Removal of Sulphonamides from urban wastewaters by fungi of genus *Pleurotus*

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Abstract

Antibiotics are widespread in the environment generating multiresistant bacteria, a threat to human health. The inefficiency of the traditional wastewater cleaning processes allows them to reach rivers, lakes, groundwater, soils, croplands and finally the food chain. Finding effective and environmentally friendly techniques to remove antibiotics before getting to natural ecosystems is the utmost importance. Ligninolytic fungi are capable of degrading lignin and organic pollutants including pharmaceuticals and personal care products. Some fungi of genus *Pleurotus* are cultivated for human consumption co-generating massive amounts of spent mushroom substrate, previously proved to efficiently degrade organic pollutants thanks to the active mycelia it keeps. The assessment of *Pleurotus* to remove antibiotics becomes a valuable research topic to develop innovative technology to properly treat wastewater while an agricultural waste is valorised and re-used as a cost-effective source of ligninolytic fungi. Along this work, a family of the most prescribed antibiotics, sulfonamides (SAs), was tested to assess the removal efficiency of two species of genus *Pleurotus* (*P. ostreatus* and *P. eryngii*). Firstly, a kinetic assay was performed in liquid medium (malt extract) for 17 days evaluating extracellular enzymatic activities and the daily decrease of SAs concentrations. Secondly, antimicrobial activity of SAs residues was evaluated resulting that they allow bacterial growth, thus SAs removal was effective. Finally, an approach to a real situation was carried out by testing both *Pleurotus* grown in a semi-solid medium (spent substrate- malt agar) to remove SAs from effluent wastewater whose antibiotic concentrations were ranged between 400 and 20 ng L^{-1}. A high percentage (93%) of SAs was successfully removed in 24h by *P.ostreatus*.

Keywords: antibiotics, biodegradation, agricultural waste, wastewater.

1. Introduction

Antibiotics are widely prescribed to treat and prevent infectious diseases in humans and livestock worldwide. According to the European Medicines Agency in 2015, 8,361 tons of antibiotics were commercialized in Europe for veterinary use, while 36,982 tons were marketed in USA in 2016 [1] and 105,000 tons in China in 2015 [2]. Sulphonamides (SAs) are wide spectrum antibiotics whose sales only for veterinary use in UE were 978.4 tons in 2016, being one of the most detected antibiotics in different water ecosystems in Europe [3]. Most antibiotics are only partially metabolized, about 25–75% of the intake is released not having suffered any kind of degradation [4], so that they can be easily found in wastewater, soil, sewage sludge and cattle manure, being some of them very persistent [5].

Antibiotics reach the food chain by adding cattle manure or sewage sludge to croplands as soil organic amendments and fertilizers, as well as watering the crops with polluted water. Antibiotics have already been reported in natural water, soils and crops mainly due to the inefficiency of current treatments in wastewater treatment plants.[6]; [7];[8]. Those so widespread sub-lethal concentrations of antibiotics

promote resistant and multiresistant bacteria which are a real threat for human health [9]. Moreover, antibiotics could also affect the bacteria involved in biogeochemical cycles and the ones responsible for supporting biological processes in water and soil [5].

Pharmaceuticals usually end up in wastewater, which after being purified through a conventional wastewater treatment, remain polluted. The inefficiency of current processes allows releasing high concentrations of antibiotics, which can reach rivers, lakes, even groundwater. It is the utmost importance to find a creative, effective, and environmentally friendly way to treat properly wastewater and, ligninolytic fungi could be a good choice thanks to their powerful enzymatic system, capable to oxidize a great number of substrates. Ligninolytic fungi can segregate extracellular enzymes capable to degrade efficiently lignin and other organic compounds. Those fungi are saprophytes, some of them primary degrading fungi, that is the case of *Pleurotus* spp. [10].

Ligninolytic fungi have been used to successfully degrade organic contaminants like synthetic dyers, polycyclic aromatic hydrocarbons (PAHs), heavy oils, or pharmaceuticals including antibiotics [11]; [12-14]; [15]. Some of these fungi are cultivated for human consumption with the consequent co-generation of enormous amounts of spent mushroom substrate [16]; [17]. Previous works have proved the usefulness of spent mushroom substrate to remove organic pollutants and the key role of the active fungal mycelium in this process [18]; [13]; [19]. Therefore, the assessment of edible fungi to degrade antibiotics is a valuable research topic to develop an innovative technology to get antibiotics removed and simultaneously is the first step in the valorisation and re-use of an agricultural waste as cost-effective source of ligninolytic fungi.

The main objective of this work is to assess the potential for sulphonamides removal of two of the most cultivated edible fungi worldwide, *P. ostreatus* and *P. eryngii*. Commercial strains of *P. ostreatus* and *P. eryngii* were tested. An enzymatic kinetic assay was done to correlate fungal ligninolytic activity with antibiotic removal. Residual antibacterial activity was tried out to assess the reclaim process. Finally, an approach to a real scene was done, testing the capability of *P.ostreatus* and *P.eryngii* to remove antibiotics from the real effluent of a wastewater treatment plant.

2. Materials and methods

2.1 Chemical and reagents

Antibiotics were purchased from Sigma Aldrich (St.Louis, MO, USA). (Sulfathiazole (STZ, 99%), sulfapyridine (SP, 95%), sulfamethoxazole (SMX, 99%), Sulfathiazole -$^{13}C_6$ and Sulfamethoxypyridazine-d_3; Across Organics (Geel, Belgium) (Sulfadiazine (99%, SDZ)) and Alfa Aesar (Sulfamethazine (SMZ, 99%)). 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, methanol from Fisher Scientific (Madrid, Spain), trifluoracetic acid and acetonitrile from Scharlaub (Barcelona, Spain), ethylenedinitrilotetraacetic-acid-disodium-salt-dihydrate from Merck (Darmstadt, Germany) and ethyl acetate from VWR International (Radnor, PA, USA). All chemical reagents were of analytical grade and solvents of HPLC grade.

Malt extract and malt extract agar (MEA) were purchased from Sigma Aldrich (St.Louis, MO, USA) while tryptic soybeans extract (TSB) was from Fluka Analytical (Madrid, Spain).

2.2 Kinetic assay

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 (PAHs) [12]. A commercial strain of *P.eryngii* was purchased from Gurelan Mycelium located in Huarte (Navarra, Spain). Mycelia grew in controlled laboratory conditions in malt extract agar (MEA) for 7 days, aliquots from this culture were used as inoculum for further cultures.

A kinetic antibiotic degradation assay was carried out in triplicate with *P.ostreatus* and *P.eryngii*. Fungi were inoculated in 500 mL Erlenmeyer flasks with 125 mL of 3% malt extract and SAs 0.1 mM by adding three mycelial pieces of 5 mm of diameter from MEA culture. Aliquots of 1 mL were daily taken for 17 days to analyse antibiotics degradation kinetic and ligninolytic activity. Fungi growth was determined by dry weight at the end of the assay.

2.3 Quantification of antibiotics

2.3.1 Quantification of antibiotics in fungi kinetic degradation assay

Aliquots of growing media were centrifuged at 5000 rpm in Eppendorf tubes and then filtered through 0.45 μm nylon syringe filters. 0.5 mL of the extract were mixed with 0.5 mL of methanol in a vial and 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). Chromatographic separation of SAs (Summa et al., 2015) was achieved with a Phenomenex Luna C18 (250 mm × 4.6 mm; particle size 5 μm) column, using a gradient elution program with 20 mM ammonium acetate adjusted to pH 4.50 with acetic acid and acetonitrile:methanol (1:1) at a flow rate of 0.9 mL min^{-1} (Table 1). The column temperature was set at 40 ^{o}C. The injection volume was 20 μL. The elution profiles were monitored at 270 nm. SAs were identified based on both UV spectra and retention times of commercially available standards (Sigma-Aldrich).

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

Elution gradient for Sulphonamides			
Time (min)	Flow (mL min^{-1})	NH4Ac (%)	ACN:MeOH (1:1) (%)
0.0	0.9	85	15
17.0	0.9	71	29
23.0	0.9	67	33
26.0	0.9	67	33
30.0	0.9	85	15

NH_4Ac: Ammonium acetate 20 mM adjusted at pH 4.50 with acetic acid.

2.3.2 Quantification of antibiotics in biofilter assay for real wastewater remediation

Effluent from an urban wastewater treatment plant (WWTP), placed at the South of Spain, was sampled and analysed by UHPLC/MS/MS system consisted of an Acquity UPLC module from Waters (Mildford, MA, USA) coupled with a Waters TQD triple quadrupole detector also from Waters. The detector was worked in MS/MS mode in positive electrospray. The column used was UPLC BEH C18 (100 mm x 2,1mm; particle size 1,7 μm), the temperature was fixed at 45ºC. The injection volume was 10 μL. The gradient program is shown in table 2. Sulfathiazole -$^{13}C_6$ and Sulfamethoxypyridazine-d_3 were used as internal standards.

SAs residues concentration in sampled water ranged between 423 and 20 ng L^{-1}, since conventional wastewater treatments are not efficient enough to remove pharmaceuticals and personal care products [20]; [21]; [22].

Table 2: Gradient elution program of mobile phases for the separation of sulphonamides by UPLC-MS

Elution gradient for Sulphonamides			
Time (min)	Flow Rate (μL min-1)	A1 (%)	B2 (%)
0.0	0.500	8.0	92.0
5.00	0.500	15.0	85.0
9.00	0.500	55.0	45.0
12.0	0.500	8.0	92.0

A1: Acetonitrile 0.1% Formic acid; B2: 0.2% oxalic acid+0.2% Formic acid in MilliQ water

2.4 Enzymatic activities determination

Laccase and Mn-peroxidase (MnP) activities were measured in the fungal culture media after mycelia filtration. Laccase activity was spectrophotometrically determined by 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}) and MnP activity 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 antibiotic activity after fungal removal was based on the inhibition growth of bacteria present in wastewater collected from the WWTP of the Autonomous University of Madrid. Bacteria were firstly grown in tryptone soy broth (TSB) for 24 h at 30oC and then diluted in malt extract to reach the OD600 of 0.1. Then, 1 mL of fungal culture from the degradation assay was added to 4 mL of bacteria culture media. The absorbance increment at 600 nm was monitored after 4h of incubation at 30oC. Controls without antibiotics and with 0.1 mM of SAs were performed in parallel. The assay was done in triplicate [23].

2.6 Removal of antibiotics from the effluent of a wastewater treatment plant.

An approach to bioremediate real wastewater sampled from the urban WWTP was done to evaluate SAs degradation and ligninolytic activity of *P.ostreatus* and *P.eryngii.* The assay was carried out on a semi-solid medium, straw-based spent substrate with MEA. Fungi were previously cultivated in malt extract in Erlenmeyer flasks of 1000 mL, in orbital shaking at 160 rpm for 15 days at 28ºC, in darkness. Then, five fungal pellets were grown in Teflon containers filled with their own spent substrate and 250 mL of malt-agar for 3 days. Teflon containers were sterilized in autoclave at 121ºC for 20 min before inoculation. A control without fungi was carried out in the same conditions. 400 mL of sampled water were introduced in the containers by a peristaltic pump at a flow of 10 mL min^{-1}. The assay took 48 h and aliquots of 30 mL were taken, the first one when water started to flow out, which took 30 min, and 1h, 2h, 4h, 6h, 24h, 30h and 48h. Then they were concentrated and purified by a SPE procedure using lipophilic/hydrophilic balanced Oasis HLB cartridges and AEDT-McIlvaine buffer (50/50) at pH 4. Analytes were eluted with ethyl acetate, then evaporated to dryness under 12 psi N_2 flow and 42ºC, and finally reconstituted in 0.25 mL of methanol/water (15/85) to be analysed by UHPLC-MS. Ligninolytic enzymes activity was analysed when the aliquots were extracted.

3. Results and discussion

3.1 Kinetic assay of antibiotic degradation

Both *Pleurotus* underwent the kinetic assay for 17 days. *P.eryngii* showed a higher growth than *P.ostreatus* at day 17 when fungal mass was measured. There was no significant difference between controls and antibiotic samples (Fig 1) however, there seemed to be a slightly higher growth of *P.ostreatus* in presence of SAs, which agreed with the results obtained by de Araujo et al [24].

Lacasse was the most produced extracellular enzyme during the kinetic assay by both fungi (Fig. 2). *P.ostreatus* showed a lower laccase expression than *P.eryngii. P. ostreatus* reached the activity peak at day 4, showing higher levels in control, 68 UL^{-1} than in presence of SAs (Fig.2). Other researchers have reported the maximum activity at day 5 in malt extract, but in presence of oxytetracycline, 110 UL^{-1}[15]. Meanwhile *P.eryngii* showed higher activity in presence of SAs reaching the greatest peak of 215 UL^{-1} at day 7. Laccase was not detected at day 14 in both fungi neither in presence of SAs nor in absence (Fig 2). However, de Araujo et al reported 410 UL^{-1} after 15 days in a 1% wheat bran extract liquid medium [24], or Grupta et al who obtained 3976 UL^{-1} in a semisolid fermentation with wheat straw substrate after 15 days as well [25]. In contrast, MnP activity was significantly lower for both *Pleurotus* studied.

P.ostreatus was able to remove SAs very efficiently (Fig.3). It is remarkable that the decrease in concentration of all the five SAs started at day 4, coinciding with the maximum peak of laccase. When its activity was almost undetectable around day 10 (1 UL^{-1}), the rate of removal was still high, a 72% of

SDZ, a 62% of STZ, a 77% of SP, a 54% of SMZ and a 63% of SMX. Those removals might be attributable to laccase activity, since laccase has been reported to degrade SAs by other researchers [26], and the degradation seems to be dependent on the N-heterocyclic moiety attached to the sulphonamide core structure [27], but the degradation occurred from day 10 until the end of the assay might be attributable to other enzymes including the intracellular system CYP 450.

Despite showing a different pattern of SAs degradation, *P.eryngii* removed the antibiotics efficiently as well; SDZ and STZ concentrations had a low and progressive decrease until day 5, having a period of hardly any degradation until day 12 followed by a sudden drop until day 14, when the removal rate was 65 and 78 % respectively. Meanwhile SP, SMZ and SMX showed a slow decrease until day 12, then the same sudden drop until day 14 occurred, reaching removals of 89, 74 and 71% (Fig 3). Laccase production seemed to be enhanced by SAs, thus it might be responsible for the slow and progressive removal of SAs, but it seemed not to be related to the sudden drop which started day 12 since its levels were extremely low at day 12, when other enzymes could be acting (Fig 2).

On the other hand, MnP could not be correlated with any antibiotic degradation because of its low activity and irregular behaviour along the assay. This fact made difficult to know the role of MnP in SAs degradation (Fig 3). Although it might be involved in any way, since it is a powerful oxidase. Some researchers have reported similar MnP behaviour in *Pleurotus spp* in aqueous media [28]. De Araujo et al. could not correlate extracellular laccase and MnP activity with the degradation of trimethoprim and sulfamethoxazole, however they concluded that biodegradation by extracellular ligninolytic enzymes seems to be the main pathway to remove antibiotics, since the biosorption of trimethoprim and sulfamethoxazole by *P.ostreatus* were 14% and 11% respectively[24].

MnP is very powerful thanks to its high redox potential, all the antibiotics tested have simpler structures than lignin, so that, its irregular expression could also be due to the expression of other enzymes with lower redox potential such as Laccase, or even due to CYP 450 expression since malt extract is a rich-nutrient medium [29] . What is more, some earlier works have demonstrated PAHs degradation correlated with some other hydrolases and transferases [30]. In addition, other enzymes segregated by ligninolytic fungi might also be involved, such as the case of aryl-alcohol oxidase, aryl-alcohol dehydrogenase, quinone reductase or glyoxal oxidase [31].

It might be concluded that both *Pleurotus* degraded effectively SAs, reaching concentrations very near LOD (SMX 12 µg L^{-1}, STZ 11 µg L^{-1}, SP 4 µg L^{-1}, SDZ 3 µg L^{-1} and SMZ 21 µg L^{-1}) and even though *P.eryngii* started degrading all the SAs slower than *P.ostreatus*, at the end of the assay the removal rates were slightly higher.

3.2. Residual antibacterial activity

The efficiency of both Pleurotus removing SAs was evaluated by a bacterial growth inhibition test, showing that bacterial growth was inhibited a 25% by SAs residues of *P.eryngii* and around a 50% in the case of *P.ostreatus*, compared to controls without antibiotics (Fig.4). So that, *P.eryngii* seemed to be more efficient degradant since 75 % of the bacterial activity disappeared although *P.ostreatus* seemed to be faster.

3.3. Degradation of antibiotics from the effluent of a real wastewater treatment plant.

Spent substrate with agar-malt biofilter model assay showed very promising results to remove SAs from the effluent of the WWTP, since some of the antibiotics found in the water analysis were removed in 24 h, especially sulfamethoxazole (SMX) which was the most abundant in the sampled water. SMX was found in a concentration of 423.9 ng L^{-1}, being the concentration of the other antibiotics below 50 ng L^{-1}. Laccase production of *P.ostreatus* was progressively increasing from 23 UL^{-1} to 510 UL^{-1} after 48 h (fig 4), which was much higher than the activities occurred in the kinetic assay (68 UL^{-1} at day 4) (fig 4) due to the presence of wheat straw, which is the fungi own substrate, in agreement with Grupta et al.[25]

P.ostreatus was able to remove the 93% of SMX in 24 h, showing the highest removal rate in the first two hours (Fig. 5). 79 % of SP was removed in the same period from an initial concentration of 20.8 ng L^{-1}. Such a fast removal means that *P.ostreatus* might be an excellent choice to bioremediate polluted water before releasing to the environment, however further research must be done.

Some other researchers have done similar experiments to remove other pharmaceuticals from the effluent of WWTP such as Křesinová Z. et al (2017), who studied the removal of some endocrine disruptors using fortified effluent WWTP water, both at laboratory scale and pilot bioreactor scale. They concluded that *P.ostreatus* could remove endocrine disruptors achieving removal rates of 41% in 3h at laboratory scale and 78% in 2h at pilot bioreactor scale [32]. The removal rates obtained in this work (Fig.6) were of 83% of SMX in 4h. Palli et al (2017) worked with a hospital wastewater and three pharmaceuticals, atenolol, diclofenac and ketoprofen using to assess the removal by *P.ostreatus* in a fluidized bed bioreactor. They obtained a completely removal of diclofenac in 18h while a 36% of ketoprofen and an 8% of atenolol in 42h [33]. As a result, *P. ostreatus* could be a proper choice to be used in water cleaning systems, but as not all the pharmaceuticals are removed the same, much more research must be done in this topic.

Meanwhile, *P.eryngii* had satisfactory results but not as effective as *P.ostreaus*. The removal rate of SMX was 67 % in 24 h, getting 79 % in 30 h, while the 94 % of SP was removed in the same period. SDZ and STZ were undetected at 30 h. *P.eryngii* seemed to be slower SAs degradant, despite being more efficient to remove SP. Laccase production was lower as well, even though it was increasing progressively until 41 UL^{-1} at 24h but reaching 60 UL^{-1} at 30h, coinciding with the highest rate of SP and SMX degradation. MnP was hardly segregated by both fungi in this assay, so laccase showed a key role regarding SAs removal.

Control biofilter showed some decrease in antibiotics concentration owed to the adsorption on the substrate, so further research should be done to study this mechanism. It is really interesting the fact that these fungi together with their substrate, which is a very abundant organic residue, could efficiently remove antibiotics from polluted water.

Those findings are really promising since the removal of SAs at real concentrations in treated wastewater happened in a really short period, so biofilters with ligninolytic fungi to reclaim water before releasing to the environment could be an outstanding choice, mainly *P.ostreatus* which seems to be the most powerful degradant fungus, which agrees with other researchers results [32];[15].

4. Conclusions

Both *P.ostreatus* and *P.eryngii* were able to remove the sulphonamides used in this work fast and efficiently. Laccase could be correlated to SAs degradation in the conditions of this work, either in the kinetic assay or biofilters ones.

P.ostreatus appeared to be the most powerful fungus of both *Pleurotus*, not only in the kinetic assay but in the biofilter to remove SAs from the effluent water of a WWTP, removing them at high and low concentrations. *P.ostreatus* was able to remove most of SAs found in the effluent water of a WWTP in a period of 24h time.

A biofilter with its own commercial spent substrate could be an effective and environmentally friendly way to clean wastewater and valorise an organic residue coming from the commercial cultivation of the fungi.

5. Acknowledgements

This work was supported by the Ministry of Science and Innovation of Spain (Project AGL2016-78490-R). Dr. García-Delgado thanks the Spanish Ministry of Economy and Competitiveness for his post-doctoral contract (JCFI-2015-23543). We thank the Laboratorio Regional de Salud Pública Comunidad de Madrid, for their analytical support and knowledge.

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Figure caption

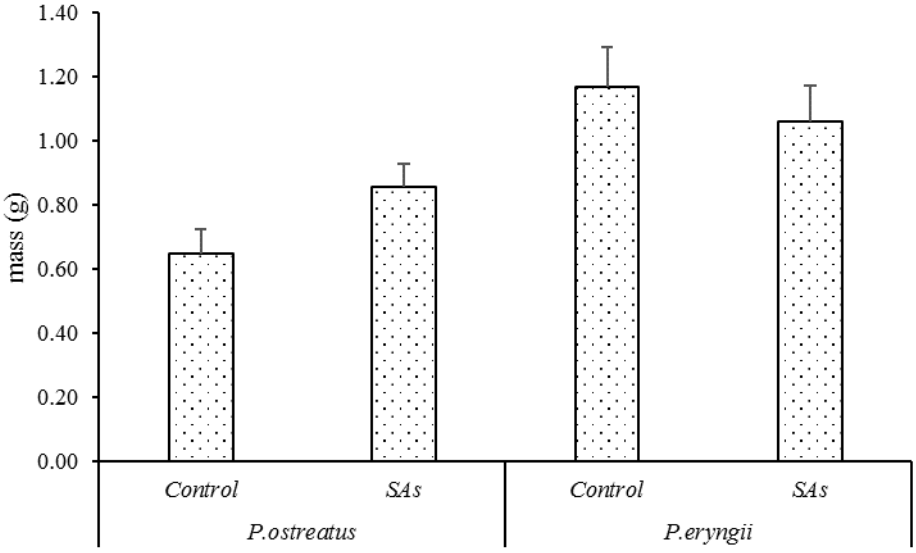
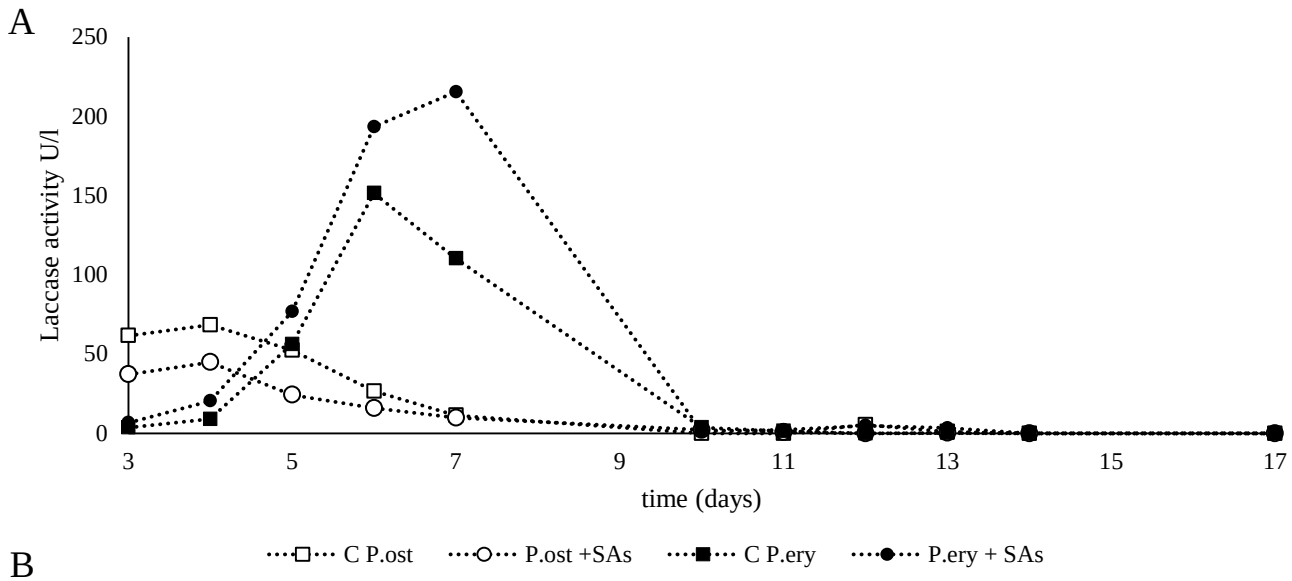


Fig 1 Fungi dry mass (g) of *Pleurotus ostreatus* (a) and *Pleurotus eryngii* (b) at the end of the assay. Error bars represent the deviation of repetitions



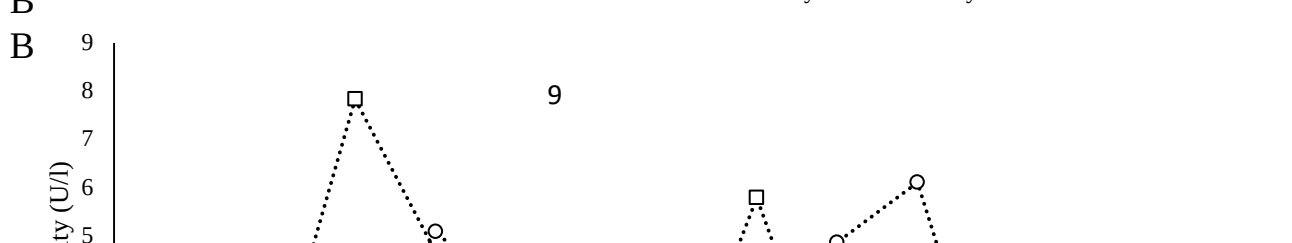


Fig 2 Enzymatic activity Laccase (a) and Mn-Peroxidase (b) of *Pleurotus ostreatus* and *Pleurotus eryngii* during the assay

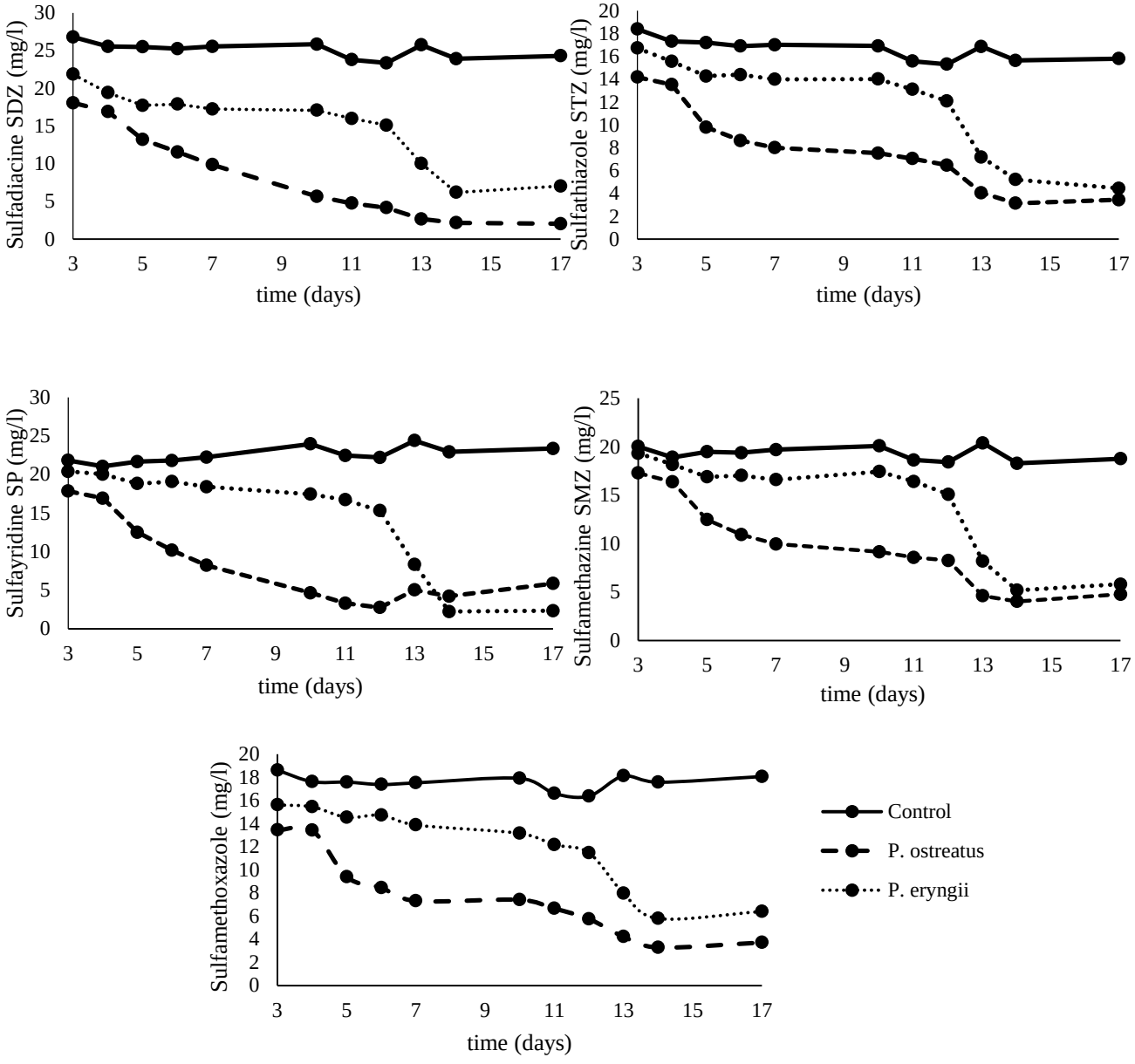


Fig 3 Daily evolution of sulfonamides concentrations for 17 days of kinetic assay. A) Sulfadiacine (SDZ). B) Sulfathiazole (STZ). C) Sulfapyridine (SP). D) Sulfamethazine (SMZ). E) Sulfamethoxazole (SMX).

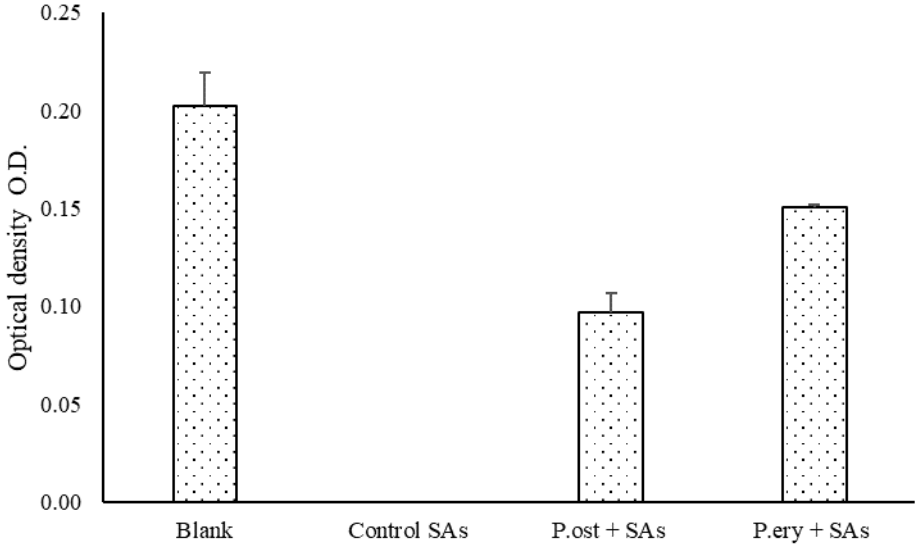


Fig 4 Residual antibacterial activity in SAs samples after their degradation by *Pleurotus ostreatus* and *Pleurotus eryngii*

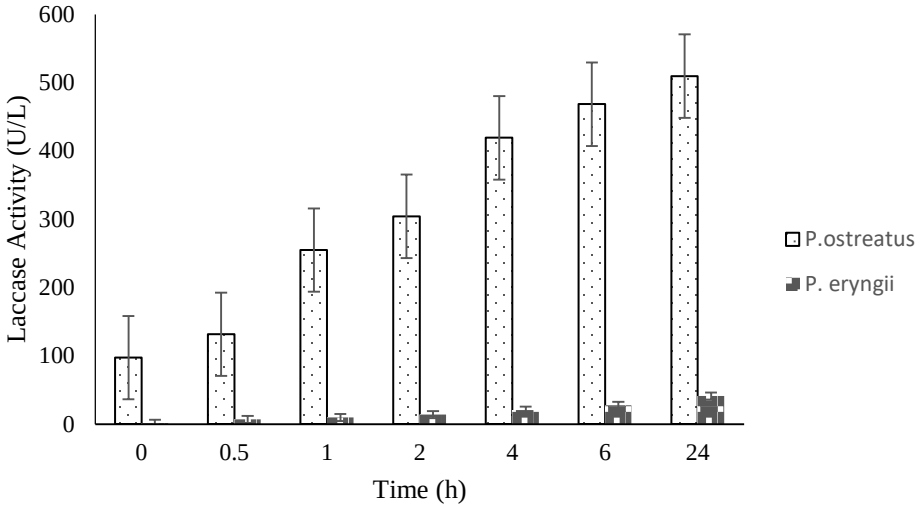


Fig 5 Laccase enzymatic activity of the studied fungi during the biofilter assay

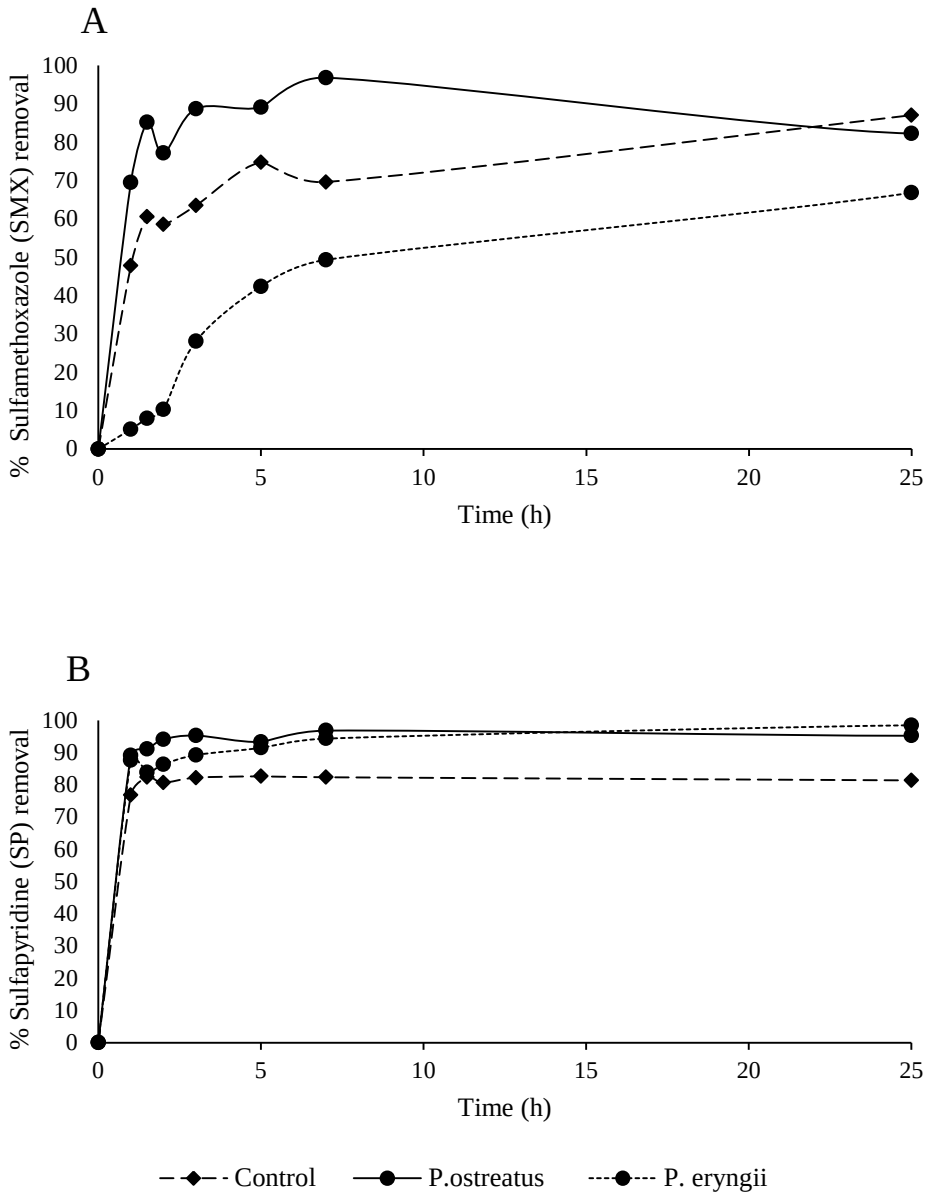


Fig 6 Removal of sulfonamides: A) sulfamethoxazole (SMX); B) sulfapyridine (SP)