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An assessment of *Pleurotus ostreatus* to remove sulfonamides, and its role as a biofilter based on its own spent mushroom substrate

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Abstract

A double strategy based on the removal of sulfonamide antibiotics by *Pleurotus ostreatus* and adsorption on spent mushroom substrate was assessed to reclaim contaminated wastewater. *P. ostreatus* was firstly tested a in liquid medium fortified with five sulfonamides: sulfamethoxazole, sulfadiazine, sulfathiazole, sulfapyridine and sulfamethazine, to evaluate its capacity to remove them, to test for any adverse effects on fungal growth and for any reduction in residual antibiotic activity. *P. ostreatus* was effective in removing sulfonamides up to 83 to 91 % of the applied doses over 14 days. The antibiotic activity of the sulfonamide residues was reduced by 50 %. Sulfamethoxazole transformation products by laccase were identified and the degradation pathway was proposed. In addition, *P. ostreatus* growth on a semi-solid medium of spent mushroom substrate and malt extract agar was used to develop a biofilter for the removal of sulfonamides from real wastewater. The biofilter was able to remove more than 90% of the sulfonamide concentrations over 24 hours by combining adsorption and biodegradation mechanisms.

Keywords: antibiotics; emerging pollutants; biodegradation; adsorption, fungi; laccase, wastewater

Abbreviation list: wastewater treatment plants (WWTPs), sulfonamides (SAs), spent mushroom substrate (SMS), sulfathiazole (STZ), sulfapyridine (SP), sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamethazine (SMZ), malt extract agar (MEA), malt extract (ME), optical density at 600 nm (OD_{600}), bacterial growth inhibition (GI%), Mn-Peroxidase (MnP), unit of enzyme activity (IU), sulfamerazine (SM), sulfamonometoxin (SX), hazard quotient (HQ), predicted no-effect concentration (PNEC),

1. Introduction

The spread of antibiotics into the environment is a global concern because of their potential ecological risk, including the generation of antibiotic resistant bacteria and their impact on the bacterial community (Rizzo et al. 2013; Santás-Miguel et al. 2020). Wastewater is an important source of antibiotics in the environment (Rizzo et al. 2013; Ben et al. 2018). The main reason is the inefficiency of wastewater treatment plants (WWTPs) to remove antibiotics or other organic pollutants because they were not designed to remove micro-pollutants (Michael et al. 2013; Berendonk et al. 2015; Tran et al. 2018). Antibiotics occur in wastewater because they are only partially metabolized by humans and animals and are excreted in the urine and faeces as parent compound in a high proportion of the given dose (Van der Ven et al. 1994; Halling-Sørensen et al. 2002).

Sulfonamides (SAs) are wide spectrum antibiotics highly prescribed for animal breeding and as growth promoters in animal husbandry whose sales for veterinary use in UE were 608.3 tons in 2017 (European Medicines Agency 2019). Sulfonamides adsorb weakly to sediments or sludge and can quickly contaminate groundwater (Reis et al. 2020). They are some of the most commonly detected antibiotics in wastewater and different natural water ecosystems in Europe at a concentration of up to $\mu g\ L^{-1}$ (Carvalho and Santos 2016). As a result of the ubiquity of this family of antibiotics, sulfonamide resistant genes have been detected in the environment (Sharma et al. 2016). SAs can also reach the food chain by the addition of cattle manure or sewage sludge to croplands as soil organic amendments and fertilizers, as well as by irrigation using polluted wastewater (Conde-Cid et al. 2018; Picó et al. 2019; Zhao et al. 2019). Hence, the removal of sulfonamides from wastewater is an imperative action both for the environment and for human health.

The removal of sulfonamides in WWTP follows abiotic and biotic pathways. Natural photodegradation has been shown to be ineffective or can result in the production of toxic compounds (Reis et al. 2020). Their adsorption in sludge can reach appreciable rates; however, this process is highly dependent on the particular operational conditions of the WWTP and is unfavourable due to their chemical properties such as low K_{ow} values (Tran et al. 2018). Advanced treatments such as membrane processes, activated carbon adsorption or advanced oxidation processes downstream of conventional biological processes, can significantly improve antibiotics removal despite an increment in capital and operational costs (Michael et al. 2013). Hence, the optimization, or new approaches to the biological degradation of sulfonamides could represent a novel improvement in the treatment of wastewater. The biodegradation of SAs by bacteria in WWTPs appears to

take place via co-metabolism although some bacteria use SAs as their sole source of energy. The co-existence of both a degradation mechanism and a resistance gene in the same bacteria raises important questions concerning the co-evolution of these traits. Since the link between antibiotic degradation and resistance remains unexplored, the direct application of these degraders may promote the undesirable spread of resistance (Reis et al. 2020). In this respect, the use of white rot fungi and their ligninolytic enzymes has emerged as an interesting alternative to bacterial biodegradation (Čvančarová et al. 2015; Lucas et al. 2016; Navada and Kulal 2019). Several biotechnological approaches have been successfully tested to determine the biodegradation of sulfonamides by white rot fungi (Rodríguez-Rodríguez et al. 2012; de Araujo et al. 2017), using free or immobilized laccase enzymes (Schwarz et al. 2010; Rahmani et al. 2015; García-Delgado et al. 2018; Alharbi et al. 2019) or using spent mushroom substrate (SMS) (Chang et al. 2018b).

Several ligninolytic fungi with the potential to degrade sulfonamides are cultivated for human consumption (Chang et al. 2018b) with the consequent co-generation of large quantities of SMS. For example, China produces about 4 million tons of SMS annually (Mohd Hanafi et al. 2018) and Europe produces more than 3.5 million tons (Gea et al. 2014). The re-use of this agricultural waste is a promising issue due to its wide applications for environmental remediation based on the degradation and adsorption of pollutants. The adsorption of organic and inorganic pollutants on SMS occurs as a results of its high organic carbon content and as functional groups that are able to interact with pollutants (Álvarez-Martín et al. 2016; Frutos et al. 2016; García-Delgado et al. 2020). The degradation of organic pollutants is carried out by the active fungal mycelium, the ligninolytic enzymes and the inherent microbiota of the SMS (García-Delgado et al. 2015b; Di Gregorio et al. 2016; Siracusa et al. 2017; Chang et al. 2018a). Therefore, the design of novel devices combining the removal of sulfonamides by ligninolytic fungi and adsorption on SMS could represent an interesting approach to reclaiming wastewater, and a reduction of the exposure of bacteria to these antibiotics, resulting in a reduction in the acquisition of specific sulfonamide resistant genes and their spread into the environment. In addition, it is a further step more in the re-use of an agricultural waste to promote a circular economy.

The aim of this work was to remove sulfonamides from real wastewater using a novel biofilter based on *Pleurotus ostreatus* and its spent mushroom substrate as a support. Previously, the ability of *P. ostreatus* to remove five sulfonamides in liquid media was tested based on fungal growth, ligninolytic activity, antibiotics dissipation, and residual antibiotic activity.

2. Materials and methods

2.1 Sulfonamides and fungal material

Sulfonamides and the internal standards were purchased from Sigma Aldrich (St. Louis, 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 (Haverhill, USA), (Sulfamethazine (SMZ, 99%)).

The strain of *P. ostreatus* was isolated from SMS collected from a commercial mushroom cultivation farm in Quintanar del Rey (Cuenca, Spain) and successfully tested for polycyclic aromatic hydrocarbons degradation in a previous work (García-Delgado et al. 2015a). The fungus was maintained on malt extract agar (MEA) plates at 4 ºC and sub-cultured every 28 days.

2.2 Assessment of *P. ostreatus* to remove sulfonamides in liquid cultures

P.ostreatus was inoculated in 500 mL Erlenmeyer flasks with 125 mL of 3% malt extract (ME) in the presence, or absence of 0.1 mM of each of the SAs (SDZ, SP, STZ, SMZ and SMX) by adding three 5 mm diameter fungal plugs from a 7 days old MEA culture . The cultures were incubated for 14 days at 28 ºC, under orbital agitation (120 rpm) in the dark. Abiotic controls without *P. ostreatus* were prepared and incubated under the same conditions. The assay was performed in triplicate. At the end of the assay, the fungal biomass was separated from the liquid medium using a Büchner funnel, and rinsed four times with sterile water to remove all traces of the medium. The fungal mass was dried at 65 ºC for two days to determine the dry weight. One mL aliquots were removed daily to analyse the kinetics of the antibiotic degradation and ligninolytic activity. The concentration of the remaining SAs was determined by placing 1 mL aliquots of liquid broth in Eppendorf tubes and centrifuged at 5000 rpm for 1 min and the supernatant was filtered through 0.45 µm nylon syringe filters. Then 0.5 mL of the filtrate was mixed with 0.5 mL methanol in HPLC vials and stored at -18ºC until analysis. The concentration of SAs was determine by HPLC coupled to a photodiode array detector according to Summa et al. (2015)A detailed description of the analytical method is included in the Supplementary Information.

The removal of SAs by *P. ostreatus* was adjusted to a pseudo-first order kinetic model.

$$C = C_0 e^{-kt} \qquad (1)$$

Where: C_0 was the initial concentration of SAs, C is the concentration of the target antibiotic at t time (days) and k is the degradation constant (d^{-1}).

The half-lives of SAs were calculated using the Eq. 2:

$$t_{1/2} = \frac{\ln 2}{k} \qquad (2)$$

The residual antibiotic activity after fungal removal of SAs was based on growth inhibition of bacteria present in real wastewater collected from the WWTP at the Autonomous University of Madrid according to García-Delgado et al. (2018). Briefly, 5.0 mL of wastewater was added to 500 mL sterilized tryptic soy broth and incubated at 30ºC in the dark under orbital shaking (120 rpm) for 24 h. The optical density at 600 nm (OD_{600}) of the resulting bacterial culture was adjusted to 0.100 by dilution with sterilized tryptic soy broth. Then, 1 mL obtained from the mycodegradation assay of SAs was mixed with 4 mL bacteria culture media. The absorbance increment at 600 nm was monitored after 4 h of incubation at 30 ºC. Controls were prepared without antibiotics, and analysis of samples with 0.1 mM of each SA was performed in parallel. The assay was performed in triplicate. The percentage of bacterial growth inhibition (GI%) was calculated by Eq. 3:

$$GI\% = (1 - \frac{OD_{600s}}{OD_{600c}}) \cdot 100 \qquad (3)$$

where: OD_{600s} and OD_{600c} are the optical densities of the sample and the controls without antibiotics, respectively.

2.3 Ligninolytic enzyme analysis

Laccase activity was spectrophotometrically determined by oxidation of 10 mM 2,6-dimethoxy phenol in 50 mM sodium acetate pH 5.0 at 477 nm (ε = 14600 M^{-1} cm^{-1}) (García-Delgado et al. 2018). Mn-peroxidase (MnP) activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2. Manganic ions, by Mn^{3+} forms a complex with malonate, which absorb at 270 nm (ε =11590 M^{-1} cm^{-1}) (Wariishi et al. 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme that produces 1 μmol of product per minute, under the assay conditions.

2.4 Degradation of SMX by commercial laccase and identification of the transformation products

Ten mL of 0.05 mM SMX were incubated together with 5 U mL^{-1} of commercial laccase from *Aspergillus* sp. over 24 h at 28 ºC in the dark. The reaction was stopped by adding MeOH 50%. The controls without laccase were performed in parallel. Then 1 mL of each sample was filtered through 0.45 µm nylon syringe filters. The degradation products were separated by UPLC (Acquity Series, Waters). Mass spectra were performed using an ultra-high-resolution QTOF instrument (MAXIS II, Bruker, Bremen-Germany). A detailed description of the analytical method is included in the Supplementary Information.

2.5 Removal of sulfonamides from real wastewater by biofilter

Wastewater was obtained from an urban WWTP in Almería (Spain) in April 2017. The wastewater contained SMX, (423 ng L^{-1}), SP (72.5 ng L^{-1}), sulfamerazine (SM, 13.5 ng L^{-1}) and sulfamonomethoxine (SX, 21.9 ng L^{-1}). The biofilter was based in a sterilized (121 ºC, 20 min) mixture of SMS (60 g) and MEA (250 mL) as a support and, as a nutrient source for *P. ostreatus*. The SMS-MEA mixture was placed in Teflon containers and after solidification, five plugs of 0.5 mm *P. ostreatus* were inoculated and grown for three days at 25 ºC in the dark. Following this, 400 mL of wastewater were introduced into the biofilter using a peristaltic pump at a flow rate of 10 mL min^{-1}. The assay took 24 h and 30 mL aliquots were taken at 30 min, 1, 2, 4, 6 and 24 h. The abiotic control consisting of the sterilized SMS-MEA mixture, without fungal inoculation, was carried out under the same operational conditions. All the experiments were performed in triplicate.

Laccase and MnP activities were measured in all the aliquots according to methods described above. The concentration of SAs in wastewater before and after the biofilter treatment was determined by UPLC-MS/MS after a concentration and purification step by solid phase extraction. A detailed description of the analytical procedure is included in the Supplementary Information.

An environmental risk assessment was determined using the hazard quotient (HQ) of the SAs in the water samples following the equation:

$$\mathrm{HQ} = \frac{[\mathrm{SAs}]}{\mathrm{PNEC}} \qquad (4)$$

where: [SAs] is the concentration of the antibiotics (µg L^{-1}) and the predicted no-effect concentration (PNEC) values were 0.027 µg L^{-1} for SMX and 21.61 µg L^{-1} for SP according to Verlicchi et al. (2012).

2.6 Statistical analysis

All statistical tests were carried out using the IBM SPSS Statistics v25 software package. Comparisons of the differences between treatments were determining using the Duncan post hoc test (according to variance homogeneity) at $p < 0.05$ was used. The Pearson correlation coefficient was calculated between the increments of laccase activity and removal rates SAs, by *P.ostreatus* in the biofilter assay.

3 Results

3.1 Degradation of sulfonamides by *P. ostreatus* in liquid cultures

Figure 1 shows the dry fungal biomass and laccase activity profile of the *P. ostreatus* culture in the presence and absence of SAs. There were no significant differences ($p > 0.05$) in the dried fungal biomass of the control (0.65 ±0.05 g) and the culture with SAs (0.85±0.05 g) after 14 days of growth (Fig. 1A). These results indicate that the SAs were neither toxic nor altered the fungal growth under these conditions. Laccase was the extracellular enzyme most expressed in both the presence and absence of SAs. Peak laccase activity (Fig. 1B) was achieved at day 4, and was higher in the control (68 U L^{-1}) than in the presence of SAs (45 U L^{-1}). From day 4, laccase activity decreased progressively until it become undetectable at day 14 in both the control and the SAs cultures. MnP was detected during the incubation period with its maximum activity (8 U L^{-1}) at day 6.

The remaining concentrations of SAs in the liquid media during treatment by *P. ostre*atus over a period of 14 days are shown in Fig. 2. The abiotic control produced a lower removal rate of SAs than the mycodegradation by *P. ostreatus*. Hence, *P. ostreatus* played a key role in the degradation of selected SAs. The highest removal rates by *P. ostreatus* took place during the first week of incubation showing rates of between 60 and 71 % of the initial concentration of SAs (Table 1) with a peak occurring between the 4^{th} and 5^{th} days of incubation, coinciding with the maximum activity of laccase. However, degradation of SAs by laccase was not the sole mechanism of degradation as shown by the extensive removal of SAs from day 10 to 14 when laccase activity was very low. Thus, the removal mechanism of SAs is probably a combination of extracellular and intracellular degradation and mycelial adsorption. At the end of the incubation period, *P. ostreatus* achieved removal rates of SAs of between 83 and 91 % denoting the effectivity of *P. ostreatus* to remove SAs antibiotics (Table 1). The pseudo-first order kinetic model adequately outlined the experimental data of SAs removal by *P. ostreatus* (Table 1). All the five SAs tested presented a good adjustment to the pseudo-first kinetic model. The removal rate of SAs by *P. ostreatus* was determined by the dissipation constant (k), of between 0.1253 and 0.1506. Four of the five SAs (SDZ, STZ, SP, SMX) showed half-lives of

less than 5 days. SMZ showed the lowest removal speed with a half-life of 5.5 days. It is clear that the chemical structure of the SAs has an effect on their dissipation kinetics.

The residual antibacterial activity of remaining SAs and their transformation products in liquid cultures was determined using a bacterial consortium from the WWTP from the Autonomous University of Madrid. No bacterial growth was observed in the presence of the initial mixture of the 5 SAs at 0.1 mM of each antibiotic. Hence, no resistance towards SAs was detected by the bacterial consortium used. Meanwhile, bacterial growth was partially inhibited by residues of SAs and their transformation products after *P. ostreatus* treatment compared with the negative controls (without SAs), showing a bacterial growth inhibition rate of 50 %. The extensive SAs removal rates, which ranged from 83 to 91 %, were not proportional to the bacterial activity inhibition rate. Hence, the transformation products of SAs produced by *P. ostreatus* retained its antimicrobial activity.

3.2 Identification of SMX transformation products by laccase

Commercial laccase was tested to degrade SMX in order to determine the role of the main ligninolytic enzyme in the mycodegradation mechanism of SMX. The most important transformation products obtained after SMX oxidation by laccase were identified by QTOF-MS (Fig. S2). The parental compound (SMX) with the molecular ion [M+H]+ of *m/z* 254.0596 as well as the characteristic SMX transformation product corresponding to 3-amino-5-methylisoxazole (*m/z* 99.0557) protonated ion were observed. Other transformation products common to the aminobenzenesulfonamide moiety were identified as: *p*-aminobenzenesulfone (*m/z* 156.0117), aniline (*m/z* 92.0591) or its typical transformation product, *p*-aminophenol (*m/z* 108.0441). Hence, laccase has a clear role in the biochemical mechanisms of SMX mycodegradation. The biodegradation pathway of SMX by laccase is schematized in Fig. 3.

3.3 Removal of sulfonamides from wastewater by biofilter

The laccase activity and the percentage of SAs removal from real wastewater by the biofilter are shown in Fig 4. Laccase was produced at high rates by *P. ostreatus* growing on a sterilized SMS based on wheat straw, mixed with MEA (SMS-MEA), reaching 510 U L^{-1}. The increment of laccase activity was sustained with growth over time. Meanwhile, MnP exhibited a noticeably lower activity, as had been observed in the previous liquid medium experiments, and an irregular pattern reaching a peak of activity of 2.06 U L^{-1} in 24 h.

The *P.ostreatus* biofilter showed high removal rates of SAs from real urban wastewater in a short period (24 h). SM and SX were not detected after wastewater treatment by the *P.ostreatus* biofilter at any sampling time denoting excellent rates of removal (100 %). SMX and SP showed high removal rates from the first sampling time. After 4 h of wastewater treatment, the removal rate of both SAs reached the maximum with the final removal of SMX and SP of 93 % and 95 %, respectively over a24 h period (Fig 3). SMX and SP fitted well with the pseudo-first order kinetic model, showing short half-lives of 1.3 h and 0.5 h respectively (Table 2). SP presented a higher kinetic constant of15.163 h^{-1} than SMX with a constant of 0.5350 h^{-1} after *P.ostreatus* biofilter performance. In addition, laccase activity and SP and SMX removal rates (Fig. 4) exhibited a statistically significant negative correlation, with a Pearson´s correlation coefficient value of -0.71 ($p < 0.05$).Initially, wastewater showed a HQ_{SMX} of 15.7, which was reduced to 0.2 by the *P.ostreatus* biofilter; meanwhile HQ_{SP} was reduced from 2.7 to 0.5.

The roles of SMS-MEA and *P. ostreatus* in the biofilter were determined by an abiotic control. The filter without *P. ostreatus* reached a percentage removal of 48 and 77 % for SMX and SP respectively and the complete removal of SM and SX after 1 h treatment. At the same time, the *P.ostreatus* biofilter reached the same removal of SM and SX, but higher removal rates of SMX and SP reaching 70 and 89 % respectively, demonstrating the quick adsorption-mycodegradation ability of the biofilter with *P. ostreatus*. After 24 h, *P. ostreatus*, removed 95 and 93 % of SP and SMX whilst the abiotic control adsorbed 81 and 87 % respectively. Based on a mass balance, the adsorption process on SMS-MEA was responsible for the removal of 91.9 and 85.6 % of SP and SMX respectively, whereas *P. ostreatus* was responsible for the removal of 8.1 and 14.4 % of SP and SMX, respectively.

4 Discussion

The presence of antibiotics in wastewater is a huge risk to the environment and the population (Berendonk et al. 2015). This concern has led to the search for new ways to remove them from wastewater, and has included a wide variety of strategies including chemical, physical and biological methods (Ben et al. 2018). Among the new biological strategies to remove antibiotics from wastewater, ligninolytic fungi are showing promise in biotechnology due to their high degradation and adsorption capacity and the low-cost source of the raw material (Asif et al. 2017a; Dąbrowska et al. 2018; Gao et al. 2018).

4.1 Removal of sulfonamides by *P. ostreatus* and laccase

The growth of *P. ostreatus* was not affected by the high concentration of SAs in the liquid media. In the same vein, de Araujo et al (2017) reported an increment in the growing speed of *P. ostreatus* in the presence of SMX and trimethoprim . However, by contrast Migliore et al. (2012) described a decrease in the growth rate of *P. ostreatus* in the presence of tetracyclines, illustrating the different effects on fungal growth as a function of the type of antibiotic.

The remaining concentration of SAs in the controls without *P. ostreatus* after 14 days of incubation was higher than 90 % for SDZ and SP, and between 60 and 70 % for STZ, SMZ and SMX indicating the different abiotic degradation of these antibiotics. The decrease of the concentration of SAs in the controls could have occurred as a result of their adsorption on glass and abiotic oxidation. By contrast, *P. ostreatus* treatment effectively removed all the five SAs in the liquid medium (83 % and 91 %). Thus, *P. ostreatus* produced an increment in the removal of SAs with respect to the abiotic control of between 49 and 87 %. The most recalcitrant SAs in the abiotic control (SDZ and SP) presented the highest increments of removal by *P. ostreatus* when compared with these controls. The high effectivity of *P. ostreatus* to remove SAs was achieved although the maximum activity of the ligninolytic enzymes laccase and MnP (45 and 8 U L^{-1}, respectively) was not high. This was probably due to the rich nutrient culture medium (ME). Laccase production is highly influenced by culture conditions. However, a high fungal biomass might not correspond with a high laccase production because the production of ligninolytic enzymes is often triggered by limiting concentrations of carbon or nitrogen. Čvančarová et al. (2015) reported good degradation rates for fluoroquinolones despite a lower laccase and MnP activity for *P. ostreatus* and other ligninolytic fungi in the liquid media. According to Bhattacharya et al. (2013), CYP450 could be the main enzymatic system expressed by *Phanerochaete chrysosporium* in a rich liquid medium, whilst lignocellulosic supports might trigger extracellular peroxidases. *P. ostreatus* might exhibit similar behaviour and the biochemical mechanisms used to biodegrade antibiotics could be based on a combination of extracellular enzymes mainly laccase (Fig. 1), and the intracellular CYP450 system, which might explain why the removal of SAs in the ME media did not have a significant correlation with laccase activity. de Araujo et al. (2017) reported the same lack of correlation between ligninolytic activity and antibiotic removal. By contrast, they observed a positive correlation between fungal growth and antibiotic degradation.

Ligninolytic fungi have complex extracellular and intracellular enzymatic systems. For example, *P. ostreatus* has 12 isozymes of laccase (Kempken 2013) and a CYP450 system that consists of 153 genes capable of

several chemical reactions such as hydroxylation, epoxidation, decarboxylation, and aryl-transformation (Golan-Rozen et al. 2011). When organic pollutant degradation is carried out *in vivo*, as in this work, both systems might be involved depending on the culture conditions. Some researchers have found that the inhibition of CYP450 of *P. chrysosporium* and *Pycnoporus sanguineus* growing in potato dextrose broth amended with 1-aminobenzotriazole significantly decreased the removal of SMX, whilst it had no significant effect on the removal of other antibiotics such as ciprofloxacin and norfloxacin (Gao et al. 2018). Rodríguez-Rodríguez et al. (2012) have suggested the implication of CYP 450 in STZ degradation but with regard to SP, the situation was not clear. Therefore, the mycodegradation of pharmaceuticals by ligninolytic fungi might be due to the synergetic performance of extracellular, intracellular and mycelium bound enzymes (Asif et al. 2017b).

However, the fungal adsorption of antibiotics, and specifically SAs could be an additional mechanism for the removal of pollutants from water. For instance, Gao et al. (2018) reported the relatively high biosorption of SMX on *P. chrysosporium* mycelium (20 %) whereas SMX biosorption was not detected on *P. sanguineus*. Hofmann and Schlosser (2016) did not observe any significant SMX adsorption on *Phoma* sp. dueto the low hydrophobicity of SMX. By contrast, they reported biosorption as an important mechanism in the removal of hydrophobic organic pollutants such as triclosan, 17 α-athinylestradiol, nonylphenol or bisphenol A, by fungi.

The antibacterial activity of any remaining SAs and their transformation products was tested on a bacterial consortium from a WWTP. This biological assessment is complementary to the chemical analysis of effluents, and is mandatory to perform a complete assessment of the effectiveness of the antibiotic degradation process. Despite the high biodegradation of SAs of between 83 and 91%, the remaining SAs and their transformation products led to a 50% decrease in antibacterial activity. This residual antibacterial activity was probably due to the incomplete degradation of the active site. The partial decrease in antibacterial activity has been described in other works of antibiotics degradation by fungi, ligninolytic enzymes, or even enzyme-redox mediator systems (Čvančarová et al. 2015; Rahmani et al. 2015; de Araujo et al. 2017; García-Delgado et al. 2018).

The role of laccase enzyme in SAs mycodegradation was supported by the *in vitro* assay (see section "Degradation of SMX by commercial laccase and identification of the transformation products") to investigate the transformation of SAs with purified laccase as suggested by Čvančarová et al. (2015). The

enzymatic oxidation of SMX by laccase for 24 h yielded 4 transformation products, the typical transformation products of SAs were identified and are shown in Figs S2 C, D and E, and the characteristic transformation product of SMX shown in Fig. S2 B, which is e in agreement with the results of other researchers (Majewsky et al. 2015; Huynh and Reinhold 2019; Reis et al. 2020). The proposed degradation pathway is in Fig. 3. The transformation product 3-amino-5-methylisoxazole (Fig 3 B), characteristic of SMX, was reported as a stable transformation product (Eibes et al. 2011) without antibacterial activity (Reis et al., 2020). Hence, laccase collaborates in the reduction of antibacterial activity of SMX and its transformation products. The degradation of the aminobenzenesulfonamide moiety yielded three degradation products (Fig 3 C, D and E), which are potentially common to all the SAs. The production of *p*-aminophenol could be performed by substitution of the sulfonamide group by a hydroxyl group. The transformation of aniline into *p*-aminophenol by laccase is impaired. Although aniline is a putative laccase substrate, aniline is not susceptible to direct oxidation by laccase due to its high ionization energy (7.72 eV) (NIST 2018) with respect to the ionization potential of laccase (≤ 7.45 eV) (Haritash and Kaushik 2009).

The key role of laccase in the removal of SMX and its transformation products is clear. Previous works have reported the importance of laccase and MnP in antibiotic mycodegradation (Prieto et al. 2011; Čvančarová et al. 2015; Alharbi et al. 2019). Hence, in view of present and previous works, mycodegradation processes able to produce abundant and constant ligninolytic activity have a greater possibility of achieving successful antibiotic degradation than those processes with low ligninolytic activity.

4.2 Assessment of *P. ostreatus* biofilter to remove sulfonamides from wastewater

The *P. ostreatus* biofilter was based on SMS as a way to reuse this abundant and cheap agricultural waste product and assure a substrate compatible with the fungus. SMS has valuable microbiota for the biodegradation of organic pollutants, including antibiotics (Chang et al. 2018b). However, in this work the SMS was previously sterilized in order to eliminate its microbiota and subsequently inoculate it with the same fungal strain used in the degradation assay of SAs in a liquid medium. Therefore, the role of SMS in the biofilter with respect to *P. ostreatus* was purely that of a support and nutrient source.

Real urban wastewater without the addition of antibiotics was used to test the biofilter toensure that the biofilter could work in the laboratory conditions, but with a real matrix and SAs concentration. This is important because the chemical and biological composition of real wastewater could interact with the

biofilter reducing its effectivity in comparison with artificial effluent. For example, the fungal growth or enzymatic activity could be negatively affected by real wastewater (Asif et al. 2017b). Laccase activity was appreciable from the first sampling time reaching a high and constant laccase activity after 6 h (Fig. 3). The presence of lignin in the SMS could induce and increase the production of laccase with respect to the previous assay liquid media (Kunamneni et al. 2007). The activity of *P. ostreatus* and the adsorption capacity of the biofilter resulted in an effective removal rate of SAs of between 93 and100 % in 24 h. The role of laccase in this process was suggested by the significant negative correlation between the removal rates of SMX and SP and laccase activity (R^2 = - 0.71 and - 0.64, $p < 0.05$). Gao et al. (2018) correlated laccase activity with SMX removal, using crude laccase from *P. sanguineus,* especially when a redox mediator such as ABTS was added which resulted in the complete removal of SMX within 12 h. SAs and other antibiotics or organic pollutants are usually degraded at higher rate by adding redox mediators (Yang et al. 2017; Navada and Kulal 2019). In this work, the biofilter worked in the absence of redox mediators with optimal results. This is a very important fact because it minimized the use of additional chemicals with a subsequent reduction in cost and operational optimization.

The role of SMS-MAE in the removal of SAs was clear because more than 85 % SP and SMX adsorbed on it. The adsorption of SAs is not easy due to the anionic nature of SAs in environmental conditions (Reis et al. 2020). Zhou et al. (2016) described SMS as an effective adsorbent for the removal of trace concentrations of SAs with a higher SAs adsorption capacity than recognized adsorbents such as activated carbon. Frutos et al. (2016) reported that more than 80% of the organic carbon from the SMS of *P. ostreatus* is in the form of O-alkyl groups, such as carbohydrates. This fact is reasonable because the basic component of *P. ostreatus* SMS is wheat straw. The high carbohydrate content of *P. ostreatus* SMS could favour the interaction of hydrogen bonds between SAs and SMS and result in the high adsorption ability of this biofilter. García-Delgado et al. (2020) reported the importance of O-alkyl groups in enhancing the hysteresis of organic pollutants.

Adsorption of SAs provided a suitable environment for fungal enzymatic performance. The fungi grown on SMS-MAE secreted laccase to degrade the lignocellulosic compounds from straw and as a result laccase also degradeed the adsorbed and dissolved SAs. In addition to this process, some lignin degradation products can act as laccase mediators (Zheng et al. 2019), enhancing the oxidation of the adsorbed and dissolved molecules of SAs.

Different kinds of dispositive based on ligninolytic fungi have been tested to evaluate the removal of different pharmaceuticals from the effluents of WWTPs and some of these have also been based on *P. ostreatus*. Křesinová et al. (2018) tested the capacity of *P. ostreatus* to remove endocrine disruptors from spiked wastewater achieving removal rates of 41% in 3 h on a laboratory scale, and 78 % in 2 h on a pilot bioreactor scale. Palli et al. (2017) assessed a fluidized bed bioreactor with *P. ostreatus* to reclaim hospital wastewater containing three pharmaceuticals: atenolol, diclofenac and ketoprofen. They obtained the complete removal of diclofenac in 18 h, 36 % of ketoprofen and 8 % of atenolol over a period of 42 h.

5 Conclusions

The growth of the ligninolytic fungus *P. ostreatus* was not negatively affected by sulfonamides. This fungus was able to remove sulfonamides at high rates by combining different mechanisms. The removal of sulfonamides produced a reduction in antibacterial activity. However, this reduction was lower than the percentage of sulfonamides removed suggesting the antibacterial activity of the sulfonamide transformation products. The biofilter based on *P. ostreatus* and its own spent substrate is an effective and sustainable way to reclaim wastewater and reuse this abundant agricultural waste. The device can remove sulfonamides at rates higher than 90 % from real wastewater by combining adsorption and mycodegradation processes.

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Table 1. Pseudo first order parameters estimated during the fungal removal of sulfonamides in liquid medium.

	k (d^{-1})	r	$t_{1/2}$ (d)	% Removal	
				day 7	day 14
SDZ	0.145	0.989	4.8	61	91
STZ	0.142	0.969	4.9	69	88
SP	0.151	0.981	4.6	67	83
SMZ	0.125	0.976	5.5	64	85
SMX	0.146	0.968	4.7	71	87

k: rate constant; $t_{1/2}$: Half life

Table 2. Pseudo first order parameters estimated during the fungal removal of sulfonamides in the biofilter.

		k (h^{-1})	r	$t_{1/2}$ (h)	Removal (%)		
					1h	6h	24h
SP	***P. ostreatus***	15.16	0.948	0.5	89	93	95
	Abiotic control	10.79	0.880	0.6	77	83	81
SMX	***P. ostreatus***	0.535	0.901	1.3	70	97	93
	Abiotic control	0.460	0.839	1.5	48	70	87

k: rate constant; $t_{1/2}$: Half life

Figure Captions:

Figure 1: Final dry mass of *P. ostreatus* (A) in the presence and absence of sulfonamides and laccase activity (B) incubated for 14 days in malt extract liquid media. Error bars represent the standard deviation (n = 3).

Figure 2: Remaining concentration of sulfonamides incubated in absence (control) or presence of *P. ostreatus* incubated for 14 days in malt extract liquid media. A) sulfadiazine (SDZ), B) sulfapyridine (SP), C) sulfathiazole (STZ), D) sulfamethazine (SMZ), E) sulfamethoxazole (SMX). Error bars represent the standard deviation (n = 3).

Figure 3: Proposed metabolic pathway of sulfamethoxazole by laccase.

Figure 4: Laccase activity and percentage of sulfamethoxazole (SMX) and sulfapyridine (SP) removal by the *P. ostreatus* biofilter using real urban wastewater.

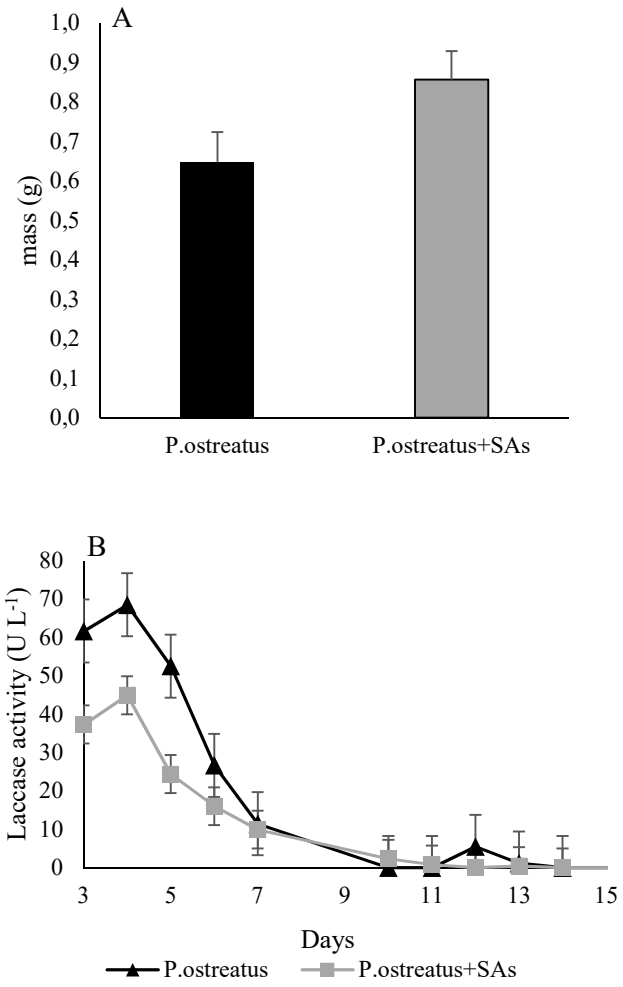
A
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
mass (g)
P.ostreatus
P.ostreatus+SAs
B
80
70
60
50
40
30
20
10
0
Laccase activity (U L-1)
3
5
7
9
11
13
15
Days
P.ostreatus
P.ostreatus+SAs

Fig. 1

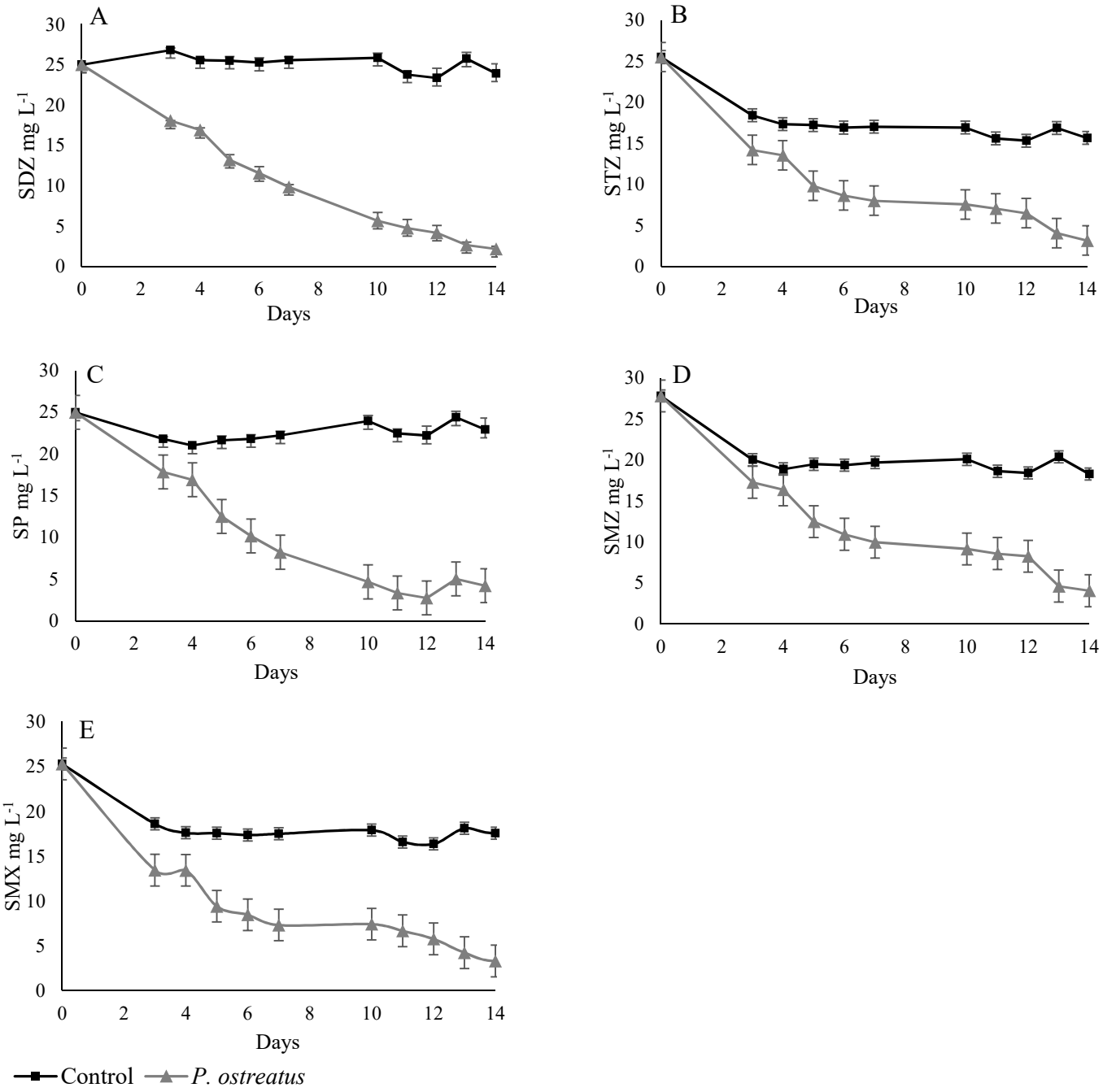
A
B
C
D
E
SDZ mg L-1
STZ mg L-1
SP mg L-1
SMZ mg L-1
SMX mg L-1
Days
Control
P. ostreatus

Fig. 2

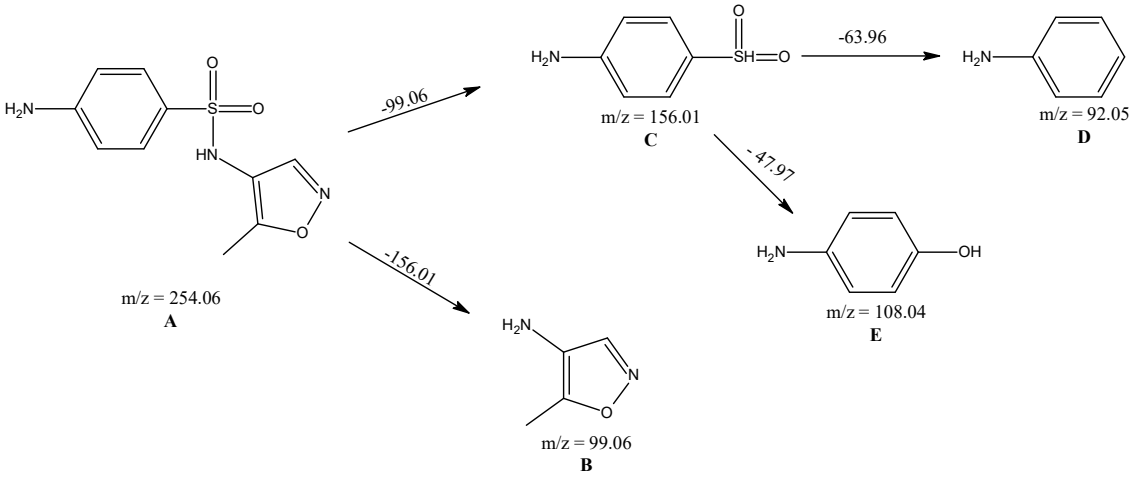
-99.06
-156.01
-63.96
- 47.97
m/z = 254.06
A
m/z = 156.01
C
m/z = 92.05
D
m/z = 108.04
E
m/z = 99.06
B

Fig. 3

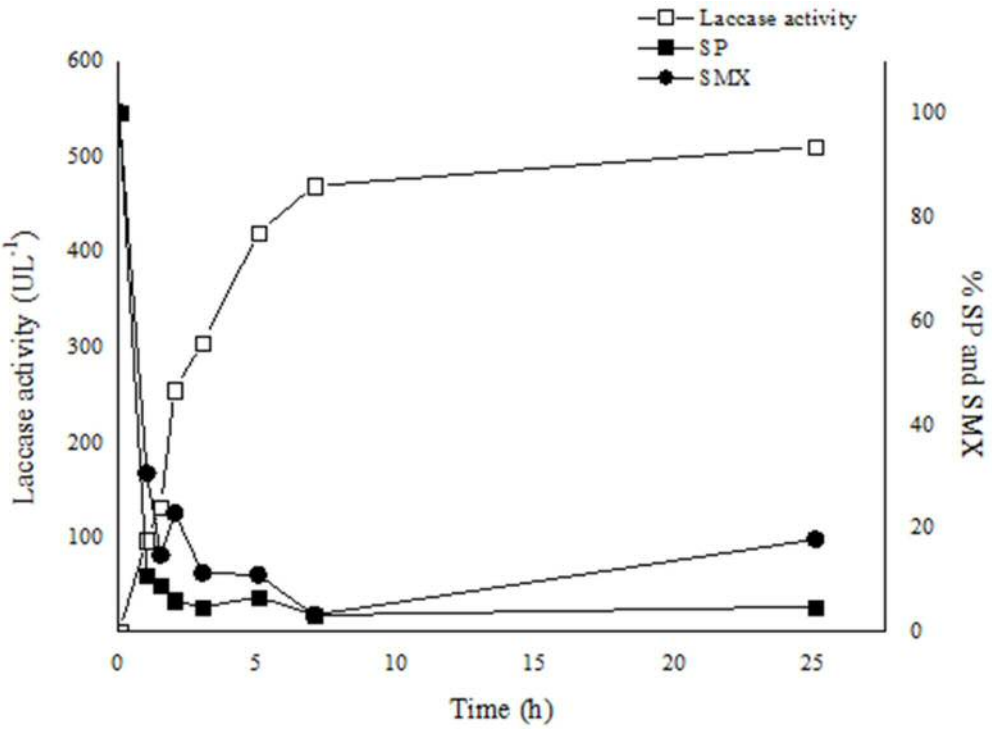
Laccase activity
SP
SMX
600
500
400
300
200
100
Laccase activity (UL-1)
100
80
60
40
20
0
% SP and SMX
0
5
10
15
20
25
Time (h)

Fig. 4