*Article*

# Biodegradation of Olive Mill Effluent by White-Rot Fungi

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**Abstract:** The liquid fraction from the two-phase extraction process in the olive industry (alperujo), is a waste that contains lignocellulosic organic matter and phenolic compounds, difficult to treat by conventional biological methods. Lignocellulosic enzymes from white-rot fungi can be an interesting solution to break down these recalcitrant compounds and advance the treatment of that waste. In the present work the ability of *Phanerochaete chrysosporium* to degrade the abovementioned liquid waste (AL) was studied. Experiments were carried out at 26 °C within the optimal pH range 4–6 for 10 days and with and without the addition of glucose, measuring the evolution of COD, $BOD_5$, biodegradability index, reducing sugars, total phenolic compounds, and colour. The results obtained in this study revealed the interest of *Phanerochaete chrysosporium* for an economical and eco-friendly treatment of alperujo, achieving COD and colour removals around 60%, and 32% of total phenolic compounds degradation, regardless of glucose addition.

**Keywords:** alperujo; olive mill waste; bioremediation; *Phanerochaete chrysosporium*; fungal treatment

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## 1. Introduction

The Mediterranean region is the main producer of olive oil, concentrating more than 95% of the world's olive trees. Within this area, Spain represents around half of total world manufacturing, considering this industry as one of the most important agri-food sectors for this country [1,2].

Presently, the main industrial process to obtain olive oil is continuous extraction by two-phase or three-phase systems. Depending on the oil extraction system used, considerable amounts of solid and liquid waste as final products are generated. Although the three-phase extraction process mainly generates alpechin and pomace as final residues, the main waste stream from two-phase extraction system is the alperujo (AL) [3]. The pomace is a solid waste composed of the pulp and pits of the olive, commonly employed as fertilizer, biofuel production, or animals feed. The alpechin corresponds with the liquid effluent, composed of water and minerals and characterized by a high organic matter load. This waste stream is considered, together with the AL, a highly polluting residue, for which reuse is not an easy task so its revalorization is still being investigated [4,5]. Due to the global increase of olive oil demand, the excessive amount of waste streams generated throughout the olive oil industry is a growing problem that poses an environmental challenge [6].

In Spain, the two-phase olive oil extraction system is used in approximately 90% of olive mills [4]. The application of this extraction system generates about 800 kg of AL per ton of processed olive, which represents an annual production of around four million tons for the Spanish oil industry [3]. The AL obtained is a semi-solid waste stream composed of vegetable water and olive pomace with high moisture content (60%) that still contains a certain amount of oil [7]. The AL is subjected to a second centrifugation, to obtain a pomace oil. The resulting residue is usually dried in rotary heat dryers at high temperatures and the by-product is subjected to an extraction with hexane to recover more oil, which

requires a large amount of energy and incurs high costs [8,9]. Dried or wet AL can also be used in composting processes. However, due to its low porosity, the addition of bulking agents such as bark chips or cotton gin is necessary [9,10]. In addition, its high content of lignocellulosic compounds and polyphenols, which are toxic to animal cells, plants, insects, and microorganisms, is an important drawback [11]. Discharging these wastes without treatment would cause serious damage to aquatic systems, such as the reduction of soluble oxygen. Furthermore, its strong odour would also cause serious problems for the population living near the discharge area [12,13]. Therefore, the removal of the pollutant compounds of the AL, and therefore favouring of the subsequent biological/physical treatment, is one of the main problems that the olive oil industry must confront.

The literature has been mainly focused on the treatment of olive mill wastes coming from the three-phase extraction system, with the aim of removing the organic and phenolic compounds and improving the biodegradability of the effluent. Regarding biological methods, anaerobic digestion or aerobic activated sludge processes have mainly been applied for the treatment of olive mill wastewater (OMW). However, these methods are not usually applied directly to the effluent due to the presence of recalcitrant molecules, polyphenols, the low nutrient load, and the acidic pH of the waste, which make treatment difficult [14]. Previous studies have reported an improvement in the efficiency of these processes reducing the acidity, adding nutrients such as cobalt, or extracting polyphenols before biodegradation [15]. Due to the high level of antimicrobial compounds present in OMW, the acclimatization of biomass or the use of physical $CaCO_3$ supports has also been required to improve the biodegradation and methanization process [16]. Therefore, the traditional biological methods are not as effective as would be desirable. Physical–chemical methods such as nanofiltration, ultrafiltration, ultrasound, hydrothermal carbonization, and different advances oxidation processes have been reported for the treatment of OMW reducing its chemical oxygen demand (COD) and phenol content [17,18]. However, these techniques have several drawbacks, such as the addition of chemicals, fouling of the membrane, and high pressure and temperature conditions [5]. Therefore, the search for alternative methods that allow the treatment of OMW and AL in an economic and eco-friendly way is crucial.

The use of fungi has been described as a promising alternative over the use of bacteria for OMW treatment due to its ability to grow under adverse conditions and to produce a great variety of extracellular enzymes that make possible the degradation of recalcitrant compounds [19]. In this way, fungi can break down the complex recalcitrant compounds making them more assimilable to be used by themselves or by the bacteria in a subsequent treatment [20,21]. The OMW treatment by fungi has mainly focused of removing COD, phenolic content, and colour, as well as obtaining by-products with biotechnological interest, such as fungal enzymes [22,23]. White-rot fungi can degrade the lignin present in lignocellulosic wastes due to the release of enzymes, mainly lignin peroxidase and manganese peroxidase [24]. These fungi have been investigated for the treatment of recalcitrant compounds and colour degradation of OMW, obtaining good results. For example, Ntougias et al. [25], who studied the capacity of several strains of *Pleurotus* and *Ganoderma* fungi to treat OMW, reported significant removals of COD, TOC, and phenolic compounds, as well as a reduction of the toxicity of the effluent. COD degradations around 50% have been achieved by the fungus *Phanerochaete chrysosporium* immobilized on loofah [26]. Great removals of colour, phenolic compounds and COD have also been obtained when OMW was treated with fungi from genus *Aspergillus* [22,27].

As far as we know, the bioremediation with fungi has been mainly applied to treat OMW or pomace coming from the three-extraction system. However, its application to AL waste, obtained from the two-phase extraction system, has been hardly studied. Therefore, the main objective of this study was to investigate the capability of the white-rot fungus *Phanerochaete chrysosporium* to treat AL waste, to reduce its COD, colour, and phenolic compounds.

## 2. Materials and Methods

### 2.1. Sample Description

The AL used for this work corresponds with the semi-solid effluent generated during olive oil extraction by a two-phase extraction system. The sample was collected from an olive oil factory sited in Sevilla, Spain. For the fungal treatment, the sample was mixed with distilled water in a ratio 1:20 (*p*/*v*). The mix was filtered using a 1.5 mm mesh sieve to remove the rest of the peel and pit of the olives. After that, the effluent was centrifuged for 10 min at 9000× *g* and the supernatant was filtered by a cellulose filter (10–20 µm). This diluted AL was used for the subsequent fungal treatments. The characteristics of diluted AL are shown in Table 1.

**Table 1.** Characteristics of the diluted AL.

| Parameter | Value |
|---|---|
| pH | 4.6 ± 0.01 |
| sCOD (mg $O_2$/L) | 4854 ± 19 |
| sBOD (mg $O_2$/L) | 408 ± 14 |
| Biodegradability Index (B.I.) | 0.080 ± 0.003 |
| Reducing sugars (mg/L) | 578 ± 24 |
| Total phenolic compounds (mg/L) | 134 ± 4 |
| Colour index (C.I.) | 1.60 ± 0.04 |
| Total Suspended Solids (mg/L) | 2475 ± 21 |
| Fixed Suspended Solids (mg/L) | 375 ± 12 |
| Volatile Suspended Solids (mg/L) | 2100 ± 28 |

### 2.2. Fungal Pellet Obtention

The white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 was used. The freeze-dried strain (CECT 2798 from Spanish Type Culture Collection) was recovered in aseptic conditions by adding 100 µL of the resuspended fungus to 10 mL of malt extract (ME). Then, a Petri plate of 1.5% malt extract agar (MEA) was inoculated with 100 µL of this suspension and incubated at 26 °C for 6 days. Two subcultures of the fungus were necessary before use in the biological treatment. Fungal subcultures were routinely made every month to conserve the strain.

The methodology described by Díaz et al. [28] was followed to obtain the fungus pellets. To this aim, five cylinders of 1 cm diameter from the growing zone of inoculated plates were used to inoculate 500 mL Erlenmeyer flasks containing 150 mL of sterilised malt extract broth (VWR Chemicals BDH), with a pH between 4.5 and 5. The inoculated flasks were incubated at 26 °C and 135 rpm for 6 days. The fungal mycelial obtained after this process was separated with a sieve and homogenized with 0.8% NaCl (*w*/*v*) in a ratio of 1:3 (*w*/*v*). An amount of 600 µL of resulting suspension was used to inoculate a 1 L Erlenmeyer flask with 250 mL of sterilised ME. Finally, the inoculated ME was incubated at 26 °C and 135 rpm for 6 days. After that time, pellets were obtained, removed with a sieve, and preserved in 0.8% NaCl (*w*/*v*) solution at 4 °C until use.

### 2.3. Fungal Treatment

Several batch tests were carried out to treat diluted AL with *P. chrysosporium*. All the experiments were performed using 1 L Erlenmeyer flask with 250 mL of AL effluent.

- Test E1 and E2 were inoculated with the fungus pellet (3 g/L of dry matter), with the only difference that E2 was supplied with 3 g/L of glucose.
- Test C1 and C2 were used as control without fungus inoculation, without and with glucose addition, respectively.

The flasks were incubated at 26 °C and in an orbital shaking (150 rpm) for 10 days. During the treatment, the pH values were maintained within the range 5–7 by adding NaOH 0.5 M or HCl 0.5 M to ensure the optimal range for the fungus enzymatic system. Samples taken periodically were centrifuged at 15,000× *g* for 15 min and the supernatant

were conserved at 4 °C until analysed. The experiments were carried out in duplicate. Data shown in Results and Discussion section are the average values of both experiments. In all cases, standard deviations were lower than 15% with respect to average value.

### 2.4. Analytical Methods

#### 2.4.1. Determination of sCOD, $sBOD_5$, and Biodegradability Index

The concentration of soluble COD (sCOD) was spectrophotometrically measured (at 600 nm) by dichromate method according to Standard Methods [29], using a DR2500 spectrophotometer (Hach Company). Soluble biochemical oxygen demand ($sBOD_5$) was determined using a manometric respirometry measurement system (Lovibond Water Testing BD 600) and biodegradability index (BI) was calculated as the ratio of $sBOD_5$ over sCOD.

#### 2.4.2. Determination of Colour and pH

The change in the colour of the AL was determined by means of the colour index (CI), which is defined according to Equation (1) [30].

$$CI = \frac{SAC_{436}^2 + SAC_{525}^2 + SAC_{620}^2}{SAC_{436} + SAC_{525} + SAC_{620}} \quad (1)$$

Spectral absorbance coefficients (SAC) are defined as the ratio of the values of the respective absorbance over the cell thickness. The absorbances were measured at 436, 525 and 620 nm using a UV/vis spectrophotometer (Thermo Scientific, He$\lambda$ios $\gamma$). The value of pH was measured by means of a pH-meter (Basic-20 Dilabo).

#### 2.4.3. Determination of Total Reducing Sugars

The total reducing sugars concentration was determined by the dinitrosalicylic acid (DNS) method with glucose as standard, according to the Miller's method [31]. The absorbance of samples was measured at 540 nm. The glucose was used as standard.

#### 2.4.4. Determination of Total Phenolic Compounds

The total phenolic compounds were determined by the Folin–Ciocalteu method in dark conditions, according to Moussi et al. [32], using gallic acid as standard. In this procedure, 400 µL of sample were mixed with 3 mL of Folin–Ciocalteu reagent (previously diluted 1:10 with distilled water). This mixture was maintained at 22 °C for 5 min. After that, 3 mL of sodium bicarbonate ($NaHCO_3$ 6 g/100 mL) were added, and the sample was again incubated at 22 °C for 90 min. After incubation, the absorbance was measured at 725 nm.

#### 2.4.5. Determination of Moisture, TSS, FSS, and VSS

Total suspended solids (TSS), fixed suspended solids (FSS) and moisture were measured according to Standard Methods [29]. The volatile suspended solids were calculated as the difference between TSS and FSS.

## 3. Results and Discussion

### 3.1. Removal of Organic Matter

The evolution of sCOD concentration during the fungal treatments is shown in Figure 1.

The initial sCOD value in the AL effluent was 4854 mg/L, which increased to 9243 mg/L after glucose addition. For C1 and C2 test, which were carried out without fungal inoculation, minor sCOD removals were observed. This degradation was carried out by the endogenous microbiota present in the effluent. With respect to test C1, no change was observed during the first two days and a 27% elimination of sCOD was achieved after four days of incubation. Afterwards, the sCOD value remained almost constant, reaching a final sCOD degradation of 30%. In the case of C2, where glucose where added, sCOD

degradation did not occur until the 4th day. The final percentage of sCOD removal was similar to that achieved with C1. However, it is necessary to point out that in C2 the final sCOD concentration was higher than the sCOD of AL before being supplemented with glucose. Therefore, the endogenous microorganisms were not able to assimilate even the sCOD provided by glucose added.

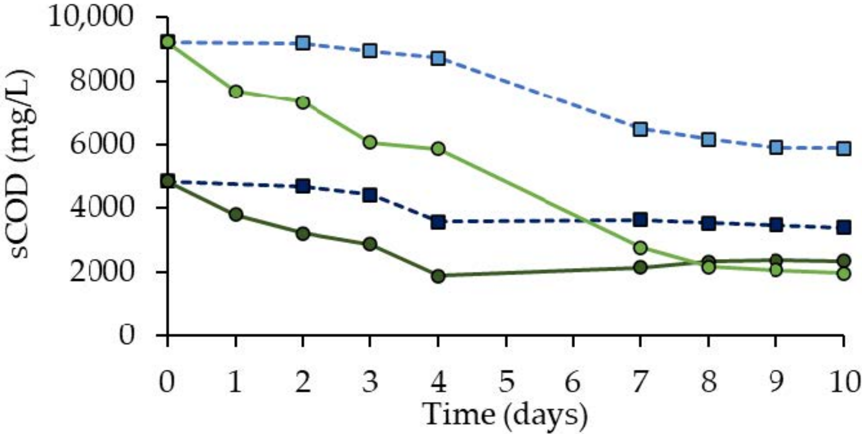


**Figure 1.** Changes in sCOD concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 6.5% of mean value.

The addition of *P. chrysosporium* in E1 and E2, caused a fast decrease in sCOD from the beginning of the treatment, obtaining sCOD removals of 51% and 59%, respectively, after 10 days of treatment. In the experiment E1, carried out without the addition of glucose, 61% of the initial sCOD was degraded in only 4 days of treatment, which duplicate the degradation efficiency reached in C1 by endogenous microorganisms. Thus, the fungal inoculation gave an average rate of sCOD degradation of 0.51 mg/(L min) during the first 4 days, whereas the average rate in C1 was only 0.22 mg/(L min). Regarding test E2, again higher sCOD removals were obtained compared with the non-inoculated test C2. As in E1, the inoculation of the fungus duplicated the sCOD removal rate, which increased up to 0.61 mg/(L min) during first 8 days, whereas in C2 it was only of 0.27 mg/(L min).

To estimate the fungus growth, the TSS at the beginning of the experiments and after 10 days were measured. Data are shown in Table 2.

**Table 2.** TSS for control (C1 and C2) and inoculated (E1 and E2) tests at initial and final times of the fungal treatment.

| Sample | Initial TSS (g/L) | Final TSS (g/L) | Increase (g/L) |
|---|---|---|---|
| C1 | 2.47 ± 0.01 | 3.06 ± 0.01 | 0.61 ± 0.01 |
| E1 | 6.87 ± 0.01 | 8.21 ± 0.01 | 1.34 ± 0.01 |
| C2 | 2.47 ± 0.01 | 5.16 ± 0.01 | 2.71 ± 0.01 |
| E2 | 6.87 ± 0.02 | 10.16 ± 0.02 | 3.29 ± 0.02 |

As can be seen, in the control tests (C1 and C2), the supplementation with glucose increased the growth of the endogenous microbiota. Moreover, in the inoculated tests (E1 and E2), the TSS increases were higher than in the controls, which can be explained by the fungus growth. Comparing the increase in TSS observed in controls and inoculated tests, it can be estimated that fungus growth was similar in E1 and E2, around 0.6–0.7 g/L (dry matter), which is in agreement with the fact that final sCOD removals were also similar. Therefore, in this case, the addition of glucose was not effective for the AL treatment.

Results for sCOD removals here obtained were higher than have been previously reported. Aloui et al. [33], reported that a 44% of COD removal was achieved by a solid-state fermentation of AL using *P. chrysosporium* in a support of sugarcane bagasse. Ahmadi et al. [26] achieved a COD degradation around 50% using this fungus immobilized on loofah. Nogueira et al. [34] reported COD removals efficiencies lower than 44% for *P. chrysosporium* for an OMW pre-treat by photocatalytic oxidation.

The initial concentration of $sBOD_5$ was 408 mg/L, with a biodegradability index of 0.08 (See Figure 2), which means that AL effluent has very low biodegradability. In the experiments C2 and E2, the initial biodegradability index (BI) was higher (0.13), as a consequence of the glucose addition.

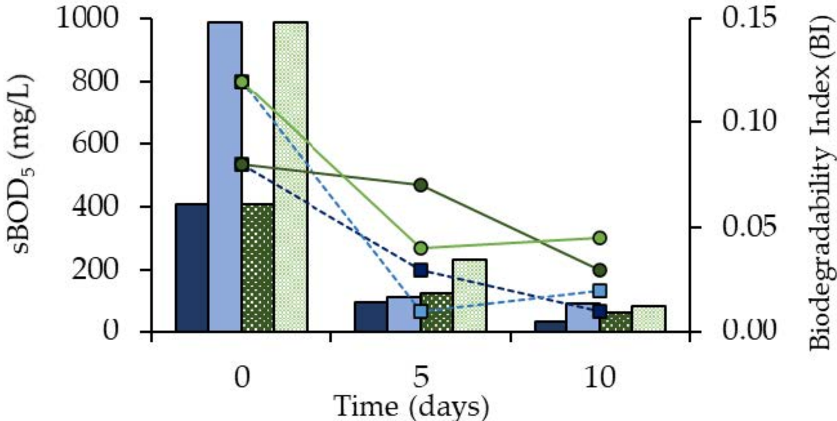


**Figure 2.** Changes in $sBOD_5$ concentration and biodegradability index for the different experiments at initial, intermediate, and final time of the treatment. Bars corresponds with $sBOD_5$ concentration for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The dashed lines shown the biodegradability index (BI) for non-inoculated tests C1 (■) and C2 (■), used as controls, whereas the solid lines represent the inoculated tests E1 (•) and E2 (•).

In all cases, the $sBOD_5$ concentration decreased throughout the fungal treatment, with final values lower than 100 mg/L. Moreover, the BI decreases with the treatment since biodegradable matter was consumed. The *P. chrysosporium* inoculated in E1 and E2 released enzymes able to break down recalcitrant organic matter into compounds more biodegradable. However, the fungus, as well as the endogenous microorganisms, consumed these compounds as they were produced, reducing the sCOD, the $sBOD_5$, and the BI. Regardless, the addition of the fungus gave final BI higher than in the controls, even though it was low. If the enhancing of biodegradability were the objective, for example, as the previous step for the biomethanization process, an alternative could be to directly use the enzymes produced by the fungus instead of inoculating the fungus strain. In this way, the recalcitrant compounds present in the AL effluent could be broken down without the fungus using this organic matter as a nutrient source [35,36]. A sterilisation process may also be necessary to inactivate the endogenous microflora.

The evolution of reducing carbohydrates has been also measured, and results are shown in Figure 3. The reducing sugar concentration of the initial sample was 563 mg/L, and the ratio $sBOD_5$/reducing sugars was 0.7, indicating that a great part of the $sBOD_5$ measured is due to the reducing sugars. As expected, in the samples supplemented with 3 g/L of glucose, the initial concentration increased until 3662 mg/L. For the non-inoculated sample C1, the amount of reducing sugars remained practically stable during the treatment. In contrast, the inoculated samples (E1 and E2) showed a significant decrease in the reducing sugars concentration, with final values of 176 and 140 mg/L, respectively. The initial reducing sugars concentration dropped abruptly in the experiments supplemented with glucose, especially in the one that had been inoculated with the fungus. In this sense, all the glucose that was practically added to test E2 was consumed during the first 24 h, whereas in the supplemented control (C2), the amount of reducing sugars dropped from

3663 mg/L to 1772 mg/L in 24 h, and afterwards remained almost constant, indicating that the endogenous microbiota was not able to degrade all the glucose added. Although the enzymatic activities of the fungus were not measured in this study, the literature has widely reported that the addition of glucose favours the synthesis of fungal enzymes, which in turn are related to the elimination of colour, COD and recalcitrant compounds [37–40]. This fact was reflected in E2, which showed a rapid degradation of reducing sugars, whereas sCOD removal was slower. Probably the fungus decomposed recalcitrant compounds that increased sCOD and, simultaneously, consumed them.

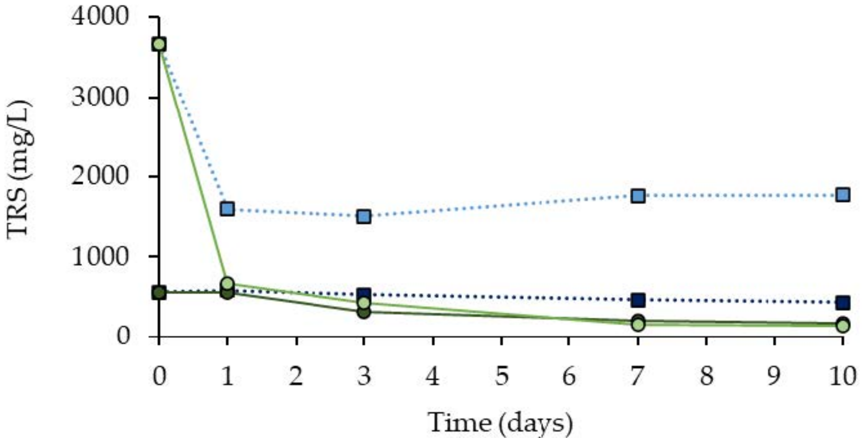


**Figure 3.** Changes in reducing sugars concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (•) and E2 (•). The standard deviation (SD) of the experimental data were in all cases less than 11% of mean value.

### *3.2. Removal of Phenolic Compounds*

The concentration of total phenolic compounds in the initial AL effluent and in AL effluent after fungal treatment was analysed. The phenolic compounds cause severe pollution of surface and ground water, soils, and vegetation. Its presence has a negative effect on microorganisms due to its high antibacterial activity [35,36].

As is shown in Figure 4, the best efficiencies of phenolic compound removal were reached with the inoculation of *P. chrysosporium* in E1, where around 30% of phenolic compounds were degraded after 10 days of treatment reaching values of 91 mg/L. This percentage of removal was slightly lower when glucose was added (E2), obtaining final removals of 25%. For the non-inoculated samples with fungus (C1 and C2), the amount of phenolic compounds removed was lower, with removal percentage of 12% in both cases. Results proved that the fungus inoculation increases the degradation of phenolic compounds with removal percentages almost three times greater than in the non-inoculated samples. However, higher efficiencies have been reported by other authors when the AL effluent was previously sterilised.

Elisashvili et al. [23], who treated a diluted and sterilised olive pomace effluent by submerged fermentation with *Cerrena unicolor*, reported a removal of phenolic content of around 80%. Additionally, this fungus showed a good capacity to release laccases, which are involved in the degradation of phenolic compounds. The low removals achieved in this study it could be because a non-sterilised AL effluent. Moreover, low laccase activity has been reported for *P. chrysosporium* [24]. García et al. [35] reported a 92% total phenol degradation using *P. chrysosporium* to treat a sterilised OMW supplied with a nitrogen source. Additionally, great phenolic removals were obtained when AL was dried, and the concentrate was treated. For example, Sampedro et al. [37] reported removals around 85% using the fungus *Phlebia* sp. immobilized in polyurethane sponge, while 43% was achieved when the effluent was treated by free mycelia.

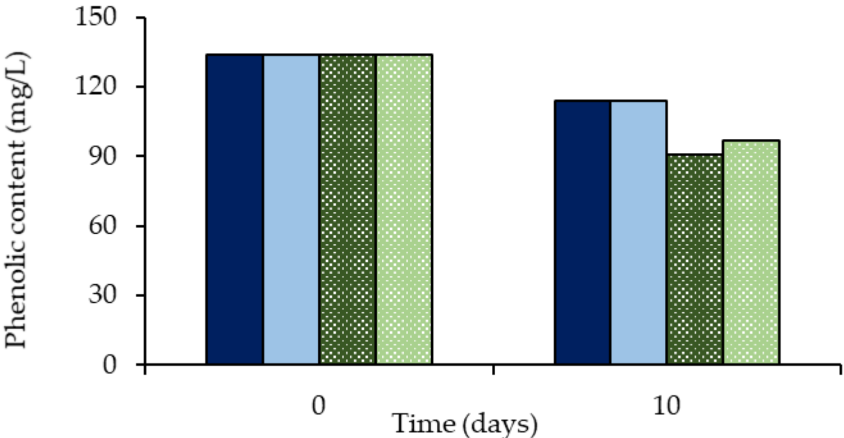


**Figure 4.** Changes in total phenolic content for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value.

### *3.3. Removal of Colour*

AL waste has a dark brown colour, so its decolourization is important to avoid negative environmental and visual effects. Highly coloured wastewater reduces the passage of light through the water, causing a reduction in photosynthetic activity and, therefore, altering the flora and fauna of the water [38]. The colour index profile is shown in Figure 5. Greater removals were obtained when the fungus was added.

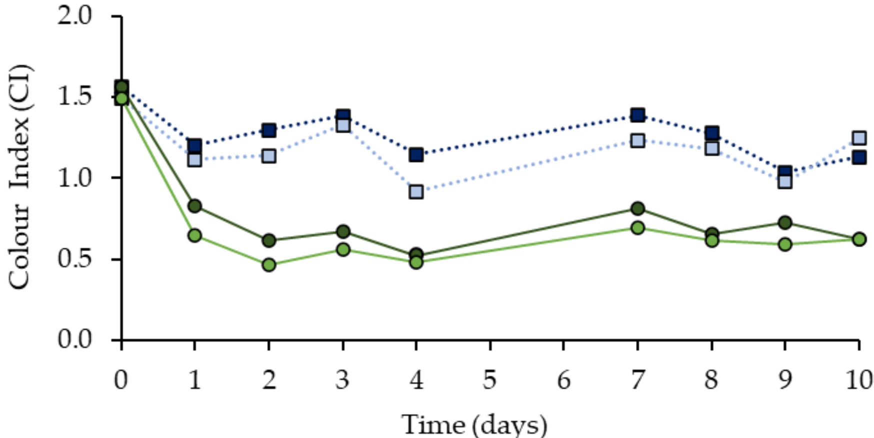


**Figure 5.** Changes in colour index during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 11.5% of mean value.

In the non-inoculate experiments (C1 and C2), the colour index was also reduced by endogenous microflora, especially during the first 24 h. Afterwards, the C1 slightly changed up and down, reaching final percentages of removal around 20%. When the fungus was inoculated (E1 and E2) the colour index decreased more abruptly especially during the first 24 h. Removals around 70% were obtained in both cases after 4 days. Then, the colour removal slightly increased and remained approximately stable, finally reaching a 60% reduction. The obtained results were in accordance with those found in the literature regarding the colour removals in recalcitrant wastewaters using white-rot fungi. Pakshirajan and Kheria [39] reported colour degradations of 64% after continuous fungal treatment with *P. chrysosporium* of industrial textile wastewaters. Ntougias et al. [25] reported colour removals around 60–65% in OMW using basidiomycetes fungus *Pleurotus*

spp. Similar reductions were reported for olive mill effluent treated by an adapted strain of *Trametes versicolor* [40].

Taking into account data reported by the literature and results obtained in this work, the use of white-rot fungi to treat AL could be considered to be a promising treatment technology. Although the removal efficiencies of sCOD, colour and phenolic compounds after treatment with fungi were slightly lower than those reported for OMW treated with physical–chemical treatments, it should be considered that these processes usually present serious drawbacks such as their high cost, bad odour, addition of chemicals, or fouling of the membrane [4,18]. Additionally, chemical oxidation treatments can produce more recalcitrant or toxic intermediate compounds, reducing the effectiveness of the treatment. In contrast, the use of white-rot fungus allows the degradation of a wide range of recalcitrant contaminants due to its ability to release extracellular enzymes, as well as lead to the detoxification of wastewater [25].

## 4. Conclusions

Biotreatment with white-rot fungus *Phanerochaete chrysosporium* is useful to degrade AL waste. When the non-inoculate AL was incubated at 26 °C, 27% of the sCOD was removed in 4 days, whereas the inoculation of fungus allowed the achievement of a sCOD degradation of 60% over the same time. The addition of glucose as an easy carbon source did not enhance the sCOD degradation. The addition of *P. chrysosporium* also allowed a reduction of the colour index of the residue close to 60%. In all the conditions tested, including a control test without inoculation, a reduction in the $sBOD_5$ and in the reducing sugar content was observed after the treatment. However, the biodegradability index decreased, more highly in the inoculated effluent than those in the absence of inoculation. Finally, the treatment of the diluted AL with the fungus allowed degradation of 32% of the total phenols initially present in the effluent, whereas the endogenous microflora could only degrade around 15% of phenolic content. Results obtained in this study open the possibility of using *P. chrysosporium* fungus in the bioremediation of low-biodegradable wastes from the olive oil industry.

**Author Contributions:** Conceptualization: A.I.D., M.I., A.L. and M.D.; methodology: A.I.D., M.I., A.L. and M.D.; validation: A.I.D., M.I. and A.L.; formal analysis: A.I.D., M.I. and A.L.; investigation: A.I.D. and M.I.; data curation: A.I.D. and M.I.; writing—original draft preparation: A.I.D.; writing—review and editing: A.L. and M.D.; visualization: A.I.D. and M.I.; supervision: A.L. and M.D.; project administration, M.D.; funding acquisition, M.D. All authors have read and agreed to the published version of the manuscript.

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