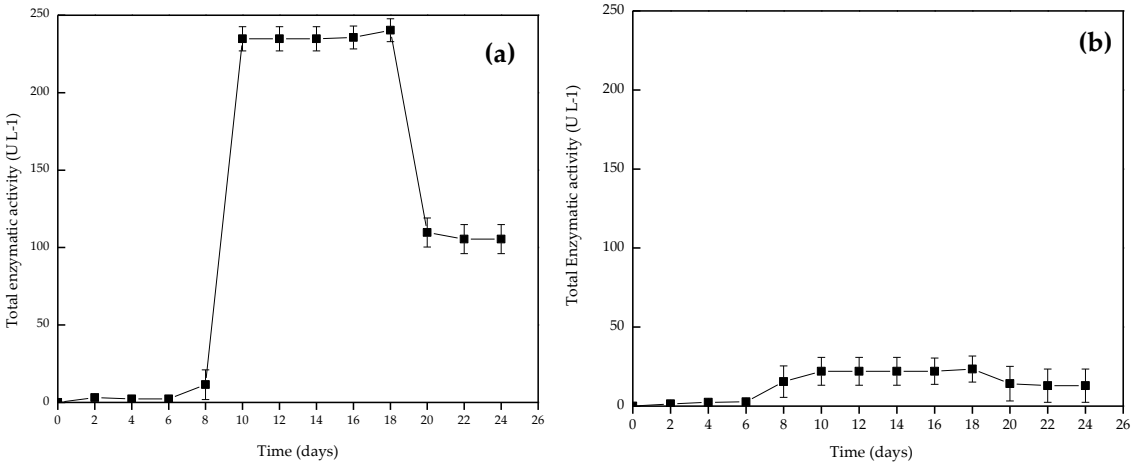

Figure 3. Ligninolytic enzymes activity (represented as the sum of Lac, MnP and MniP), as a function of time using olive mill solid waste (OMSW), OMSW + Kirk's medium and Kirk's medium as the substrate for *A. discolor* (a) and *S. hirsutum* (b).

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-1 for *A. discolor* and 22.1 U L^{-1} for *S. hirsutum* on day 8 (Figure 3). 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 [47, 48]. The most likely reason for increasing their production is the presence of phenols such as ferulic, caffeic, coumaric and chlorogenic acids [49, 50]. Furthermore, the fungus 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 2, Table 6), this condition was the optimal to maximize the enzymatic activity (Figure 3).

Figure 4 shows the individual enzyme activity for Lac, MnP and MniP using *A. discolor* (4a) and *S. hirsutum* (3b) 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, 56.1 and 20.9 U L-1, respectively, for each enzyme, i.e., MniP was produced by *A. discolor* 3 and 8 times more than MnP and Lac, respectively. *S. hirsutum* reported higher Lac productivity over MnP and MniP, reaching 15.5, 5.2 and 1.5 U L-1, respectively. The higher presence of MniP may be related to its high selectivity in the degradation of lignin, and also due to

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the low concentration of manganese in the OMSW (< 13 mg kg-1 OMSW, Table 4), which would have limited the MnP activity in favor of the MniP activity [51, 52].

The increase in the activity of MniP and MnP enzymes was previously reported by Reina *et al.* (2013) [53], who described an increase in peroxidase activity from 0% to 19% in two weeks 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 [53, 54]. Although the Lac activity was detected, its low production may be explained by the C/N ratio of the OMSW, i.e., 60 (Table 4), since some experimental works suggested that substrates with a C/N ratio of less than 16 are the most suitable condition for Lac production [47]. Additionally, substrates with a ratio greater than 40 can strongly limit Lac production by WRF [55, 56].

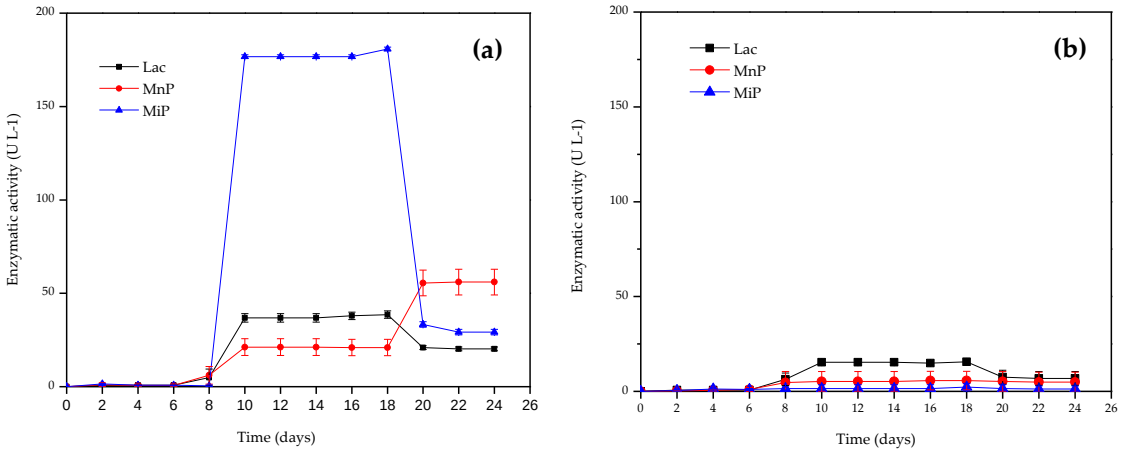

Figure 4. Lac, MnP and MniP activity variation as a function of culture time using OMSW as substrate for both strains studied; *A. discolor* (a) *S. hirsutum* (b).

Although the obtained results are interesting, the Lac and MnP values reported in the present work were low compared to 600 and 163 U L-1 of Lac and MnP, respectively, produced at day 15 for *A. discolor* using potato peel waste reported by Schalchli *et al.* (2017) [27]. That could be explained by the higher lignin content in the potato peel waste (around 20 % on a dry basis) [57] compared to OMSW. However, further research on strategies for enhancing the enzymatic activity would be interesting, such as the addition of supplements for enhancement in production of certain ligninolytic enzymes by the fungal strains [26, 58, 59]. In this context, Mishra *et al.* (2017) [60] increased Lac production in the *C. versicolor* strain from 11.9 to 58.2 g U-1 g by adding $CuSO_4$ using sweet sorghum bagasse as the substrate. Knežević *et al.* (2014) [52] reported that MnP and MniP were strongly stimulated by Zn and Mn supplementation, whereas the addition of 0.5 mM of Fe favored lignin degradation in wheat straw by *Trametes gibbosa*.

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 5). 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 [61]. The phenol removal efficiencies achieved were slightly higher than those reported by Reina *et al.* (2013) [53], who described a final phenol content varying between 20% to 40% compared to the initial concentration after 4 weeks for *Auricularia auricula-judae*, *Bjerkandera adusta*, and *Coprinellus radians* during the solid-state fermentation of olive mill residues. On another hand, the removal efficiency achieved in the present

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study was higher than that obtained through the combination of thermal treatments and a further extraction system previously proposed for OMSW, which depending on the thermal treatment conditions varied from around 45% to 75% [23, 61]. It is worth noting that the present fungal treatment allowed the phenolic detoxification of the OMSW, but not the recovery of the valuable phenolic compounds. However, despite this drawback, the fungal treatment does not have high energy requirements in comparison with the thermal treatments proposed for the recovery of phenols [62]. Therefore, the proposed fungal treatments using *A. discolor* and *S. hirsutum* would be a suitable strategy for the detoxification of OMSW, allowing the subsequent valorization of this biomass without a high energy cost.

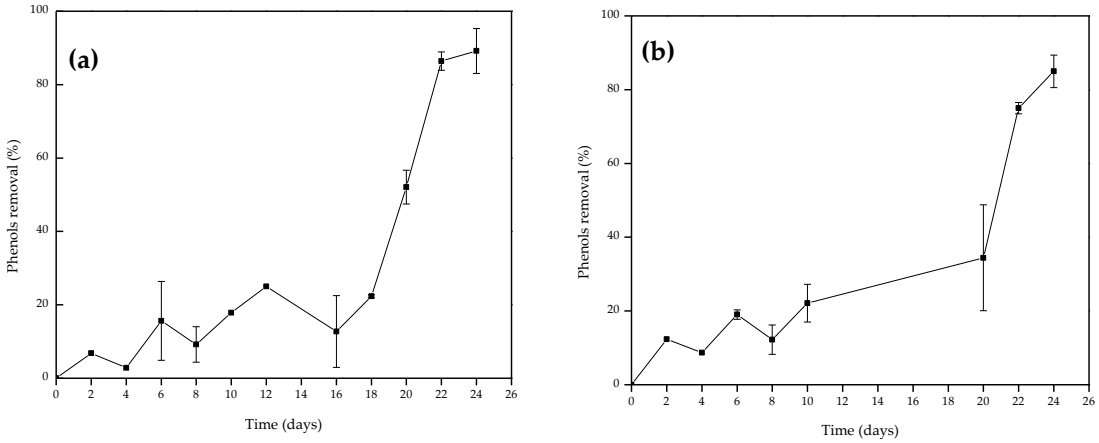

Figure 5. Phenol removal as a function of culture time using OMSW as substrate for both strains studied; *A. discolor* (a) *S. hirsutum* (b).

3.6. Microstructure analysis

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

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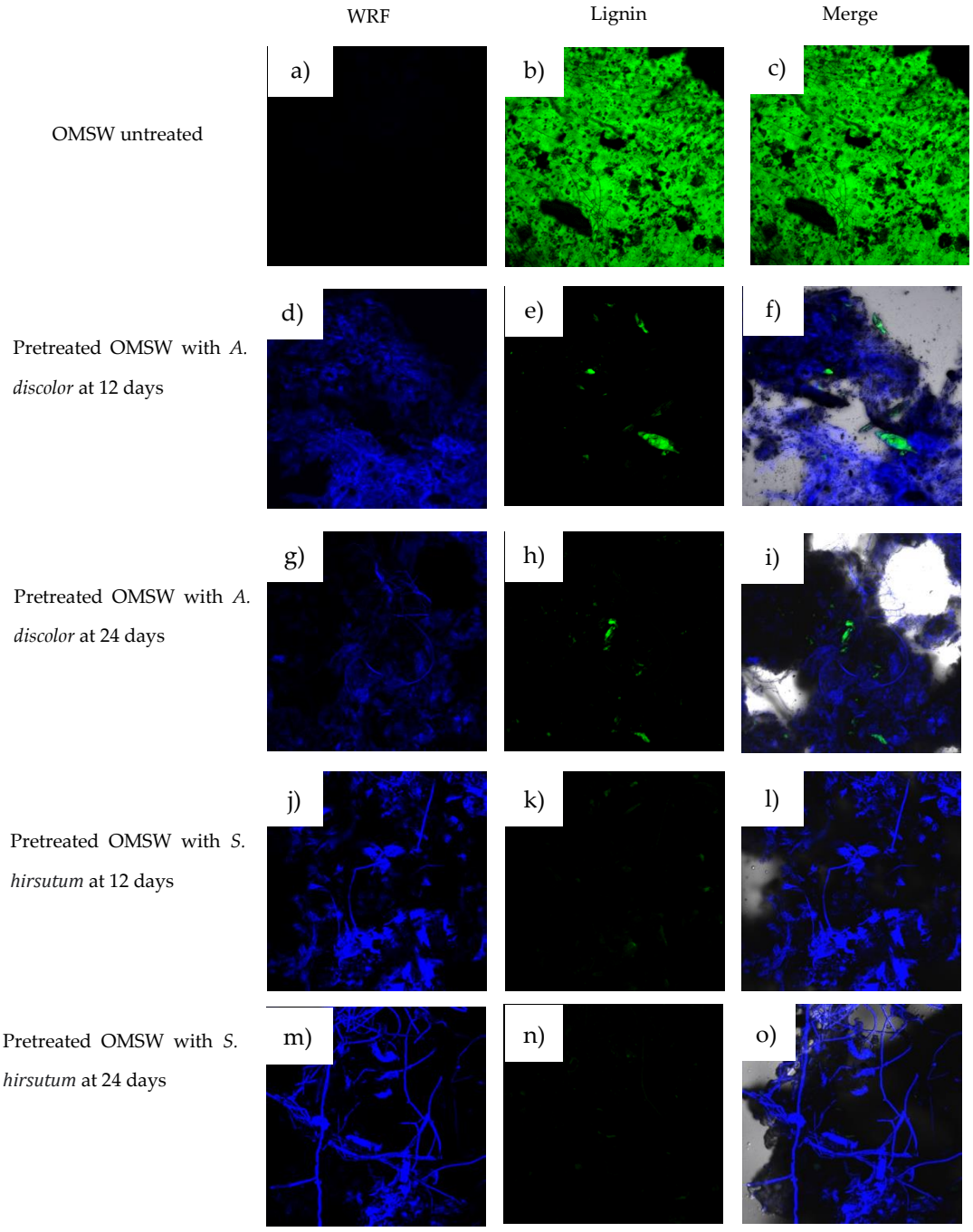

Figure 6. Confocal images of untreated OMSW (a-c); pretreated OMSW with *A. discolor* at 12 (d-f) and 24 (g-i) days; pretreated OMSW with *S. hirsutum* at 12 (j-l) and 24 (m,o) days.

The relative fluorescence units (RFU) obtained from the CLSM analysis are provided in Table 7. The lignin fluorescence decreased from 3967 for untreated OMSW to 235 and 221 RFU after 24 days of treatment using *A. discolor* and *S. hirsutum* respectively. At the same time, the fungi fluorescence increases 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

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Table 7. Relative fluorescence units (RFU) of untreated OMSW and pretreated OMSW at 12 and 24 days using *A. discolor* and *S. hirsutum*.

Treatment	WRF (RFU)	Lignin (RFU)
OMSW untreated	**291**	**3967**
Pretreated OMSW with *A. discolor* at 12 days	2816	1077
Pretreated OMSW with *A. discolor* at 24 days	3548	235
Pretreated OMSW with *S. hirsutum* at 12 days	2091	1140
Pretreated OMSW with *S. hirsutum* at 24 days	3432	221

The morphology of the untreated OMSW and fungal pretreated OMSW at 12 and 24 days of degradation is shown in Figure 7. The untreated OMSW (Figure 7a) shows clearly defined fibers. By contrast, in Figure 7 (b-g), fibers are not distinguished. Particularly in Figure 7b, 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 [64], who reported the formation of holes and crevices on the biomass surfaces in pretreated sweet sorghum bagasse. These results contribute to the understanding of the effects of WRF on degradative abilities and extracellular activities of *A. discolor* and *S. hirsutum* for the pretreatment of OMSW. Moreover, it made it possible to find the potential of this fungi for the pretreatment of OMSW, enhancing its digestibility.

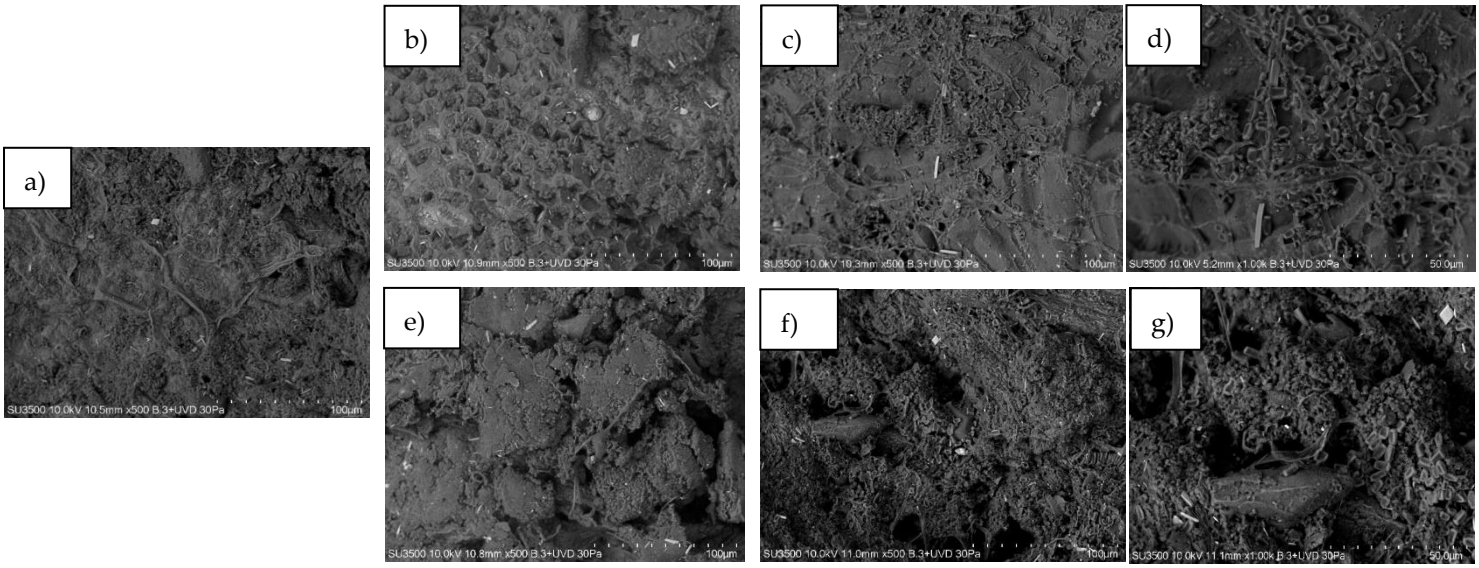

Figure 7. SEM images of (a) untreated OMSW; (b) pretreated OMSW with *A. discolor* at 12 (b) and 24 (c,d) days; pretreated OMSW with *S. hirsutum* at 12 (e) and 24 (f,g) days.

4. Conclusion

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.

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