Spent mushroom substrates biofilters: degradation of antibiotics by ligninolytic fungi

R. Camacho-Arévalo[1], B. Mayans[12], C. García-Delgado[3], R. Antón[1], C. Escolástico[2], E. Eymar[1]
[1]Department of Agricultural Chemistry and Food Sciences, University Autónoma of Madrid, 28049 Madrid, Spain
[2]Department of Organic and Biorganic Chemistry. National Distance Education University (UNED)
[3] Institute of Natural Resources and Agrobiology of Salamanca (IRNASA) Spain
Corresponding author email: enrique.eymar@uam.es ; Phone: 0034914975010; Fax: 0034914973826

Abstract

Antibiotics are emergent pollutants, their presence in the environment in low concentrations is generating multiresistant bacteria which represent a very important threat to human health. A high proportion of the consumed antibiotics are not metabolized, they end in low doses polluting waters and the environment. Ligninolytic fungi had already been used to remove organic pollutants due to their enzymatic activity, during this work several biofilters with this kind of fungi had been developed. *Pleurotus ostreatus* and *Pleurotus eryngii* were the species chosen to be grown in straw-based media with and without malt extract agar as a source of nutrients. Straw present in the spent mushrooms substrates assayed also contributes to the antibiotic removal by adsorption. Effluent from a wastewater treatment plant (WWTP) was introduced in the biofilters where the concentration of several sulfonamides and tetracyclines were detected. The evolution of extracellular activity (laccase, manganese peroxidase and versatile peroxidase) were analyzed taking aliquots over 48 hours. The antibiotics were removed in all the biofilters, with more effectivity and extracellular enzyme activities in the biofilters without malt extract and with no significant different between the two *Pleurotus.* This result agrees with the hypothesis that relates higher extracellular enzymatic activities in poor nutrient conditions. Further research is needed to optimize the biofilters and adequate them to their future application.

Keywords: tetracyclines, sulfonamides, bioremediation, wastewaters, biofilter

1. Introduction

The presence of antibiotics in the environment, even in very low concentrations, is generating multiresistant bacteria which represent a very important threat to human health. The latest WHO report predicts that in 2050 there will be more deaths caused by multiresistant bacteria than by cancer [1].

Antibiotics are essential to maintain public health and to increase the quality and production of animal source foods. In 2014, 8,935 tons of antibiotics were commercialized in the European Union (EU) for veterinary use [2], nearly 105,000 tons in China in 2015 and in the same year the US consumption rose to 15,600 tons.

A high percentage of antibiotics are excreted in the same way they were consumed which is the reason why they end polluting waters. Antibiotics have been reported in natural waters, soils and crops mainly due the inefficiency of current treatments in wastewater treatment plants which are not able to remove 30 % of them [3]. Antibiotics are emerging pollutants, their residues are not legislated, which makes them to end contaminating waters and the environment. Continuous exposure to doses below the minimal inhibitory concentration generate multiresistant bacteria.

Sulfonamides (SAs) and tetracyclines (TCs) were chosen for the assay because they are the two groups of antibiotics more employed for veterinary use in the EU with a percentage of 9,6 % and 36,7 % respectively [2].

Some fungi could be a good option to degrade antibiotics in wastewaters thanks to their ligninolytic enzymatic system, capable to oxidize a great number of substrates by radical formation. The main extracellular enzymes are laccase, Mn-peroxidase (MnP), Lignin peroxidase (LiP) and Versatile peroxidase (VP), depending of the medium composition. Intracellular enzymes can also intervene in the degradation by the action of cytochrome P450 [4]. Several white rot fungi, such as genus *Pleurotus* have already been used effectively to degrade organic pollutants such as synthetic dyers, polycyclic aromatic hydrocarbons or heavy oils [5].

Some kinds of ligninolytic fungi are cultivated for human consumption generating huge amounts of spent mushroom substrate. In addition, they are cultivated in straw which can also retain the antibiotics, especially TCs due to their naphthalene nucleus structure, bigger than the sulfonamides that consist in a benzene ring with sulfonyl and amino groups. Successful studies had been made employing lignocellulose material such as sawdust to remove tetracyclines [6].

The objective of this paper is to re-use the agricultural waste generated by edible fungi, which still contain mycelium, to remove the antibiotics of wastewaters combining the action of the fungi and the straw adsorption. To achieve this goal, several biofilters have been developed in different conditions, employing different mushrooms and media to degrade the antibiotics of a real wastewater.

According to Hatakka and Hammel [4], the ligninolytic fungi segregate more extracellular enzymes when there are no easy nutrients available to degrade big molecules into smaller ones. When they are in a rich nutrient medium the fungi tend to use the cytochrome P450 instead of the extracellular activity to degrade the organic compounds. To compare which kind of degradation is better to remove the antibiotic pollutants of the wastewater, two different mediums were prepared. The rich nutrient medium was a semi solid medium whit malt agar extract (MEA) while the poor nutrient medium was based on straw from spent-mushroom substrates.

The three edible fungi assayed were: *Pleurotus ostreatus (P.ostreatus)* and *Pleurotus eryngii (P.eryngii).* These fungi were chosen due to their high human consumption and their capacity previously tested to degrade several organic compounds [7].

2. Material and Methods

2.1 Materials

P. ostreatus was isolated from spent mushroom substrate collected in a commercial crop located in Quintanar del Rey (Cuenca, Spain). This fungal strain was previously successfully tested for polycyclic aromatic hydrocarbons degradation [8] and for tetracyclines and sulfonamides degradation. Commercial strains of *P. eryngii* were purchased from Gurelan Mycelium located in Huarte (Navarra, Spain). The mycelium grew in the controlled laboratory conditions in malt extract agar (MEA) for 7 days, and then aliquots of this culture were used as inoculum.

Wastewater was obtained from an urban sewage treatment plant located in University Autónoma of Madrid (Spain).

2.2. Inoculation and biofilter design

Spent mushroom substrate was autoclaved for 15 minutes at 121 ^{o}C, some of them were added malt extract agar. With this two different approaches there was a semi-solid medium, spent substrate with MEA, and a straw based culture medium. Several mushroom substrates were inoculated with *P. ostreatus* and *P. eryngii* separately. Controls without fungi were also made to evaluate the influence of the straw adsorption in the removal.

In the semi-solid medium, the fungi were previously cultivated in malt extract in Erlenmeyer flaks of 1 L, in orbital shaking at 160 rpm for 15 days at 28 ^{o}C in darkness. Afterwards, in teflon containers with 500 ml capacity that were used as support to create the biofilter, five fungal pellets were grown. The teflon containers filled with the spent substrate and 250 ml of MEA were previously sterilized in autoclave.

In the straw based medium, 80 g of the sterilized spent mushroom was introduced in the Teflon containers after been inoculated for periods of time between four and five weeks with three mycelial pieces of 5 mm from MEA culture.

400 mL of sample water were introduced in the containers using a peristaltic pump with a flow of 8 ± 2 ml. The assay lasted 24 h, in the meantime aliquots of 25 mL were taken at different moments: at the beginning, at 30 min,1h, 2h, 4h, 6h and 24h. These aliquots were used to determine the enzymatic activity and to quantify TCs and SAs. All the biofilters were made in triplicate.

2.3. Quantification of antibiotics

To quantify SAs and TCs, the samples were extracted by a Solid Phase Extraction procedure using lipophilic/hydrophilic balanced Oasis HLB cartridges. The analytes were eluted with ethyl acetate, then evaporated to dryness under 12 psi N_2 flow and 42ºC. Finally, they were reconstituted in methanol/water (15/85) and analyzed.

The analysis of antibiotics, sulfonamides (SAs) and tetracyclines (TCs), were performed using a UHPLC/MS/MS system consisted of an Acquity UHPLC module from Waters (Mildford, MA, USA) coupled with a Waters TQD triple quadrupole detector also from Waters. The detector was operated in MS/MS mode in positive electrospray. The column was a UHPLC BEH C18 (100 mm x 2.1mm; particle size 1.7 µm), the temperature was fixed at 45ºC and the injection volume was 10 µL. The gradient program is shown in table 1.

Table 1: *Gradient elution program of mobile phases for the separation of sulphonamides and tetracyclines by UHPLC-MS/MS*

Time (min)	**Flow Rate (µL min^{-1})**	**A1 (%)**	**B2 (%)**
0.00	0.500	8.0	92.0
5.00	0.500	15.0	85.0
9.00	0.500	55.0	45.0
12.00	0.500	8.0	92.0

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

SAs and TCs residues concentration in sampled water ranged between 20 and 423 ng L^{-1}, since conventional wastewater treatments are not efficient enough to remove pharmaceuticals and personal care products [9-11]. After analyzing the wastewater only the SAs were inside the range of concentration previously described, while the TCs were in lower concentrations so it was necessary to raise them by the addition to samples of known concentrations of TCs until they reached similar concentrations to those detected for SAs.

The sulfonamides found in the wastewater were: sulfadiazine, sulfathiazole, sulfapyridine, sulfametazine, sulfamethoxazole and sulfisoxazole. The tetracyclines present were: tetracycline, oxitetracycline, chlorotetracycline and doxycycline.

2.4. Enzymatic Activity

Ligninolytic enzymes activity were analyzed when the aliquots were extracted. Extracellular enzymatic activity of laccase, Mn-peroxidase (MnP) and Versatile Peroxidase (VP) were analyzed. 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}). VP activity was determined by oxidation of 40 mM veratryl alcohol in 100 mM tartaric acid/ sodium tartrate buffer in presence of 0.4 mM H_2O_2 at 310 nm (ϵ = 9300 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 [8].

3. Results and Discussion

3.1. Antibiotic Degradation

The removal of most antibiotics occurred very fast, obtaining slightly better results with *P.ostreatus* and in poor nutrient media.

3.1.1. Sulfonamides

The percentages of degradation of sulfamethoxazole (SMX) and sulfapyridine (SP) in the *P.ostreatus* and *P.eryngii* biofilters are shown in fig 1 a and b respectively. Both sulfonamides were present in the wastewater with a concentration of 510 ng L^{-1} for SMX and a concentration of 147 ng L^{-1} for SP. From the group of sulfonamides previously identified they were the only which maintained their order of concentration without variation in all the WWTP samplings. The other sulfonamides identified were in a range of concentrations between 15 and 30 ng L^{-1} too low to study the effect of the fungi during the assay because in less than half an hour their concentration decreases to levels under the limit of detection. In addition, SP and SMX allowed us to compare the ligninolytic fungi impact in conditions of different concentrations.

The fig 1 a, shows that all the biofilters assayed were able to remove the SMX, reaching more than 80 % of removal at 24 hours. *P. eryngii* in the MEA medium was the only exception removing the 67 % of SMX. The controls showed that the straw achieved a good SMX removal, although the additional presence of fungi accelerated the disappearance of this antibiotic from the wastewater. Both *Pleurotus* were able to degrade the antibiotic in less than 24 hours. In 5 hours *P.ostreatus* in both treatments degraded the SMX in more than 90 % while *P.eryngii* reached these percentages only in MEA absence, when nutrient availability is lower. In contrast, *P.eryngii* in MEA presence required more time to degrade SMX, after 5 hours it degraded the 42 %.

According to the fig 1 b, the biofilters without fungi removed the 81 % of SP in less 24 hours, they reached this retention percentage during the first 1.5 hour but were not able to remove more SP. The presence of both *Pleurotus* improved the SP removal. Its degradation with both *Pleurotus* and in the two media after 4 hours was higher than 90 %. In the poor nutrient media both *Pleurotus* removed SP slightly faster, surpassing the 90 % of SP degradation in 2 hours.

It can be observed a different behavior of *P. eryngii* with regard to the removal of SAs at higher (SMX) versus lower (SP) concentrations. The presence of MEA in the medium had not effect on SP removal, however, at higher concentrations (SMX) produced a slower pattern of disappearance. Degradation of SAs by *P.ostreatus* was not affected nor by the presence of nutrients in the medium nor the concentrations of SAs.

The use of spent-mushroom substrate to clear away sulfonamides was effective, reaching 90 % of removal in less than 24 h for higher and lower concentrations of SAs with both *Pleurotus*.

3.1.2. Tetracyclines

The TCs were present in the wastewater but in very low concentrations. In the biofilters with MEA it was not possible to analyze its effect so it was decided to dope the wastewater to perform the study with the straw biofilters. The three tetracyclines added were tetracycline (TC), oxitetracycline (Ox-TC) and chlorotetracycline (Cl-TC). These antibiotics were chosen because they were present in the wastewater even if they were in low concentration (10-30 ng L^{-1}). They were added in a concentration of 1.75 ± 0.05 mg/L, three times higher than the level of the antibiotic with highest real concentration (SMX) to analyze the effect of the biofilters in higher concentrations.

The figure 2 shows that TC and Ox-TC were degraded in more than 95% in less than an hour with and without fungi. Cl-TC results were not shown because the obtained concentration was under the determination limit. There were no appreciable difference between both *Pleurotus* tested. Results indicated the necessity to check intermediate points at shorter periods of time to study the kinetics of the biofilters, which would bring information about the influence of the fungi in the antibiotic removal.

Despite the more complex chemical structure of the tetracyclines, they were better removed than the sulfonamides. The controls without fungi show similar results, which suggest that TCs are better adsorbed by the straw than SAs.

Even though the results did not allow the analysis of the influence of the fungi, it was clear that the biofilters were more efficient in the TCs removal than in the SAs, employing less than an hour to remove TCs above the 95 %. Although further research is needed to determine the degradation of antibiotics by the fungi once they have been adsorbed in the straw and also to obtain the effective live time of the biofilter; the spent mushroom substrate could be used to remove both antibiotic families.

3.2. Enzymatic activities

The ligninolytic enzymes analyzed showed that laccase was the most expressed enzyme in both *Pleurotus*. There were more enzymatic activities in the medium with less nutrient available, which is correlated with other studies such as Gupta and Jana, 2018 [12]. The enzymatic activity pattern tended to increase over time but they experiment ups and downs, especially in the peroxidases.

3.2.1. Laccase

The results of laccase activity are shown in the table 2.

Table 2. *Laccase activity in biofilters over 24 hours. Activity is expressed in U L^{-1}. Treatments described as P.ostreatus + MEA and P.eryngii + MEA correspond to P.ostreatus and P.eryngii with malt extract agar. P.ostreatus and P.eryngii in straw medium are represented as P.ostreatus and P.eryngii respectively.*

Time (h)	0	0,5	1	2	4	6	24
***P.ostreatus* + MEA**	6	14	10	21	14	11	7
***P.eryngii* + MEA**	4	20	14	18	32	51	37
P.ostreatus	-	-	517	800	506	1170	1457
P.eryngii	-	-	109	163	216	226	456

The levels of laccase were significantly different in the presence and absence of MEA. When there were no easy nutrients available both *Pleurotus* increased their laccase activity level in some cases above 100 %. In all the samples of straw based medium the laccase activity was higher than 100 U L^{-1} for *P. eryngii* between 109 and 456 U L^{-1} and for *P. ostreatus* even higher levels, between 506 and 1457 U L^{-1}, while the biofilters with MEA *P. eryngii* had more activity, reaching a maximum of 51 U L^{-1} at 6 hours.

Despite the difference of laccase activity, all the biofilters reached the same rate of removal of antibiotics even if the biofilters with malt extract agar needed more time. These results suggested that laccase was involved in the antibiotic degradation. However, there was no need to reach high levels of laccase activity (over 100 U L^{-1}) to remove the antibiotic at the antibiotic levels the wastewater presented. The amount of laccase might have been the result of the ligninolytic process and the effect of natural mediators related to lignin, hemicellulose or cellulose present in the straw [13]. It can also mean that the CYP450 was involved in the degradation in both cases. That was the reason why the differences in the laccase activity did not have a transcendent impact in the antibiotic degradation. This enzymatic activity was degrading other organic compounds of the straw or of the wastewater.

It seems necessary to clarify if the amount of laccase produced by the fungi in the biofilters with less nutrient availability is degrading the antibiotics retained by the straw. If that were the case, it would be an advantage versus the other biofilters with MEA because they would be able not only to remove the antibiotics from the water, but also to obtain a residue without antibiotic activity.

3.2.2. Peroxidases

The levels of versatile peroxidase (VP) were very low in all the biofilters, in the majority of the aliquots taken over 24 hours they were not detected. That is the reason why the results of table 3 only include the MnP activity.

Table 3. *Manganese Peroxidase activity in biofilters over 24 hours. Activity is expressed in U L^{-1}. Treatments described as P.ostreatus +MEA and P.eryngii + MEA correspond to P.ostreatus and P.eryngii with malt extract agar. P.ostreatus and P.eryngii in straw medium are represented as P.ostreatus and P.eryngii respectively. ND is not detected and NP not performed.*

Time (h)	0	0.5	1	2	4	6	24
***P.ostreatus*+ MEA**	0.5	0.2	ND	ND	0.1	0.6	2.1
***P.eryngii* + MEA**	ND	2.5	ND	ND	ND	ND	ND
P.ostreatus	NP	NP	12	29	41	33	31
P.eryngii	NP	NP	82	78	108	136	116

The results of MnP activity are lower than the laccase, but they had the same behavior, there was more MnP in the biofilters without MEA, reaching the higher enzymatic activity in *P.eryngii* that had lower levels of laccase than *P.ostreatus.*

In the *P.eryngii* with MEA, MnP was not detected in most of the samples, and the presence in the *P.ostreatus* with MEA biofilters it was present in very low concentrations or not detected. However, the antibiotic showed that in these biofilters the SAs and TCs were removed, which implies that the peroxidases did not have a direct effect in these degradations.

It is necessary to remember that those enzymes oxidized every substrate susceptible to be degraded; they could be degrading other pollutants of the water or simply the straw. That might be why the fungi was segregating them even if they have no impact in the antibiotic removal. Their presence is higher in the biofilters without MEA, where the nutrients were less available, a situation that forced the fungi to segregate more extracellular enzymatic activities to obtain nutrients from the water. However, in MEA presence, the nutrients were easily available and the fungi did not need to segregate extracellular enzymes.

An evaluation of the effect of these enzymes and a way to take advantage of this enzyme production in the removal of other emergent pollutants needs to be carried out.

3.3. Relationship between antibiotic degradation and ligninolytic activity

The straw alone was able to eliminate part of the antibiotics from the water, but the presence of fungi improved the removal and also the way to degrade the antibiotics, this is the reason why the study of their behavior became absolutely important.

The biofilters with malt extract agar segregated low doses of extracellular enzymes, however, these biofilters were able to degrade SAs with an effectivity between 80 % to 99% depending on the fungi and the sulfonamide in less than 24 hours. Biofilters without malt extract, segregated higher extracellular activities and were also able to degrade antibiotics reaching 90 % of removal in less than 5 hours in all the antibiotics.

It should be necessary to evaluate the contribution of the intracellular enzymes, such as CYP450, in the antibiotic degradation in order to improve the biofilters. To create a biofilter with longer useful life, the rich nutrient media such as MEA, could be useful if they maintain the removal of antibiotics over time with less extracellular activity. Further studies are needed to identify if there is also a parallel removal of pollutants of the wastewater and the efficiency of both kinds of biofilters.

4. Conclusions

Biofilters of spent mushrooms substrates with straw and ligninolytic fungi mycelia were effectively used to degrade sulfonamides and tetracyclines. The removal was a combination between the adsorption by the straw and the degradation by the fungi.

Several studies are being performed to relate the activity of intracellular enzymes (CYP450) in the degradation with a nutrient rich medium and the influence of extracellular enzymes (Lac, MnP and VP) when the media is only the spent mushroom substrate. They also would determine if the enzymatic activity is also degrading the antibiotic retained in the straw.

Biofilters developed in present work represent a new and feasible approach for the treatments of wastewaters and solid wastes with emergent pollutants such as antibiotics tested. Further research is needed in order to optimize them for their future application.

5. Acknowledgments

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)

6. References

[1] O'Neill J. Tackling Drug-Resistant Ingections Globally: Final Report And Recommendations. The Review On Antimicrobial Resistance. World Health Organization. (2016).

[2] European Medicines Agency. European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption, 2015. 'Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013'. (EMA/387934/2015). (2015).

[3] Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, Borrego CM, Barceló D, Balcázar JL. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res.; 69:234-42. (2015).

[4] Hatakka A, Hammel KE. Fungal Biodegradation of Lignocelluloses. In: Karl Esser, editor. The Mycota: Industrial Applications. 2 ed: Springer Berlin Heidelberg; pp. 319-339. (2010).

[5] Migliore L, Fiori M, Spadoni A, Galli E. Biodegradation of oxytetracycline by Pleurotus ostreatus mycelium: A mycoremediation technique. J Hazard Mater. 215-216:227-32; (2012).

[6] Alidadi H, Dolatabadi M, Davoudi M, Askari FB, Jamali-Behnam F, Hosseinzadeh A. Enhanced Removal of Tetracycline Using Modified Sawdust: Optimization, Isotherm, Kinetics, and Regeneration Studies. Process Saf Environ Prot. (2018).

[7] Gern RM, Wisbeck E, Rampinelli JR, Ninow JL, Furlan SA. Alternative medium for production of Pleurotus ostreatus biomass and potential antitumor polysaccharides. Bioresour Technol. 99(1),76-82. (2008).

[8] García-Delgado C, Yunta F, Eymar E. Bioremediation of multi-polluted soil by spent mushroom (Agaricus bisporus) substrate: Polycyclic aromatic hydrocarbons degradation and Pb availability. J Hazard Mater. 300:281-8. (2015).

[9] Baquero F, Martínez J, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol. 19(3):260-5. (2008)

[10] Lucas D, Barceló D, Rodriguez-Mozaz S. Removal of pharmaceuticals from wastewater by fungal treatment and reduction of hazard quotients. Sci Total Environ. 571:909-15. (2016)

[11] Manaia CM, Macedo G, Fatta-Kassinos D, Nunes OC. Antibiotic resistance in urban aquatic environments: can it be controlled?. Appl Microbiol Biotechnol. 100(4):1543-57. (2016)

[12] Gupta A, Jana AK. Effects of wheat straw solid contents in fermentation media on utilization of soluble/insoluble nutrient, fungal growth and laccase production. 3 Biotech. 8(1):35. (2018).

[13] Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimha G. Fungal laccases and their applications in bioremediation. Enzyme Res. 2014:163242. (2014).

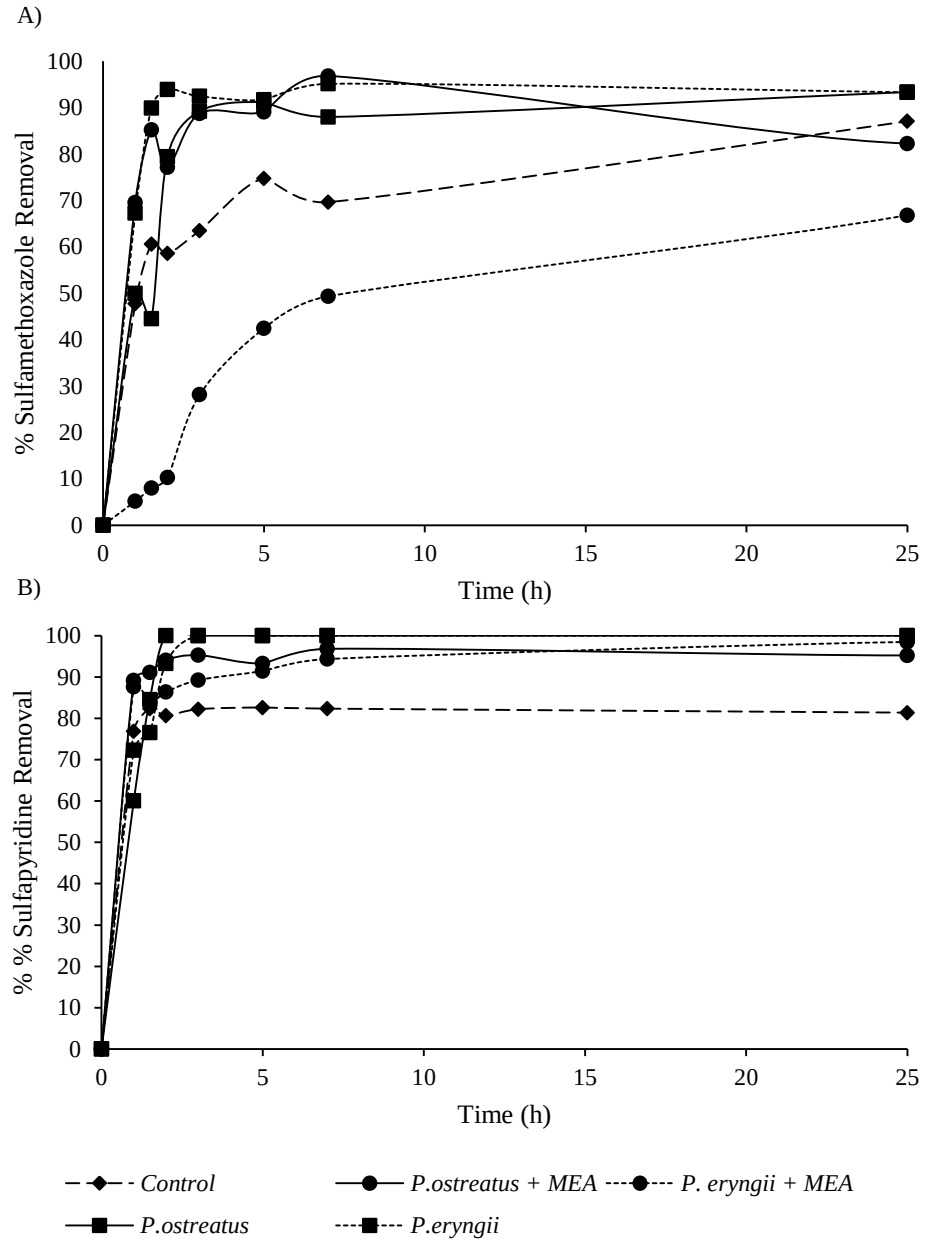

Fig 1 Percentage of removal of sulfonamides A) Sulfamethoxazole B) Sulfapyridine over time in the different biofilters. *P.ostreatus* in straw with malt extract agar is represented as *P.ostreatus* + MEA, *P.eryngii* in the same conditions as *P.eryngii* + MEA, and the biofilters with *P.ostreatus* and *P.eryngii* in straw medium as *P.ostreatus* and *P.eryngii* respectively. The control refers to the straw biofilter without fungi.

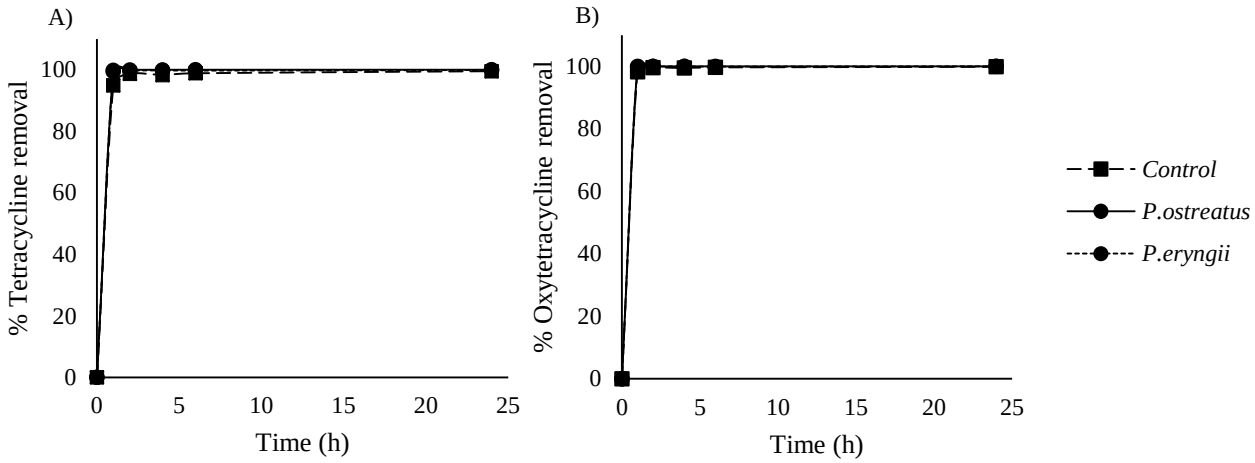

Fig 2 Percentage of removal of tetracyclines A) Tetracycline B) Oxitetracycline over 24 hours in the different biofilters. *P.ostreatus* and *P.eryngii* in straw medium are represented as *P. ostreatus* and *P. eryngii* respectively. The control refers to the straw biofilter without fungi.