Tetracyclines degradation by edible ligninolytic fungi

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Abstract

Antibiotics are widely used to treat and prevent infectious diseases in livestock all over the world. Tetracyclines (TCs) are among the most used, so they can be easily found in wastewater, soil, sewage sludge and cattle manure, being some of them very persistent. Spent mushroom substrates from edible fungi are agricultural wastes with potential usefulness to degrade organic pollutants. The goal of present work consists in the assessment of edible fungi mycelium: button mushroom (*Agaricus bisporus*), oyster mushroom, (*Pleurotus ostreatus*), king mushroom (*Pleurotus eryngii*) and shiitake (*Lentinula edodes*) to degrade TCs (Tetracycline, TC, Oxytetracycline, OTC and Chlortetracycline, CTC). Three experiments were performed. In the first one, the four ligninolytic fungi were tested to assess their ability to degrade TCs for 8 days. A subsequent experiment with those of genus *Pleurotus* was carried out for 14 days evaluating laccase and Mn Peroxidase activities and the daily decrease of TCs concentration. Finally, the antimicrobial activity of the TCs residues was evaluated. Results obtained show the high potential to degrade TCs by fungi of genus *Pleurotus*, especially *P. eryngii*. Nevertheless, the oxidizing of TCs produced metabolites with antimicrobial activity, due to degradation products could have kept intact the tetracyclic nucleus naphthacene where the antibacterial activity relies on, although its pharmacokinetic might have been affected so, consequently further studies are needed to optimize the degradation process by stimulation of *Pleurotus* growth and peroxidases activities to reduce the presence of antimicrobial activity in wastewaters. Thus, degradation products should be investigated to elucidate the mechanism of fungal degradation.

Keywords: antibiotics, tetracyclines, biodegradation, pharmaceutical compounds, wastewater.

1. Introduction

Antibiotics prescription for humans or livestock is increasing worldwide. Consequently, a huge amount of them are being released to the environment. Health authorities have started to be worried about the increasing number of multiresistant bacteria produced by antibiotic residues at non-lethal or sub-inhibitory concentrations that allow susceptible strains of bacteria to continue growing [1].Tetracyclines (TCs) are among the most used antibiotics. Their sales for veterinary use in UE were 2,983 tons 2014, whereas in USA in 2015 were 6,880 tons. Most of antibiotics are only partially metabolized, about 25–75% of the intake is released to the environment not having suffered any kind of degradation [2] , so they can be easily found in wastewater, soil, sewage sludge and cattle manure, being some of them very persistent [3].

Antibiotics in general, and especially TCs, have been found in natural water as a result of the inefficient current treatments to reclaim wastewater [4], all kind of foodstuff [5], crops [6], soil [7] , sewage sludge or cattle manure [3].

It is absolutely important to find an effective and environmentally friendly treatment to remove antibiotics from the effluent of wastewater treatment plants. Ligninolytic fungi have been reported to degrade organic contaminants such as synthetic dyers, polycyclic aromatic hydrocarbons (PAHs) [9-11]; [12], heavy oils, or pharmaceutical compounds including antibiotics [13];[14]. So that, ligninolytic fungi could be an excellent choice thanks to their powerful ligninolytic enzymatic system. Ligninolytic fungi are known to segregate a set of very powerful extracellular enzymes capable to degrade efficiently lignin and other organic compounds. Laccase, Mn-peroxidase (MnP), Lignin peroxidase (LiP) and Versatile peroxidase (VP) are the most important. *Pleurotus spp* and *Lentinula spp* are primary degrading fungi, while *Agaricus spp* are secondary degrading fungi only able to break down simpler organic substances.[8].

Some of these ligninolytic fungi are edible, so they are widely cultivated for human consumption with the consequent co-generation of vast amounts of spent mushroom substrate [15]; [16], which has been demonstrated to be useful to degrade organic pollutants thank to their active fungal mycelium[17] ; [10]; [18]. Therefore, edible fungi show high potential to develop groundbreaking and environmentally friendly technology to remove antibiotics. Besides, it is the first step in the valorization and re-use of an agricultural waste as cost-effective source of ligninolytic fungi mycelia.

Since the widespread presence of antibiotics in the environment is known to be a threat for humans and they have to be removed before releasing, the main objectives of this work were 1) to assess the potential by four of the most cultivated edible ligninolytic fungi around the world: *Pleurotus ostreatus, Pleurotus eryngii, Lentinula edodes* and *Agaricus bisporus* to degrade tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) 2) to relate enzymatic ligninolytic activities (laccase and Mn-Peroxidase) with antibiotic degradation 3) to determine the residual antibacterial activity after TCs degradation.

2. Materials and methods

2.1 Chemical and reagents

Tetracycline hydrochloride (TC, 95%), Oxytetracycline hydrochloride (OTC, 95%) and Chlortetracycline hydrochloride (CTC, 97%) were purchased from Sigma Aldrich (St.Louis, MO, USA). Veratryl alcohol 96%, 2,6-dimethoxy phenol, ammonium acetate, malic acid, $MnSO_4 \cdot H_2O$ 98% and H_2O_2 33% were purchased from PanReac (Barcelona, Spain), dimethyl sulfoxide from Sigma Aldrich (St. Louis, MO), methanol from Fisher Scientific (Madrid, Spain), trifluoracetic acid and acetonitrile from Scharlab (Barcelona, Spain), ethylenedinitrilotetraacetic-acid-disodium-salt-dihydrate from Merck (Darmstadt, Germany) and ethyl acetate from VWR International (Radnor, PA, USA). All chemicals were of analytical quality and solvents of HPLC grade. Malt extract was purchased from Sigma Aldrich (St. Louis, MO) while tryptic soybeans extract (TSB) was from Fluka Analytical (Madrid, Spain).

2.2 Assessment of ligninolytic fungi to degrade tetracyclines

Four ligninolytic fungi (*P. ostreatus*, *P. eryngii*, *L. edodes* and *A. bisporus* were tested to determine their ability to degrade TC, OTC and CTC. *P. ostreatus* was isolated from spent mushroom substrate collected in a commercial crop placed in Quintanar del Rey (Cuenca, Spain). This fungal strain was previously successfully tested for polycyclic aromatic hydrocarbons degradation [9]. Commercial strains of *P. eryngii*, *A. bisporus* and *L. edodes* were purchased from Gurelan Mycelium located in Huarte (Navarra, Spain). The mycelium grew in controlled laboratory conditions in malt extract agar (MEA) for 7 days.

The selection of the most efficient fungi to degrade TCs was carried out by incubation in presence of the two TCs for eight days in Erlenmeyer flask of 100 mL using 25 mL of 3% malt extract sterilized in autoclave at 121 ◦C for 20 min. Tetracyclines (TCs) were dissolved in dimethyl sulfoxide, which is not toxic for fungi, and immediately added to the growth media to a final concentration of 0.1 mM. Finally, fungi were inoculated by adding three mycelial pieces of 5 mm from MEA culture. Controls were performed in parallel, two of them to determine the fungal growth and activity in absence of TCs, and other two to determine the abiotic degradation.

To determine the fungi more efficient to degrade TC, the four fungi were incubated in orbital shaking at 160 rpm for 8 days at 28 ºC, in darkness. Then, the growing media was filtrated to determine the fungi growth by mycelium dry weight. 1.5 mL aliquot of the liquid phase was used to analyse the antibiotics residue by HPLC-PDA (High-Performance Liquid Chromatography–Photodiode Array Detection) and ligninolytic enzymes activity.

Then a kinetic assay was carried out in triplicate with the most efficient fungi, *P. ostreatus* and *P. eryngii*, to degrade TCs. . Fungi were inoculated in 500 mL Erlenmeyer flasks with 125 mL of 3% malt extract and TCs 0.1 mM. Aliquots of 1 mL were daily taken for 14 days to analyse TCs degradation kinetic and ligninolytic activity. Finally, fungi growth was obtained by dry weight, and culture media were stored at -18ºC to evaluate residual antibacterial activity.

The statistical analysis was done by IBM SPSS Statistics 23 and a post-hoc test.

2.3 Quantification of antibiotics by HPLC-PDA

Aliquots of growing media were centrifuged at 5000 rpm in Eppendorf tubes and then filtered through 0.45 µm nylon syringe filters. Then 0.5 mL were added to a HPLC vial and mixed with 0.5 mL of methanol, which denaturalized the ligninolytic enzymes and samples were stored at -18ºC to preserve them until analysis. The HPLC system was a Waters 2695 separation module coupled with a Waters 996 photodiode array detector (PDA) (Waters, Milford, MA, USA). Chromatographic separation of TCs was achieved with an Agilent Zorbax SB-C8 (250 x 4.6 mm, particle size 5 µm) column using a gradient elution program with trifluoroacetic acid 10 mM, acetonitrile, and methanol (Table 1). The column temperature was set at 30ºC. The injection volume was 20 µL. The elution profiles were checked at 355 nm. TCs were identified based on both UV spectra (200 – 650 nm) and matching the retention times with commercially available standards (Sigma-Aldrich, St. Louis, MO).

Table 1: Gradient elution program of mobile phases for the separation of tetracyclines by HPLC-PDA.

Time (min)	Flow (mL min^{-1})	TFA (%)	ACN (%)	MeOH (%)
0.0	1.5	95	4	1
7.5	1.5	70	24	6
13.5	1.5	65	28	7
15.0	1.5	95	4	1

TFA: trifluoroacetic acid 10 mM; ACN: acetonitrile; MeOH: methanol

2.4 Enzymatic activities determination

Laccase and Mn-peroxidase (MnP) activities were analyzed in the fungal culture media after mycelia filtration. Laccase activity was spectrophotometrically determined following the oxidation of 2 mM 2,6-dimethoxy phenol in 50 mM sodium acetate pH 5.0 at 477 nm (ϵ = 14600 M^{-1} cm^{-1}).

MnP activity was assayed by Mn^{3+}-malate complex formation in 1 mM $MnSO_4$ in 50 mM malic acid/sodium malate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2 at 270 nm (ϵ = 11590 M^{-1} cm^{-1}). One unit of enzyme activity (IU) is defined as the amount of enzyme which produces 1 μmol of product per minute under the assay conditions.

2.5 Residual antibacterial activity assay

Residual antibacterial activity after fungal removal was based on the inhibition of bacteria growth present in wastewater collected from the wastewater treatment plant of the Autonomous University of Madrid. Bacteria from wastewater was firstly cultivated in tryptone soy broth (TSB) for 24 h at 30 ^{o}C and then diluted in malt extract to reach the optical density at 600 nm (OD_{600}) of 0.1. Then, 1 mL of fungal culture holding TCs residues from the degradation assay was added to 4 mL of bacteria culture media. The increment of absorbance at 600 nm was monitored after 4h of incubation at 30 ^{o}C. Controls without antibiotics and with 0.1 mM of the TCs were performed in parallel. This procedure was performed in triplicate [19].

3. Results and discussion

3.1 Selection of the most degrading fungi

The four fungi grew in presence of TCs(Fig 1). *A. bisporu*s showed the lowest growth both conditions, probably due to culture conditions. Comparing fungi weight medians statistically by IBM SPSS Statistics 23 and the Duncan test ($p < 0,05$), there were no significant differences between fungi control and TCs samples, so it could be said that tetracyclines did not inhibit fungi growth at doses used in this work. In any case *P. ostreatus* and *P. eryngii* showed higher growth than the other two fungi.

Regarding to enzymatic activity, all the fungi produced Laccase and MnP. Despite the low growth, *A.bisporus* produced more laccase than any other fungi in presence or absence of TCs (Fig 2). Meanwhile MnP was less expressed than laccase by all the fungi, probably because of the presence of easily degradable compounds since malt extract is a nutrient-sufficient medium. *P.eryngii* showed a higher expression of MnP in presence of TCs but it was not significant (Fig 2).

Apart from ligninolytic enzymes, fungi might have been expressing other enzymatic systems such as CYP 450, which has been assessed to be involved in PAHs degradation when the experiment was performed in nutrient -sufficient media. [20].

Concerning TCs removal, *P. ostreatus* removed all the TCs under the lowest detection limit (LOD <35 μg L^{-1}) (Fig 3a). *P. eryngii* degraded TCs as well, but less efficiently than *P. ostreatus*. Meanwhile *L.edodes* and *A. bisporus* performed worse than both *Pleurotus* (Fig 3b).

Despite the fact both *Pleurotus* had lower Laccase activities, they managed to degrade TCs better than the other fungi, so that Laccase might not be the only enzyme involved in antibiotic degradation since MnP showed some activity, moreover intracellular enzymes might be contributing to TCs degradation in this assay [14]. So that, a kinetic study was performed to clarify how the extracellular enzymes are evolved into TCs degradation and the existence of a possible correlation between ligninolytic enzymes and TCs degradation.

3.2 Kinetic assay of tetracyclines degradation

Both *Pleurotus* managed to degrade TCs much better than *A. bisporus* and *L. edodes*, so that they were selected to follow the kinetic assay. The mass of both *Pleurotus* was measured at the end of the assay, (14 days) by dry weight, not showing significant difference between the two fungi, neither between controls and samples with antibiotics as was reported in the preliminary assay (Fig 4).

Laccase and Mn-Peroxidase activity is shown in figure 5A and B, respectively. *P.ostreatus* showed lower laccase activity than *P.eryngii*, having its maximum activity peak around day 4 (68 U L^{-1}) in control meanwhile the highest activity was achieved in presence of TCs (89 U L^{-1}) happened at day 3 o even before, since the first analysis was done that day based on previous experiments. From this day, laccase activity started to decrease being almost negligible at day 7. The peak of laccase activity in *P.eryngii* was at day 6 (152 U L^{-1}) both in presence or absence of TCs. So, *P. eryngii* produced 1.7 folds more laccase than P. ostreatus. In addition, P. eryngii produced laccase more time than P. ostreatus. (Fig 5)

Regarding MnP, just the opposite occurred, *P.ostreatus* showed higher levels, despite showing levels under 10 UL^{-1} both fungi. *P.ostreatus* expressed more MnP in absence of TCs, having its maximum peak at day 6 (8 U L^{-1}), while the treatment with TCs achieved the maximum peak the day before (5 U L^{-1}). This enzyme behaved irregularly along the 17 days of the assay, showing increases and decreases along it (Fig 6). The irregular behaviour of MnP has been reported before by some researchers in assays with *Pleurotus spp*, mainly in liquid media [21]. Some others authors suggested that apart from ligninolytic system, there could be also acting some other enzymatic systems like CYP 450 such as Golan-Rozen et al (2011), who tried to clarify the role of both systems degrading carbamazepine [22]. They concluded that although MnP was involved, CYP450 played a very important role in degrading carbamazepine.

Percentage of TCs degradation by *P. ostreatus* and *P. eryngii* are shown in figure 6. Both fungi, *P.ostreatus* and *P. eryngii*, degraded CTC under LOD at day 10 showing both fungi a similar ability to degrade CTC. *P. ostreatus* reached 93 and 56% of OTC and TC degradation respectively. Similar values of degradation were obtained by *P. eryngii* with complete degradation of TC and 56% of CTC degradation. However, *P. eryngii* was more effective to degrade TCs because get the greatest TC and OTC degradation before *P. ostreatus*.

Laccase levels could be correlated to TCs removal since it was secreted and present in the medium when the antibiotics were removed, especially in *P.eryngii*, although MnP was present as well but in much lower concentration. So, it was difficult to clarify which enzyme was mostly responsible for TCs degradation. Wen X et al. (2010) [23], achieved a 72% degradation of TC and 84% of OTC using crude MnP (40U/L) from *P. chrysosporium.* having the first MnP activity on day 3 and a peak on day 10. Migliore et al. (2012) reported complete degradation of OTC by *P.ostreatus* after 14 days, but they could not relate the OTC removal to laccase since they evaluated the performance of purified laccase and no significant differences were found between the degradation rate with or without laccase in the culture medium [14] . Moreover, OTC uptake was proved after day 3 of exposure followed by an efficient degradation of the drug.[14] , Sun et al. found a high rate of TC removal with commercial extracellular fungal laccase from *P. ostreatus* coupled with the redox mediator 1-hydroxybenzotriazole (HBT) [24]. Taheran et al.(2017) reported a 58.3% CTC degradation using immobilized laccase in continuous mode. [25]

3.3 Residual antibacterial activity

Residual antibacterial activity of the growing media after TCs degradation assay was done to evaluate the efficiency of both *Pleurotus* degrading TCs (Fig. 7). Tetracyclines without fungal treatment kept a high residual antibacterial activity, 95% with respect to absence of TCs. Residual antibiotics and metabolites of TCs produced by *P. eryngii* inhibited bacterial growth at the same rate than TCs without fungal treatment. In contrast, *P.ostreatus* was more efficient than *P. eriingy* to reduce the residual antibiotic activity of the growing media and inhibit the bacterial growth around 75% compared to the control.

TCs were degraded to some compounds which had antibacterial activity. Degradation products could have a very similar chemical structure that might not have lost the tetracyclic nucleus naphthacene where the antibacterial activity relies on, although its pharmacokinetic might have been affected (Yang et al., 2017). Other earlier works regarding tetracyclines degradation by laccase showed equivalent results. The residues from the TCs removal using laccase kept antibacterial activity almost as the same level as the antibiotics themselves [26]; [27]. Further research should be done concerning those compounds with antibacterial activity.

4. Conclusions

Spent mushroom substrates could be a very interesting material to remove TCs, specially *P.eryngii* seemed to be more efficient in those experimental conditions. So, it could be an environmentally friendly way to bioremediate water before being released into natural water.

The powerful enzymatic system of genus *Pleurotus* makes them an excellent option to develop an innovative technology to produce TCs degradation in liquid wastes.

Clarifying the role of each of the extracellular peroxidases is a key issue to develop new clean and environmentally friendly procedures to purify wastewater.

5. Acknowledgments

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6. References

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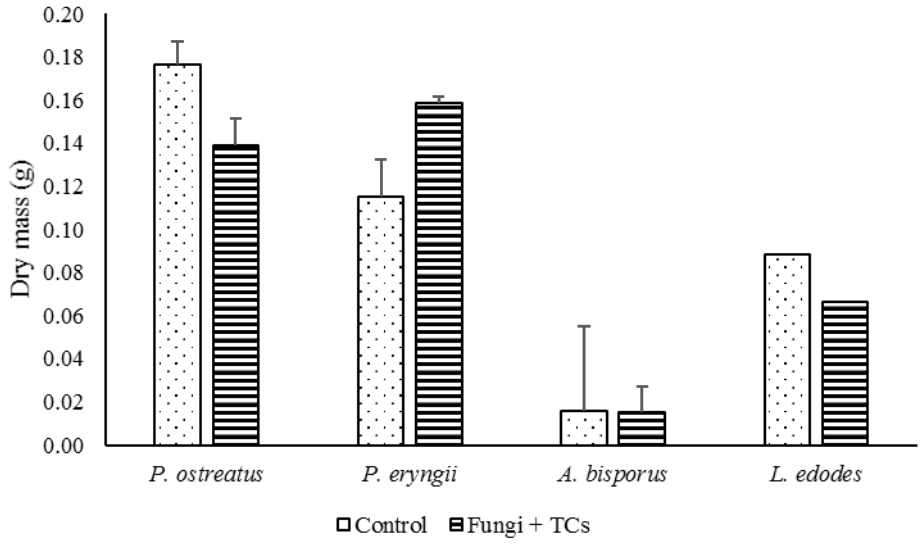

Fig 1 Fungi dry mass (g) measured after 8 days of growth in liquid medium of malt extract . Error bars represent the standard deviations of repetitions ($p < 0.05$) (n=3)

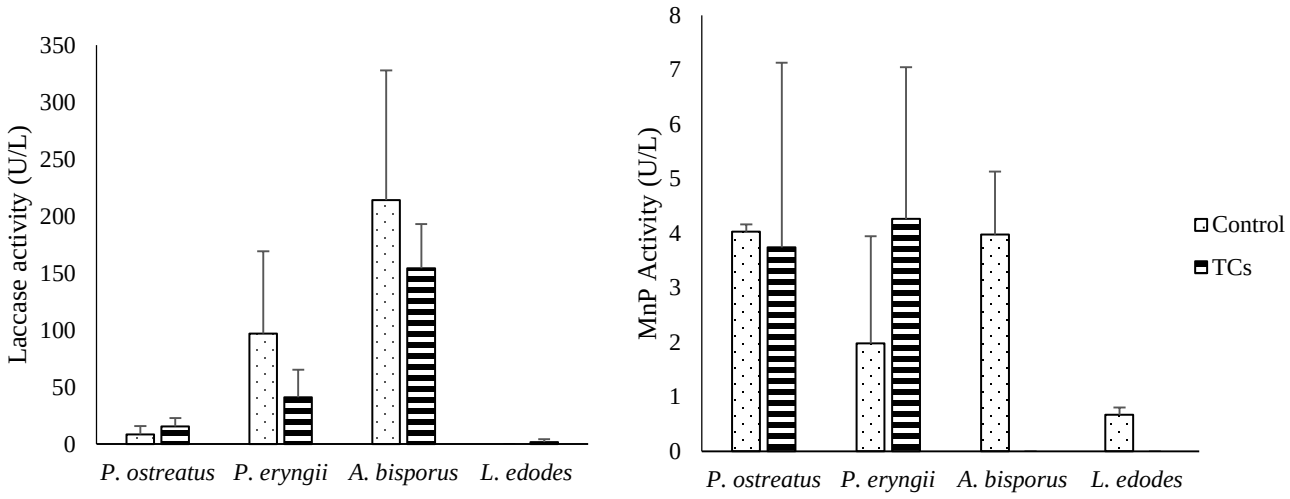

Fig 2 Laccase (a) and MnP (b) enzymatic activity of the studied fungi after 8 days. Error bars represent standard deviation

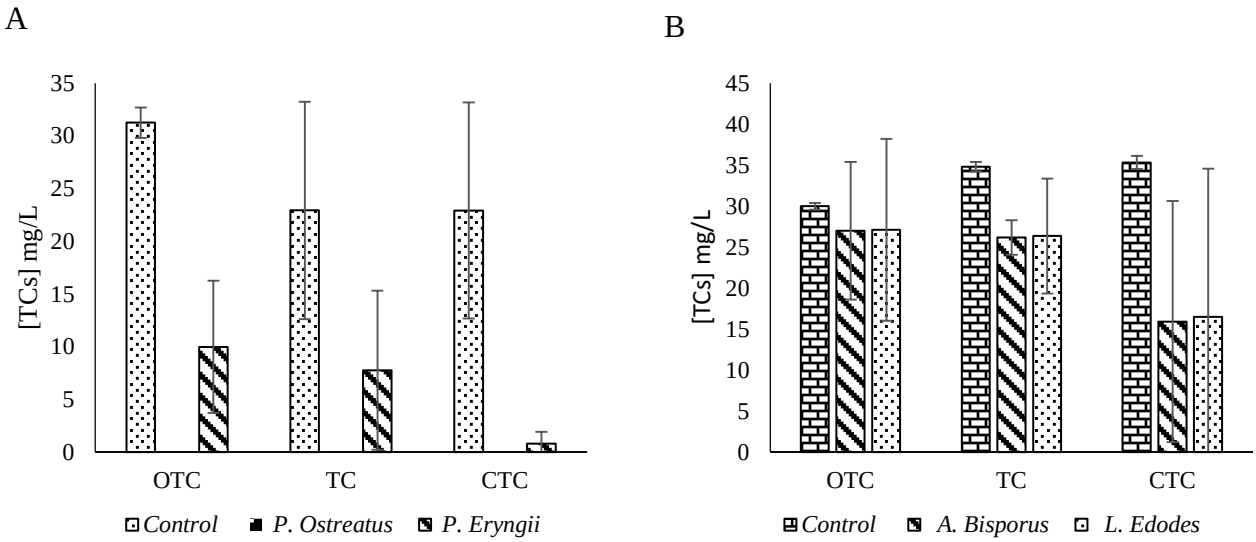

Fig 3 Tetracyclines concentrations in controls, *Pleurotus ostreatus* and *Pleurotus eryngii* presence (a), in controls, *Agaricus bisporus* and *Lentinula edodes* (b). Error bars represent standard deviations (n=3)

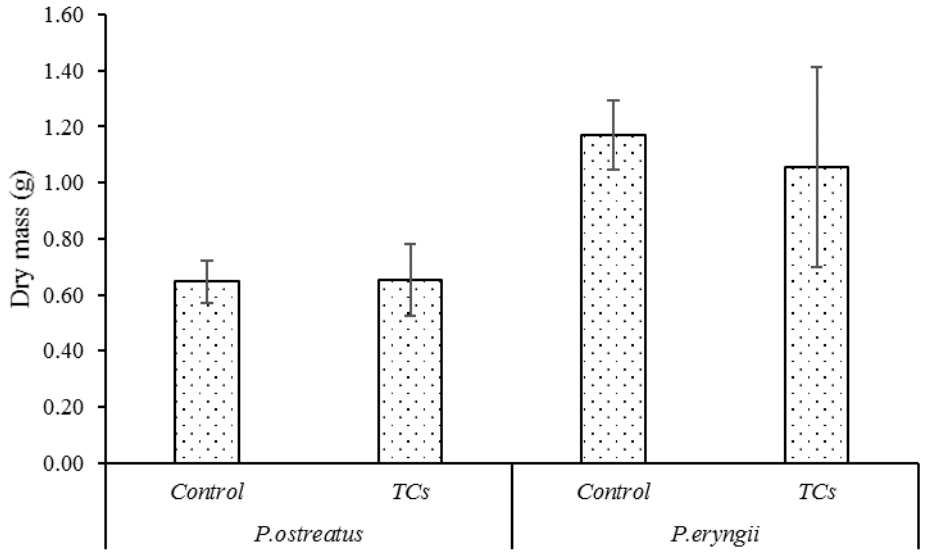

Fig 4 Fungi dry mass (g) of *Pleurotus ostreatus* and *Pleurotus eryngii* after 14 days of growth in liquid medium of malt extract. Error bars represent the standard deviation (n=3)

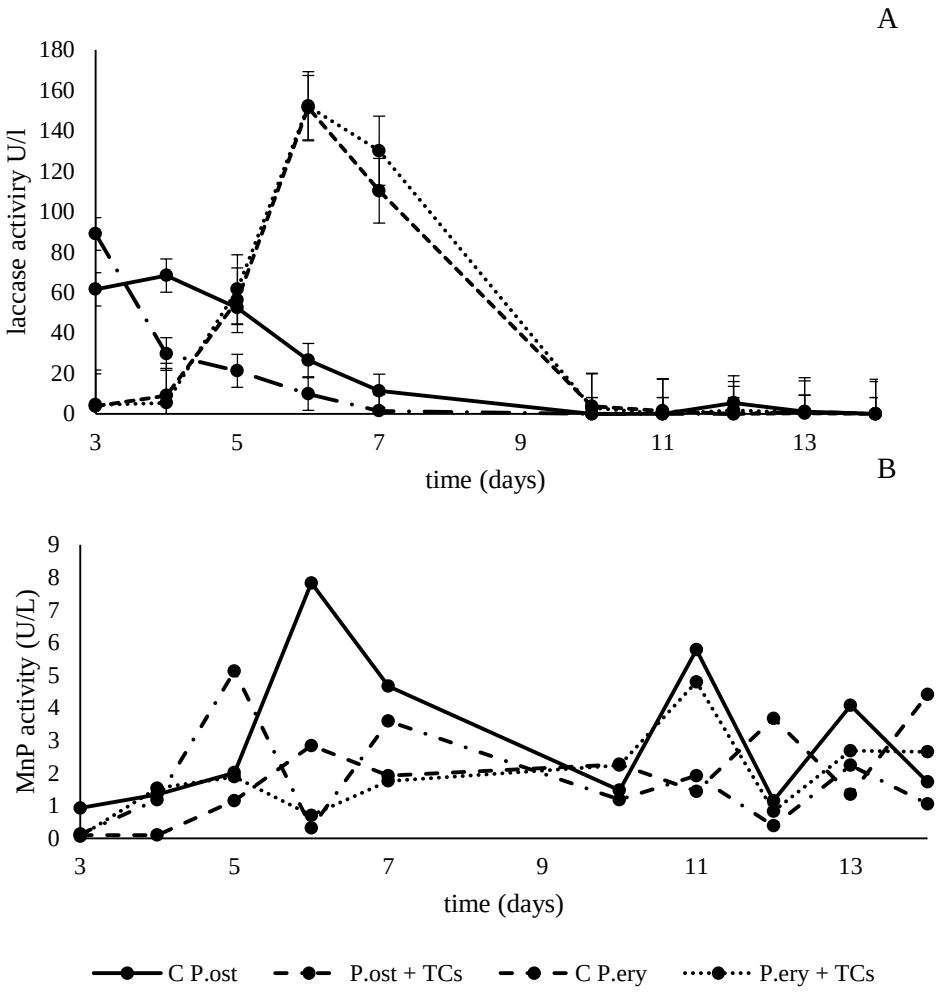

Fig 5 Laccase (A) and MnP (B) enzymatic activity of *Pleurotus ostreatus* and *Pleurotus eryngii* during 14 days of growth in liquid medium of malt extract. Error bars represent the standard deviation (n=3)

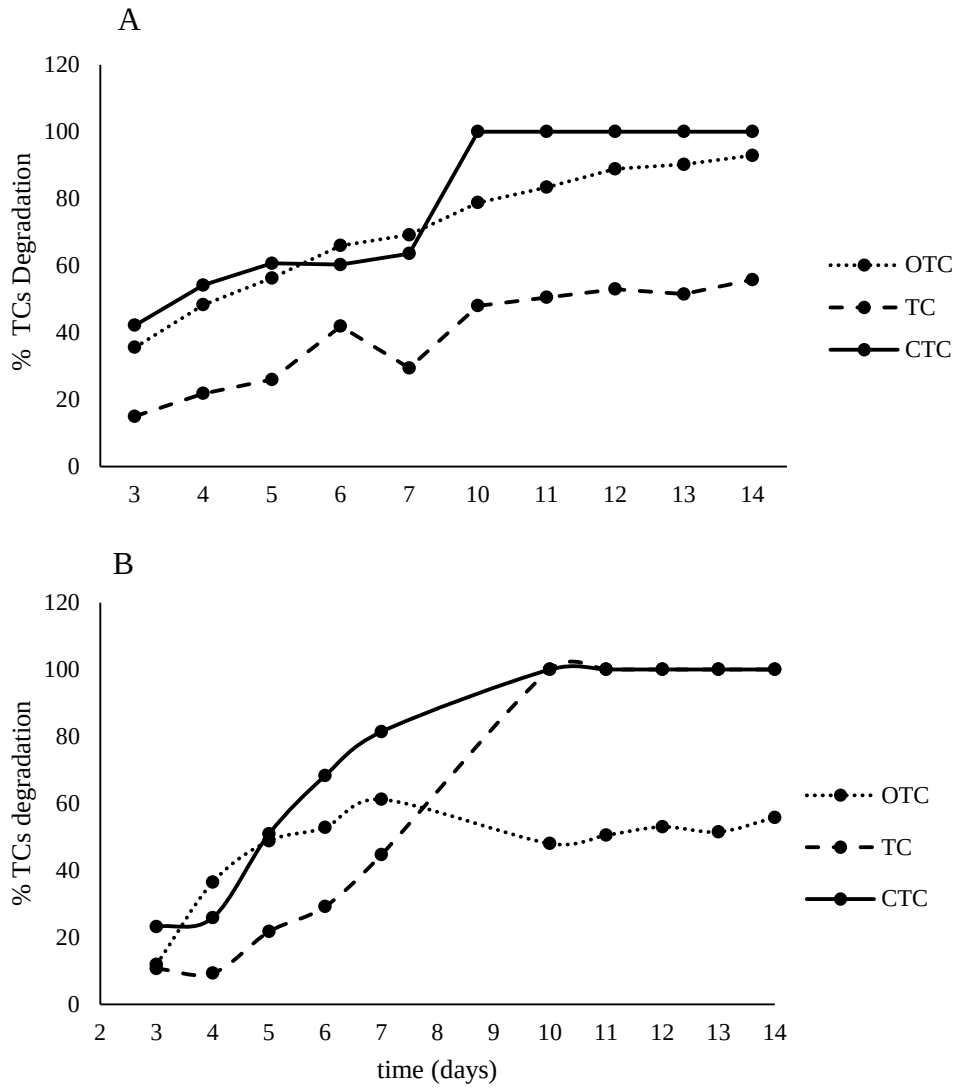

Fig 6 Degradation rate of TCs by *P.ostreatus* (A) and by *P.eryngii* (B)

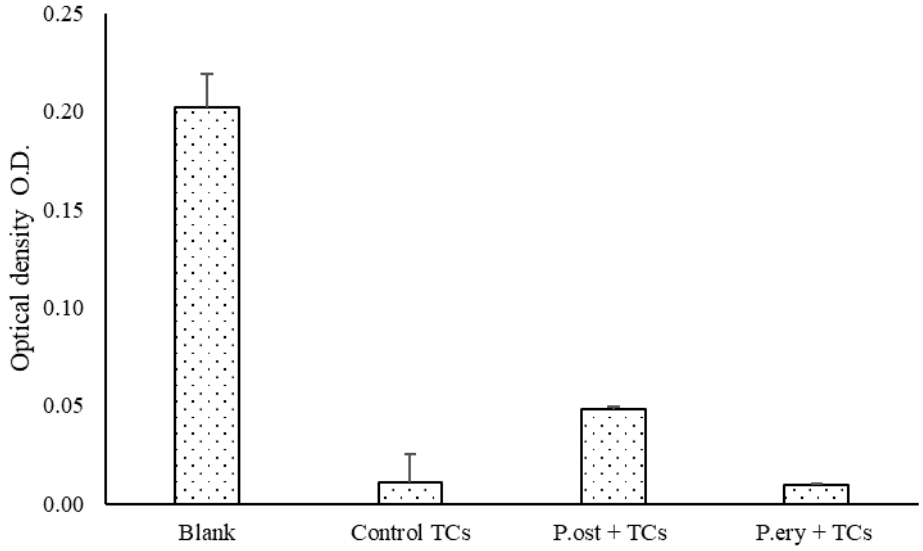

Fig 7 Residual antibacterial activity in TCs samples after the TCs degradation by *Pleurotus ostreatus* and *Pleurotus eryngii*