Research article

Approach to a fungal treatment of a biologically treated landfill leachate

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ABSTRACT

White-rot fungi (WRF) have the ability to synthetize extracellular enzymes that could degrade recalcitrant pollutants. The aim of this work was to evaluate the use of *P. chrysosporium* to treat a biologically and physically pre-treated landfill leachate which high load of refractory compounds (COD>1000 mg/L, BOD_5<50 mg/L) in order to reduce COD and colour. Batch tests were carried out at 26 °C and 135 rpm for 15 days. The soluble chemical oxygen demand (sCOD), soluble biological oxygen demand ($sBOD_5$) and colour, as well as the lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymatic activities were analysed. Besides, the effects of different operating conditions, i.e., pH control, permeate dilution and supplementation, on treatment efficacy were investigated.

The control of pH was shown to be key for fungal treatment. In addition, it was found that the addition of carbon and nitrogen sources improved the enzymatic synthesis and the removals of sCOD and colour. Data here obtained open the possibility of using fungi for reducing the amount of recalcitrant pollutants still present in treated landfill leachates or similar effluents.

1. Introduction

Municipal and industrial solid waste disposal in a landfill remains a common practice in many countries. To avoid environmental problems, the leachates generated must be collected and properly treated. Landfill leachate contains a large amount of organic contaminants such as lincomycin, acetaminophen, diclofenac, naproxen bisphenol A, endosulfan, humic and fluvic acids, nitrogenous and chlorinated organic compounds that are harmful for humans and animals, and can cause several damages to the environmental (Díaz et al., 2019; Wang et al., 2018; Yu et al., 2022). Due to the complexity of these organic compounds, the treatment of landfill leachate is difficult and usually requires different processes to reduce the concentration of these pollutants until acceptable levels.

The process employed to treat landfill leachates include physicochemical and biological treatments. So, physico-chemical methods, such as chemical oxidation, coagulation-floculation, electrolysis, membrane techniques and activated carbon adsorption, as well as the combination between them, have been studied to remove refractory pollutants from landfill leachate (Bandala et al., 2021; Tałałaj et al., 2019). Nevertheless, although these methods can reduce effectively organic and inorganic loads, their application has some drawbacks such as the high energy demand and operating cost of chemical oxidation

processes, the need to regenerate activated carbon to avoid clogging problems, or the membrane fouling in filtration processes (Torretta et al., 2016; Wiszniowski et al., 2006). Other disadvantages are the huge amount of sludge generated, in the case of coagulation-flocculation processes, or the secondary contaminants formed as result of oxidation processes (Saxena et al., 2022).

Advanced oxidation methods (AOPs), such as homogeneous Fenton (Teng et al., 2020), persulfate oxidation (Chen et al., 2021) and electrocatalytic oxidation (Zhang et al., 2020), have reported good results in the treatment of landfill leachate, however they usually require strongly acidic conditions, which is a disadvantage for their industrial application. Because AOPs methods are often costly in terms of initial plant equipment outlay, energy requirements and frequent use of additional chemicals, biological methods are often the preferred option. A significant decrease in leachate treatment cost could be obtained by combining AOPs techniques with biological methods, i.e., using ozonation as a pre-treatment for biological treatment due to ozone-based oxidation can enhance the biodegradability of landfill leachate (Chemlal et al., 2014; Fitzke et al., 2013; Renou et al., 2008).

With respect to biological techniques, nitrification-denitrification process using bacteria is the widely method used since the landfill leachate contains high concentration of nitrogen (Bove et al., 2015). However, these methods are hampered when the effluent to tread contains high load of refractory compounds such as polyaromatic

Acronym	
Description	
WRF	White-rot fungi
COD	Chemical oxygen demand
sCOD	Soluble chemical oxygen demand
BOD$_5$	Biological oxygen demand
sBOD$_5$	Soluble biological chemical demand
LiP	Lignin peroxidase
MnP	Manganese peroxidase
Lac	Laccase

hydrocarbons, halogens, polychlorinated biphenyls or humic substances. Then, the efficiency of the treatment is reduced due to the limited level of biodegradable organics. The nitrification stage could be hindered by the high concentration of heavy metals and ammonia, leading to the accumulation of toxic nitrites in the environment (Kurniawan et al., 2010). In addition, it is frequent the contradictory situation of having to add a carbon source for the denitrification step despite containing high chemical oxygen demand (COD) (Díaz et al., 2019; Oulego et al., 2015; Renou et al., 2008). Therefore, it is essential to look for alternative methods to reduce these refractory compounds and/or enhancing the biodegradability of the leachates.

In this sense, the application of white-rot fungi for the treatment and detoxification of complex industrial wastewater has been considered as one of the most promising alternatives in recent years, due to its ability to synthetize powerful extracellular oxidative enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), capable of degrading recalcitrant organic compounds (Collado et al., 2019; Ghosh and Thakur, 2017a). Furthermore, fungi have greater tolerance to adverse environmental conditions than bacterial species present in conventional treatments, withstanding higher concentration of toxic compounds and larger ranges of pH and temperature (Collado et al., 2019; Ghosh and Thakur, 2017b; Hu et al., 2017). These characteristics delineate their potential for application in wastewater treatment. Moreover, in the case of leachate treatment, the use of white-rot fungi could be an advantage due to their versatility. The fungal enzymes synthesized by these microorganisms can degrade lignin, cellulose and hemicellulose, mainly present in young leachates, and refractory organic compounds such as fulvic and humic acids, more common in mature leachates (Kalčíková et al., 2014).

Several papers have been published on the use of fungi for the treatment of concentrates from filtration processes of landfill leachate, subsequently treated by coagulation (Long et al., 2017), electrodialysis (Fu et al., 2021; Zhu et al., 2021), ozonation (Chen et al., 2019a) or other physico-chemical methods (Keyikoglu et al., 2021). Additionally, there are studies focused on the use of fungi as pre-treatment of landfill leachates, using its extracellular enzymes to break down the complex compounds, thus favouring further treatment. These works have reported successful results, with COD and colour removals between 30% - 90% (Collado et al., 2019; Ghosh and Thakur, 2017b; Islam and Yuan, 2020; Spina et al., 2018; Islam et al., 2020). However, this may be a problem for a subsequent nitrification-denitrification process because biodegradable organic matter, necessary for denitrifying bacteria, is removed by the fungi. An alternative is to employ the fungi after the conventional biological treatment; nevertheless, there is almost no published work on the use of fungi as post-treatment for effluent from a biological treatment of landfill leachates (Islam et al., 2020). These effluents usually still contain high COD values, whereas biological oxygen demand (BOD$_5$) is very low, being good candidates for fungi treatment.

The effluent considered in this work has already been subjected to a biological nitrification-denitrification process followed by an ultrafiltration stage, which separate the treated water from the biological

sludge. Although the COD and ammonia concentration have been considerably reduced after biological and physical pre-treatments, the permeate still contains refractory compounds that must be treated before discharge. In addition, the BOD$_5$ of the permeate is very low (<30 mg O$_2$/L), which indicates that most of the organic matter is resistant to a conventional biological treatment. Therefore, the use of fungi seems to be a good alternative for the degradation of recalcitrant organic matter in the permeate.

For these reasons, the aim of this work was to investigate the capacity of the white-rot fungus *Phanerochaete chrysosporium* to treat an effluent from a real nitrification-denitrification plant that treat landfill leachates, to degrade its refractory pollutants, reducing COD and colour. In addition, the effect of different operating parameters, such as pH control, degree of dilution and addition of nutrient sources, on the efficiency of fungal treatment and enzymatic synthesis has been studied.

2. Material and methods

2.1. Effluent description

The sample used in this work was a permeate coming from an ultrafiltration process (pore size 0.02 μm) that treats an average flow of 700 m^3/day of landfill leachate previously treated by a biological nitrification-denitrification process. The treatment plant is sited in COGERSA, the Central Waste Management Facility of Asturias (Spain). For more details of the process, see Díaz et al. (2019). A detailed description of the effluent is shown in Table 1.

2.2. Fungal strain and pellets obtention

The white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 was used for the fungal treatment. The freeze-dried strain CECT 2798 was provided by Spanish Type Culture Collection (CECT). The recovery of freeze-dried strain as well as the obtention of the fungus pellets used for the treatment were carried out according to the methodology described by Díaz et al. (2021).

2.3. Fungal treatment

Eight batch tests were assayed with permeate inoculated with *P. chrysosporium* to study the effect of different operational parameters on the efficacy of the fungal treatment. The influence of pH change during the fungal treatment was evaluated. With this purpose, the pH of the permeate was initially adjusted in all cases to 6.0 by using NaOH 1 M and/or HCl 1 M (Hu et al., 2017; Kim et al., 2003). Then, **F1** was carried out without pH control, whereas for **F2** the pH was manually controlled around pH 6 during all the treatment.

Tests from **F3** to **F6**, were carried out to study the influence of the addition of easily assimilable carbon and nitrogen sources on the fungal treatment, using glucose and ammonium tartrate as co-substrates (Díaz et al., 2021). The tests **F3**, **F4**, and **F5**, were supplemented with 1 g/L, 3 g/L, and 5 g/L of glucose, respectively. An additional test, **F6**, was

Table 1
Characteristics of the leachate permeate.

Parameter	Value
pH	6,78 ± 0,11
Conductivity (μS/cm)	11,723 ± 453
sCOD (mg O$_2$/L)	1265 ± 6
sBOD$_5$ (mg O$_2$/L)	28 ± 3
Biodegradability (BI)	0,02 ± 0,003
Ammonium (mg/L)	21 ± 3
Nitrate (mg/L)	469 ± 79
Nitrite (mg/L)	1,5 ± 0,3
Phosphate (mg/L)	9 ± 2
TS (mg/L)	6,8 ± 0, 1

assayed supplementing 1 g/L of glucose and 0.5 g/L of ammonium tartrate.

Finally, to assess the influence of the permeate dilution, an **F7** test was carried out using as raw material the permeate of the biologically pre-treated landfill leachate mixed with distilled water in a ratio 1:1. Supplementation with glucose (1 g/L) and ammonium tartrate (0.5 g/L) was also investigated for the diluted permeate, corresponding to test **F8**.

All the experiments were performed in 1 L Erlenmeyer flasks containing 200 mL of sample inoculated with 3 g/L (dry weight) of *P. chrysosporium* fungus. Flasks were incubated at 26 °C and 135 rpm in an orbital incubator (New Brunswick Scientific, Excella E25) for 15 days and the pH was manually controlled around pH 6 during the treatment in all cases, except for F1. For all the conditions tested, control experiments without fungus inoculation (tests from C1 to C8) were assayed to evaluate the effect of *P. chrysosporium* in relation with the activity of the endogenous microbiota.

All the experiments were carried out in duplicate. The data shown in graphs are the average of the experimental data. A summary of the experiments is shown in Table 2.

2.4. Analytical methods

Samples was taken periodically each 24 h, centrifuged at 10,000 g during 10 min, filtered by 0.45 μm filter (Millipore) and stored at −20 °C until being analysed. All analytical measurements were done at least in triplicate.

The concentration of soluble chemical oxygen demand (sCOD) and soluble biochemical oxygen demand (sBOD$_5$), as well as the biodegradability index (BI) and the change in the colour, indicated by colour number (CN), were assessed according to Díaz et al. (2021). The means of CN is defined according to Equation (1) (Tizaoui et al., 2007), were spectral absorbance coefficients (SAC) are the ratio of the values of the respective absorbance (Abs) over the cell thickness (x).

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

For the analysis of LiP and MnP enzymatic activities the methodology described by Lisboa et al. (2017) was followed. The dry weight of the fungus pellet was determined by oven-drying (105 °C) a sample to

Table 2
Experimental design for the fungal treatments (F1–F8) and the control tests (C1–C8).

Sample	pH control	*P. chrysosporium*	Glucose	Ammonium tartrate	Dilution
C1	–	Non-inoculated	–	–	–
C2	5,8–6,1	Non-inoculated	–	–	–
C3	5,7–6,1	Non-inoculated	1 g/L	–	–
C4	5,8–6,0	Non-inoculated	3 g/L	–	–
C5	5,6-6,1	Non-inoculated	5 g/L	–	–
C6	5,8–6,0	Non-inoculated	1 g/L	0,5 g/L	–
C7	5,7–6,2	Non-inoculated	–	–	50%
C8	5,8–6,1	Non-inoculated	1 g/L	0,5 g/L	50%
F1	–	3 g/L (dry matter)	–	–	–
F2	5,7–6,1	3 g/L (dry matter)	–	–	–
F3	5,5–6,0	3 g/L (dry matter)	1 g/L	–	–
F4	5,5–6,1	3 g/L (dry matter)	3 g/L	–	–
F5	5,5–6,1	3 g/L (dry matter)	5 g/L	–	–
F6	5,5–6,0	3 g/L (dry matter)	1 g/L	0,5 g/L	–
F7	5,6-6,1	3 g/L (dry matter)	–	–	50%
F8	5,7–6,1	3 g/L (dry matter)	1 g/L	0,5 g/L	50%

constant weight. Finally, total solids (TS) of the permeate were measured according to Standard Methods (Baird et al., 2017). The value of pH was measured by means of a pH-meter (Jenway 3510).

3. Results and discussion

3.1. Fungal treatment of leachate permeate with and without pH control

The evolutions of sCOD concentration and colour number during the fungal treatment of the leachate permeate with and without pH control (F2 and F1) are shown in Fig. 1, as well as the control tests without fungus inoculation (C2 and C1).

When pH was not controlled, it increased, achieving values above 7 in just 1 day. In this case, the colour remained almost constant and only 3% of sCOD degradation was obtained. Although good results were not obtained after fungal treatment without pH control, the different evolution of pH between the F1 and C1 tests is noteworthy (Fig. 1B). In the C1 test (non-inoculated), the pH showed a progressive increase during the treatment, reaching values close to pH 9. However, for F1 test, a maximum pH value of 7.8 was reached after two days of treatment. Afterwards, the pH values decreased for 48 h until reaching a pH value of 7. For times longer than 4 days, the pH remained between a pH range of 7–7.5.

Several authors have indicated the capacity of fungi to synthesize different organic acids (Liaud et al., 2014; Vylkova, 2017). Comparing the evolution of the pH in C1 with the data obtained in F1, it seems that, although not important changes were observed in colour and sCOD concentration, the fungus *P. chrysosporium* was able to produce compounds that acidified the environment, reducing the pH of the permeate during fungal treatment.

In contrast to F1 test, certain degradation of sCOD was achieved in the F2 test, which was performed at pH controlled between 5.7 and 6.1, reaching final sCOD and colour removals of 12% and 13%, respectively.

Previous studies have reported as optimal values for industrial wastewater treatment by fungi the operating conditions of temperatures between 25 and 30 °C and pH 5–6. Hu et al. (2016), who studied the effect of pH on landfill leachate treatment by *P. chrysosporium*, reported a maximum total organic carbon (TOC) removal efficiency around 73% at pH 6. Lu et al. (2009), also achieved the higher removal rates of COD and phenolics compounds at pH between 5.0 and 6.0 during the fungal treatment of a recalcitrant wastewater from the coke industry by *P. chrysosporium*. The low sCOD removal efficiencies obtained in this study in comparison with Hu et al. (2016) is due to the fact that in this work the effluent had been already treated in a biological process, so the organic matter in the effluent is highly recalcitrant. Similar data were reported by Saetang and Babel (2010) who achieved COD removals around 15% after 12 days of landfill leachate treatment by the white-rot fungus *Trametes versicolor*.

No important differences were shown in terms of sCOD, and colour removal comparing C1 with C2, probably because the presence of endogenous micro-organisms in the sample is very low in both cases. Previous works have been reported a microbial concentration lower than 10 UFL/ml for permeates coming from a landfill leachate treatment plant (Díaz et al., 2019; Sancha et al., 2014). As most endogenous microorganisms were retained during the ultrafiltration stage and BOD$_5$ is very low, the impact of the endogenous microbiota on the treatment of the leachate permeate was not considered. This reflects the degradative capacity of the fungus as can be seen by comparing C2 and F2.

The capacity of white-rot fungi to remove colour and recalcitrant organic matter is linked to its ability to synthetize extracellular enzymes such as MnP and LiP (Islam et al., 2019). As can be seen in Fig. 2, MnP and LiP enzymatic activities in F2 test were quite higher than those obtained in F1. Again, it is evident that pH values higher than 7.0 were not optimal for ligninolytic enzymes production, since in F1 test the production of MnP and LiP was very low. On the opposite, in F2 maximum activities above 70 U/L were measured for both, LiP and MnP,
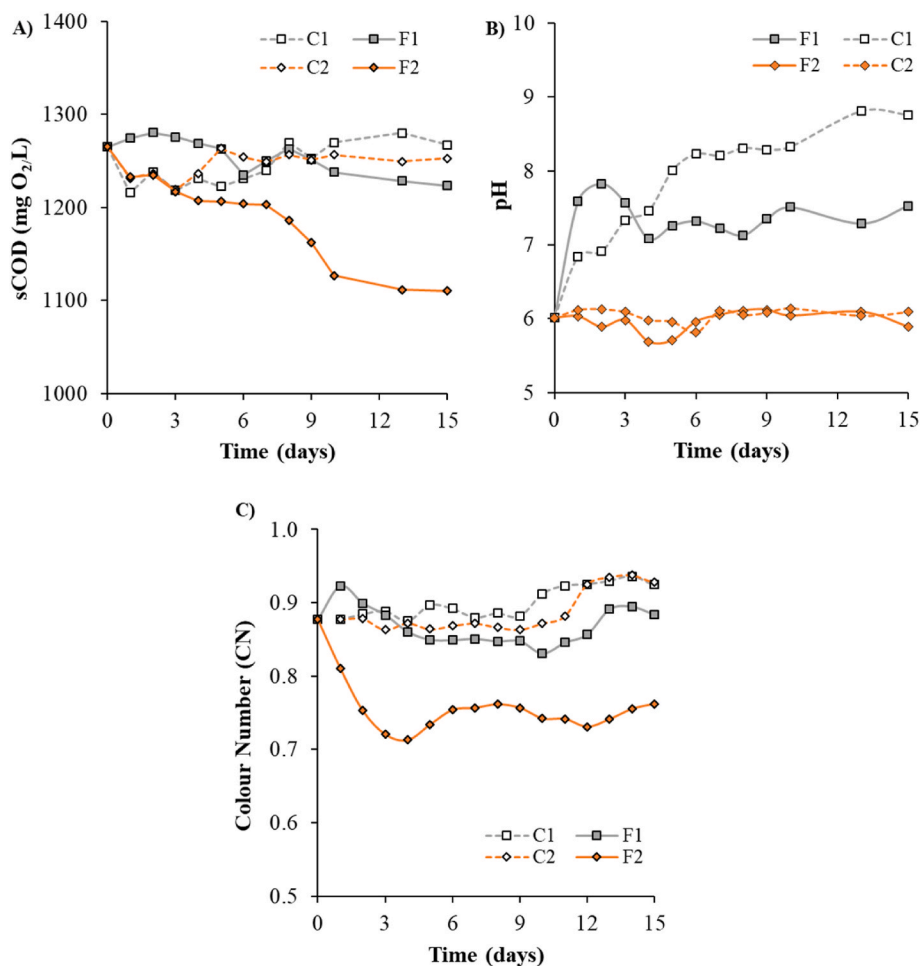
Fig. 1. Evolution of sCOD **(A)**, pH **(B)** and colour number **(C)** during the treatment of permeate by *P. chrysosporium*. Solid lines and filled markers show the data for inoculated test F1 (without pH control) and F2 (with pH control), whereas dashed lines and empty markers symbolize the non-inoculated tests C1 and C2, used as control. The standard deviations (SD) of the experimental data were in all cases lower than 9% of the mean value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
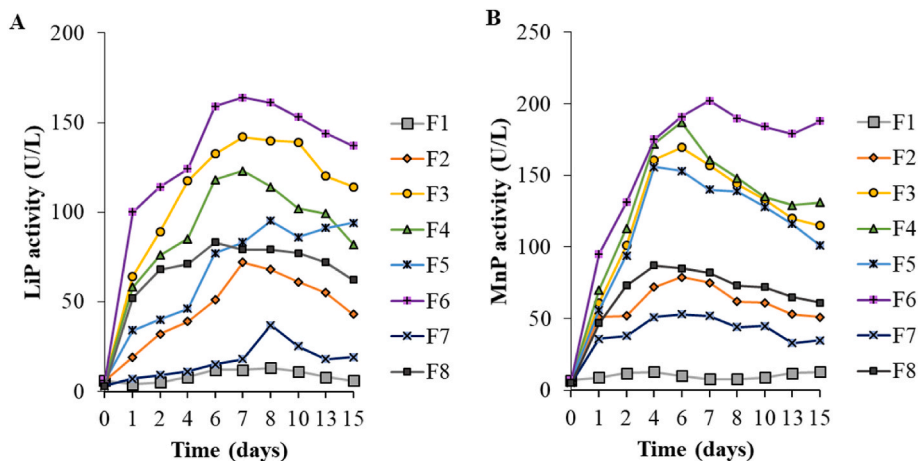

Fig. 2. Evolution of LiP **(A)** and MnP **(B)** enzymatic activities during the treatment of leachate permeate by *P. chrysosporium* for all the inoculated tests. The percentage of standard deviation (SD) of the experimental data were in all cases less than 6% of shown values.

after a week of treatment. This accorded with the time when the degradation rate of sCOD increased (See Fig. 1A). Islam et al. (2019), who investigated the effect of fungal enzymes on the treatment of mature landfill leachate reported MnP and LiP of only 12.8 U/L and 8.6 U/L, respectively, after 12 days of treatment by *P. chrysosporium*,.

The enzymatic activities measured in this work were quite higher than those previously reported in similar works. It should be noted that the ammonia concentration in the permeate used in this work was around 21 mg/L, since it was biologically treated by a nitrification-denitrification process. High concentrations of ammonia have been associated with important reductions in fungal enzyme activities (Ellouze et al., 2009), which could explain the higher values here obtained.

Concentration of sBOD$_5$ were measured at intermediate (8 days) and final (15 days) of treatment. Important changes were not observed for both control and inoculated tests after 15 days the fungal treatment,

with final values and BI similar to the initial one (sBOD$_5$: 23–29 mg/L; BI $\approx$ 0.02). It should be noted that for F2, the sBOD$_5$ values after 8 days were higher than the initial ones, reaching values of 52 $\pm$ 1 mg/L. However afterwards, the sBOD$_5$ decreased again to 23 $\pm$ 2 mg/L, which supports that the fungus, thanks to the enzymatic activity, transformed organic matter non-biodegradable into biodegradable and immediately consumed it.

Razarinah et al., (2014), who evaluated the treatment of landfill leachate by immobilized *Trametes menziesii*, reported that fungus only reduced 50% of BOD$_5$ and 32% of COD after 7 days in a batch reactor at 28 °C and 150 rpm. The fungi immobilization in different supports have been related with better decolourization and organic matter removal efficiencies (Hu et al., 2016; Sedighi et al., 2009). These results cannot be compared with the obtained in this work since the biodegradability of the permeate was much lower.

To evaluate the microbial growth, TS was measured at the beginning of the experiments and after 15 days (see Table 3). Due to the low TS increase for control tests (non-inoculated), the TS increases observed for F1 and F2 were directly related with fungal growth, dismissing the endogenous microbiota contribution. The biomass increase in F1 was also negligible, whereas certain fungus growth was observed in F2 (from 2.98 $\pm$ 0.03 g/L to 3.31 $\pm$ 0.01 g/L, dry weight) when pH was maintained between 5.7 and 6.1. Again, the convenience of controlling pH was demonstrated, so subsequent studies were carried out keeping the pH at a value around 6.

3.2. Effect of supplementation with carbon and nitrogen sources

As above commented, the initial sBOD$_5$ of the leachate permeate used in this work was low (28 $\pm$ 5 mg O$_2$/L). The presence of a readily nutrient source is key to activate fungal enzymatic synthesis and enhance the degradation of recalcitrant organic matter (Bardi et al., 2017; Díaz et al., 2021; Schneider et al., 2018). So, the addition of glucose and ammonium tartrate as co-substrates was evaluated.

For the leachate permeate, the qualitative evolution of sCOD concentration during the fungal treatment was very similar regardless the glucose concentration added (see Fig. 3A). In all cases, the sCOD degradation was progressive until 9th day of treatment, and from this time on, concentration remained almost constant. As can be observed, the amount of total sCOD removed was higher when the amount of glucose added was higher. However, for both F3 and F4 tests, final sCOD concentrations were very similar, achieving values of 979 $\pm$ 9 mg/L and 998 $\pm$ 8 mg/L after 15 days of treatment, which corresponds with around 20% of sCOD removal, calculated with respect to the sCOD

concentration of the non-supplemented (See Table 3). This removal efficiency is quite higher than that obtained in the test without glucose supplementation. When 5 g/L of glucose were added (F5) the percentage of sCOD removal was lower. In all cases, the amount of sCOD degraded in the non-inoculated test (C3, C4 and C5) was quite low, showing that the endogenous microbiota was not able to degrade even the glucose added.

Regarding colour removal, similar final decolourization efficiency, around 20% was achieved in the three cases (F3, F4, and F3). The evolution with the time of the CN was quite similar in all the test with pH control. So, CN decreased achieving a minimum value around the fourth day and then began increase. This indicated the changes suffered by the organic matter during the degradation process. As previously reported Junnarkar et al. (2016), the degradation of recalcitrant organic matter can give rise to by-products that increase the colour of the effluent.

Regarding the MnP and LiP enzymatic activities, great differences were observed according to the amount of glucose added, especially in the case of LiP. As is shown in Fig. 2, the LiP activity measured during the treatment was inversely proportional to the concentration of glucose added, obtaining the highest values for F3 test, in agreement with the higher percentage of sCOD removal observed.

In the case of MnP activity, mainly related to decolourization processes, F3 and F4 tests showed the highest values, which was correlated with the highest percentages of colour removals obtained (around 35% in the 4th day). Saetang and Babel (2010), reported a maximum LiP activity of 193 U/L and MnP activity 437 U/L, when a landfill leachate was treated without glucose addition, reaching a 12% of decolourization. Meanwhile, the supplementation with 3 g/L of glucose increased the enzymatic activities until values of 384 U/L and 1241 U/L for LiP and MnP, respectively. Consequently, a 40% of COD removal and 58% of decolourization was achieved. In this work, for the tests supplemented with glucose, the maximum enzymatic activity for LiP was 142 $\pm$ 3 U/L measured in F3 after 7 days, whereas the maximum MnP activity was 187 $\pm$ 4 U/L in F4 after 6 days, which could explain the lower degradations of sCOD and colour achieved. Enzymatic activities and also percentages of removal obtained in this work were lower than those obtained by Saetang and Babel (2010). Nevertheless, again is necessary to consider that the leachate permeate here employed has already been biologically treated.

Taking into account all above commented, it is evident that the supplementation with glucose improved the efficacy of the treatment being 1 g/L enough quantity. The addition of 5 g/L (F5) was too much, giving efficacies of sCOD removal similar to the test without supplementation. Radha et al. (2005), who investigated the treatment of an

Table 3
Percentages of sCOD and colour degradations and percentages of TS increments at 8 days and 15 days of treatment for inoculated tests (F1–F8) and non-inoculated tests used as controls (C1–C8). Negative percentages of degradation indicate that the sample suffered an increase in the value of the parameter studied.

| Test	sCOD degradation (%)		Colour (CN) degradation (%)		Total Solids increment (%)
	8 days	15 days	8 days	15 days	15 days
F1	1,9 $\pm$ 0,3	3,1 $\pm$ 0,2	3,31 $\pm$ 0,01	0,68 $\pm$ 0,05	2,64 $\pm$ 0,02
F2	3,8 $\pm$ 0,1	12,3 $\pm$ 0,5	14,37 $\pm$ 0,05	16,53 $\pm$ 0,11	11,07 $\pm$ 0,02
F3	15,3 $\pm$ 0,4	22,6 $\pm$ 0,7	23,57 $\pm$ 0,12	20,82 $\pm$ 0,09	31,56 $\pm$ 0,12
F4	1,5 $\pm$ 0,1	21,1 $\pm$ 0,9	28,38 $\pm$ 0,01	22,08 $\pm$ 0,08	36,47 $\pm$ 0,03
F5	−6,5 $\pm$ 0,3	15,9 $\pm$ 0,5	23,68 $\pm$ 0,06	17,96 $\pm$ 0,18	45,82 $\pm$ 0,11
F6	27,5 $\pm$ 0,1	33,2 $\pm$ 0,9	25,03 $\pm$ 0,01	37,72 $\pm$ 0,16	33,55 $\pm$ 0,15
F7	9,8 $\pm$ 0,4	10,5 $\pm$ 0,7	2,61 $\pm$ 0,08	12,87 $\pm$ 0,12	3,98 $\pm$ 0,02
F8	17,1 $\pm$ 0,1	17,8 $\pm$ 0,2	14,74 $\pm$ 0,11	17,54 $\pm$ 0,11	6,29 $\pm$ 0,05
C1	1,4 $\pm$ 0,1	1,6 $\pm$ 0,1	−0,51 $\pm$ 0,03	−9,24 $\pm$ 0,02	0,62 $\pm$ 0,03
C2	2,6 $\pm$ 0,2	4,9 $\pm$ 0,3	1,55 $\pm$ 0,06	−6,59 $\pm$ 0,07	0,57 $\pm$ 0,01
C3	−48,1 $\pm$ 0,4	−40,3 $\pm$ 0,5	1,54 $\pm$ 0,04	−0,68 $\pm$ 0,03	1,02 $\pm$ 0,09
C4	−219,5 $\pm$ 0,6	−217,6 $\pm$ 0,3	0,68 $\pm$ 0,15	−4,45 $\pm$ 0,13	1,36 $\pm$ 0,05
C5	−356,2 $\pm$ 0,4	−354,3 $\pm$ 0,4	5,13 $\pm$ 0,17	0,63 $\pm$ 0,15	1,55 $\pm$ 0,07
C6	−39,4 $\pm$ 0,3	−32,5 $\pm$ 0,2	2,96 $\pm$ 0,09	0,23 $\pm$ 0,08	1,48 $\pm$ 0,06
C7	3,0 $\pm$ 0,2	4,7 $\pm$ 0,1	−5,78 $\pm$ 0,03	−4,29 $\pm$ 0,01	0,43 $\pm$ 0,02
C8	−41,7 $\pm$ 0,5	−28,0 $\pm$ 0,3	−2,61 $\pm$ 0,04	−8,40 $\pm$ 0,06	0,71 $\pm$ 0,08
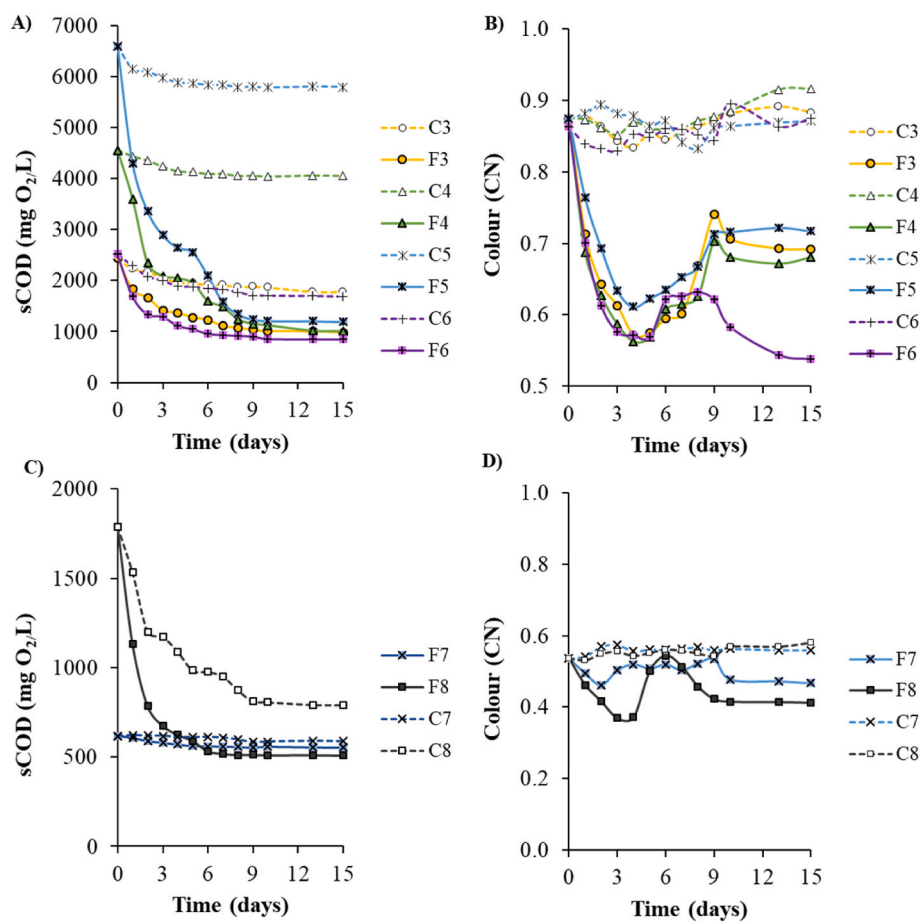
Fig. 3. Evolution of sCOD **(A)** and colour number **(B)** during the treatment of permeate by *P. chrysosporium* from test F3 to F6. Evolution of sCOD **(C)** and colour number **(D)** during the treatment of diluted permeate by *P. chrysosporium* for test F7 and F8. Solid lines and filled markers represent the data for inoculated tests from F3 to F8, whereas dashed lines and empty markers show the data for non-inoculated tests from C3 to C8, used as controls. In F3, F4 and F5 tests, 1 g/L, 3 g/L and 5 g/L of glucose was added as co-substrate, respectively. In F6 1 g/L of glucose and 0,5 g/L of ammonium tartrate were supplied. In F8, 1 g/L of glucose and 0,5 g/L of ammonium tartrate was supplied, while in F7 no external source of nutrient was added. The percentage of standard deviation (SD) of the experimental data were in all cases less than 8% of mean value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

effluent from dye-based industries by *P. chrysosporium*, reported that glucose concentrations higher than 5 g/L decreased the decolourization rate. Results here obtained indicate that when glucose concentrations are higher than 3 g/L, *P. chrysosporium* consumed the added glucose but did not break down complex compounds. This fact was reflected in a greater growth of the fungus when glucose concentrations were increased.

Regarding BOD_5 concentration, the behaviour was similar to that obtained without supplementation (F2). As soon as fungus broke recalcitrant compounds into by-products more biodegradable, they were consumed by the microorganisms. Final BI were also very low with final values between 0.02 and 0.03.

In addition to carbon sources, previous studies have reported that nitrogen and phosphorus concentrations also play a key role in the production of fungal ligninolytic enzymes (Ellouze et al., 2009; Radha et al., 2005). For aerobic biological treatments, the literature has usually reported a COD/N/P ratio around 100/5/1 as optimal to cover the nutritional requirements of the microorganisms and ensure their growth (Collado et al., 2019; Díaz et al., 2021; Ellouze et al., 2009). In F3 test, where 1 g/L of glucose was added, the final concentrations of glucose, soluble COD and nitrogen were 2512 mg/L, 7.78 mg/L and 2.93 mg/L, respectively. Therefore, the COD/N/P ratio was approximately 100/0.32/0.23, indicating that the effluent contained a very low concentration of nitrogen and phosphorus. For this reason, it was carried out an additional test (F6), supplemented with 1 g/L of glucose and 0.5 g/L of ammonium tartrate. When glucose and ammonium tartrate were supplied, the COD/N/P ratio was 100/3.34/0.12. Although the phosphorus concentration was still low, the COD/N ratio considerably improved, which improved sCOD and colour removals, as well as the fungal enzymatic activity.

Islam et al. (2020), who studied the treatment of landfill leachate using aerobic activated sludge (SBR) and continuous fungal bioreactor, reported that COD removal increased proportionally to the concentration of ammonium nitrate. This increase was in accordance with a higher enzyme synthesis by *Penicillium* sp. They reported the best results by adding ammonium nitrate concentrations of 352 mg/L, reaching COD removals close to 40%. Higher concentrations (914 mg/L) had the opposite effect, reducing COD removal to 17%.

As can be seen in Figs. 2 and 3, many differences can be observed between F3 (without N supplementation) and F6, with respect to the colour degradation and enzymatic activities synthesis, especially for MnP. Unlike F3 test, where MnP activity abruptly decreased after 6 days of treatment reaching final values of 115 U/L, the addition of a nitrogen source in F6 tests favoured the MnP activity, which remained above 180 U/L from day 7 until the end of treatment.

Comparing the experiments where an external nutrient source was added (F3, F4, F5 and F6) with F2 (without supplementation), results indicated that the addition of certain amount of easily degradable carbon and nitrogen source is required to enhance the growth of *P. chrysosporium* and the release of ligninolytic enzymes. The best results were obtained adding 1 g/L of glucose and 0.5 g/L of ammonium tartrate. This fact was reflected in the higher decolourizations and sCOD degradations obtained in F6, achieving final percentages of removal of 38% and 33% for colour and sCOD, respectively. This COD removal was similar to that reported by Chen et al. (2019b), who achieved a COD removal of 33% when treated a landfill leachate from a membrane reactor by oxidation with O_3/H_2O_2. However, higher efficacies were obtained by Zolfaghari et al. (2016) who reported a COD removal around 57%, after treating a landfill leachate by sequential membrane bioreactor and electro-oxidation processes.

Considering the capacity of *P. chrysosporium* to release LiP and MnP during the treatment of the supplemented leachate sample, the incorporation of agro-food wastes into the process as an additional nutrient source could be an interesting line of future research. Several studies have already been published on the use of organic industrial effluents as raw material for the synthesis of fungal enzymes such as cellulases, lignin peroxosidades, hydrolases, etc (Mamma et al., 2008; Srivastava et al., 2018; Teigiserova et al., 2021). These enzymes have several biotechnological applications, especially in bioremediation, biosynthesis, and many nature-inspired commercial applications (Gao et al., 2022; Sellami et al., 2022).

3.3. Effect of permeate dilution

Taking into account the possible presence of inhibitors in the leachate permeate that could hinder biological treatment (Renou et al., 2008), the dilution of the leachate permeate with distilled water was tested (tests F7 and F8). The evolutions of soluble COD and colour number during the fungal treatment were shown in Fig. 3C and Fig. 3D.

Results showed that the dilution of permeate, achieving similar efficiencies than those obtained in F2, performed with undiluted effluent. Better results were showed when the diluted permeate was supplied with 1 g/L of glucose and 0.5 g/L of ammonium tartrate, showing higher enzymatic activity than those observed for F2 test, with final decolourizations and sCOD removals around 18%. However, these removal percentages were lower than those obtained in F6, which was carried out under the same operational conditions and using undiluted permeate. For future work, the use of other non-toxic wastewater for the dilution of the leachate permeate should be considered, with a special interest in those with a high nitrogen content, thus avoiding supplementation with external sources of nitrogen, such as ammonium tartrate.

4. Conclusions

In this work, a landfill leachate pre-treated by nitrification-denitrification and ultrafiltration was treated, for first time, by *P. chrysosporium* in order to degrade the remaining recalcitrant organic matter. Results showed that the addition of glucose and ammonium tartrate as external nutrient sources allowed improving the synthesis of MnP and LiP enzymes and therefore the efficacy of the process. In this sense, it was shown that the addition of 1 g/L of glucose as co-substrate was enough to greatly improve the sCOD and colour removals, while the use of a concentration of 5 g/L abruptly decreased the effectiveness of the fungal treatment. Moreover, when 0.5 g/L of ammonium tartrate were added, the sCOD and colour degradations were notably improved, achieving in only 8 days higher percentages of removal than those obtained without supplementation in 15 days. Regarding the effect of leachate permeate dilution, lower removal efficiencies were obtained compared with the removal percentages obtained without dilution. Thus, the treatment of the leachate permeate supplemented with 1 g/L of glucose and 0.5 g/L of ammonium tartrate reported the best results, achieving final removals of 38% and 33%, for colour and sCOD, respectively. These results show the potential of *P. chrysosporium* for the treatment of effluents with high content in recalcitrant pollutants. However, it is necessary to consider that, to achieve optimal results, the operational times are longer than those used in conventional aerobic treatments, with the subsequent energy consumption.

Therefore, future work activities should be focused on the optimization of operating conditions with the objective of reducing treatment times. In view of the results obtained in this work, the C/N ratio of the effluent would be one of the key aspects to be considered to achieve good biological degradation. Likewise, the use of other organic wastes as nutrient sources for fungal treatment would be another aspect that deserves to be investigated. This aspect would make treatment cheaper and could also be an environmentally friendly way to valorise organic waste.

It is important to highlight that the implementation of the use of fungi for the treatment of recalcitrant compounds is in accordance with current environmental policies that promote treatments based on green chemistry, since it implies the reduction or elimination of the use of products that are harmful to people and the environment.

CRediT author statement

Ana Isabel Díaz: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization. Adriana Laca: Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision. Mario Díaz: Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

Data availability

All the data obtained in the research are shown in the manuscript.

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