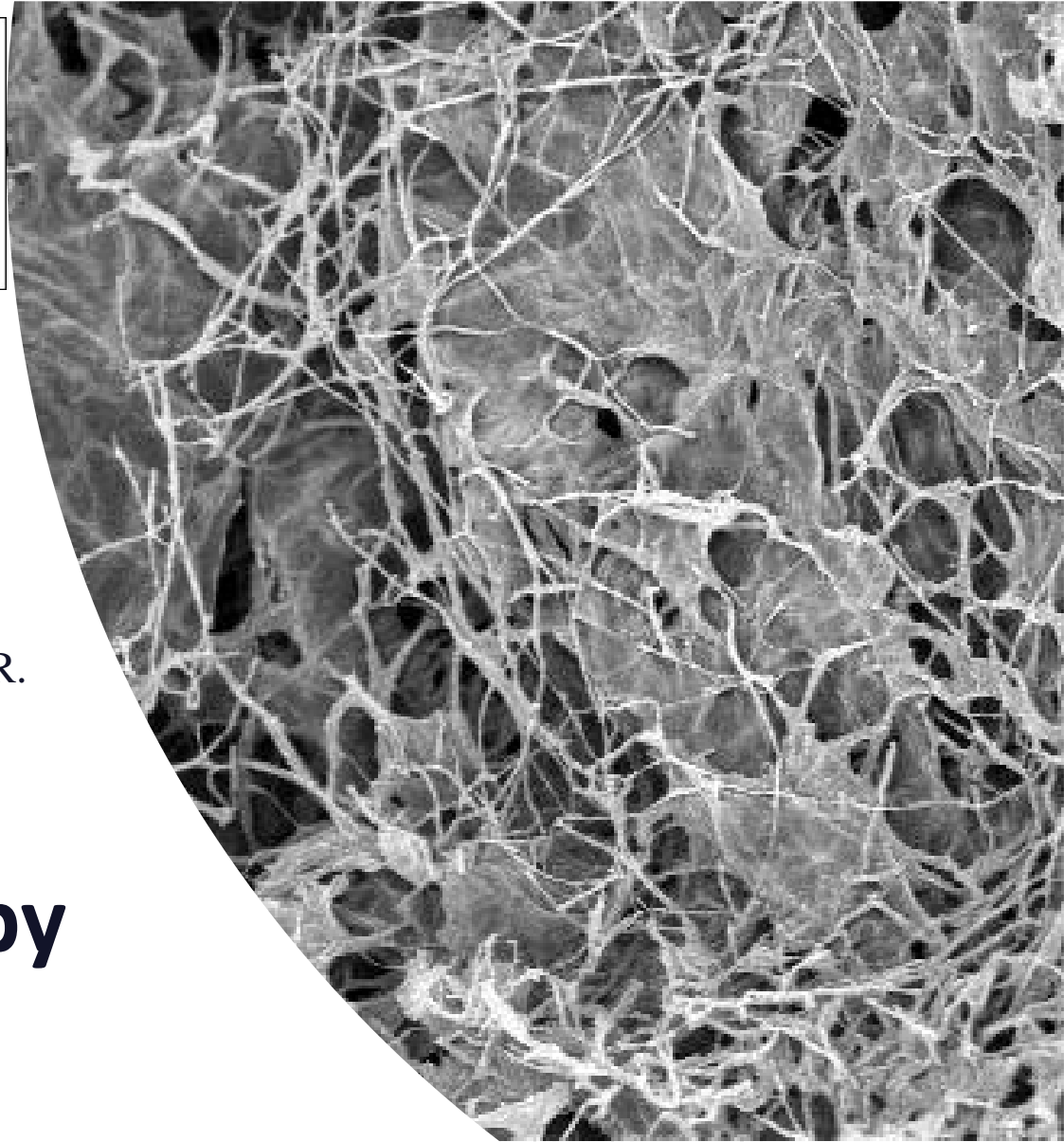


B. Mayans, R. Camacho-Arévalo, C. García-Delgado, R. Antón, C. Escolástico, ML. Segura, E. Eymar

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

6th International Conference on Sustainable Solid Waste Management, Naxos Island, Greece, 13–16 June 2018



INTRODUCTION

UE

According to the European Medicines Agency, 8,361 tons of antibiotics were sold in 2015 for veterinary use (EMA, 2017)

USA

According to US Food and Drug Administration, 36,982 tons of antibiotics were marketed in USA in 2016 for livestock (FDA, 2016)

CHINA

A 105,000 tons of antibiotics were sold in China in 2015 (Collignon P., 2015)

INTRODUCTION



Antibiotics can be easily found in wastewater, soil, sewage sludge and cattle manure, being some of them very persistent (Grenni P. et al, 2018)

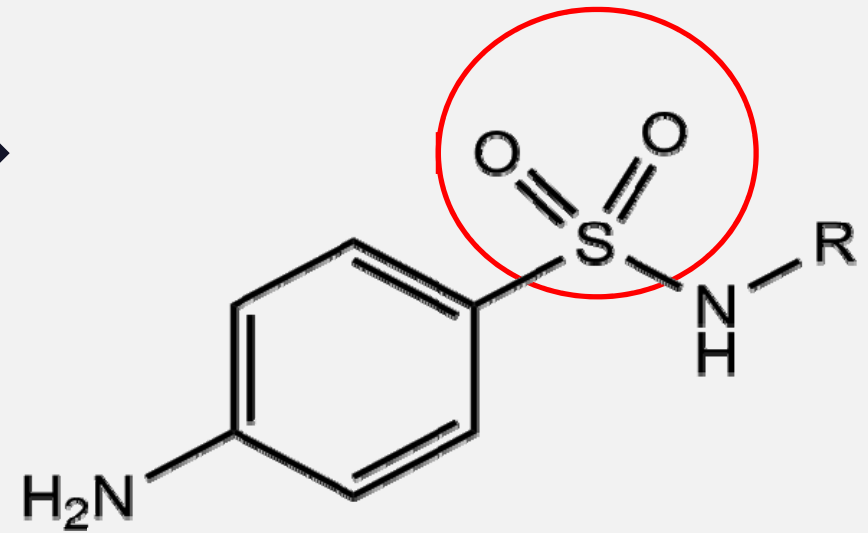
Antibiotics reach the food chain by adding cattle manure or sewage sludge to croplands as soil organic amendments and fertilizers, as well as watering the crops with polluted water. (Martínez-Carballo E. et al, 2007)

Widespread sub-lethal concentrations of antibiotics promote resistant and multiresistant bacteria which are a real threat for human health. (Berendonk TU et al, 2015)

INTRODUCTION

- Sulfonamides are a group of broad spectrum antibiotics widely prescribed for human and livestock healthcare.

- Sulfonamides have been very frequently detected in surface water in many countries since current wastewater treatments are not effective to remove antibiotics.



General chemical structure of Sulfonamides

INTRODUCTION

- Ligninolytic fungi can secrete extracellular enzymes, like laccase and MnP, capable to degrade efficiently lignin and other organic compounds.

- Some of these fungi are cultivated for human consumption with the consequent co-generation of enormous amounts of spent mushroom substrate



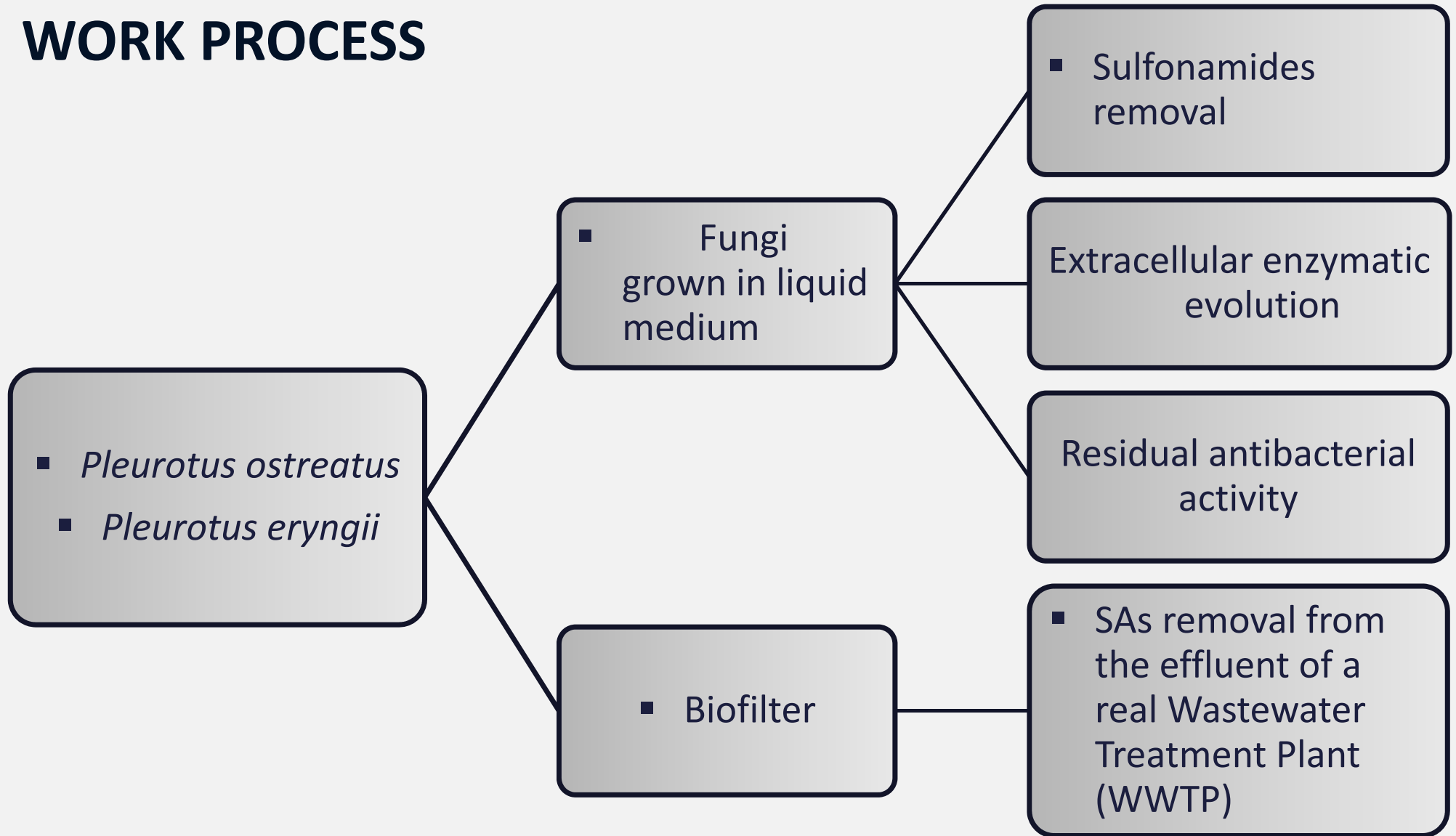
OBJECTIVES

To assess the potential for Sulfonamides removal of two of the most cultivated worldwide edible fungi, *Pleurotus ostreatus* and *Pleurotus eryngii*

To relate fungal ligninolytic activity with antibiotic removal.

To test the capability of *Pleurotus ostreatus* and *Pleurotus eryngii* to remove antibiotics from the effluent of a WWTP

WORK PROCESS

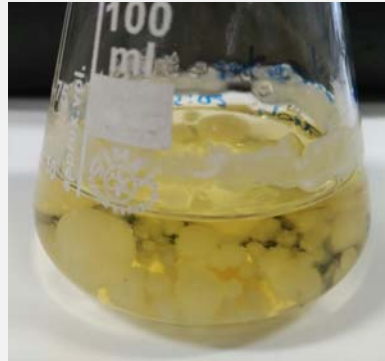


SULFONAMIDES REMOVAL ASSAY

Materials and Methods



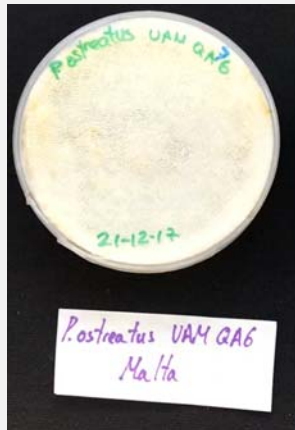
3 pieces of culture medium



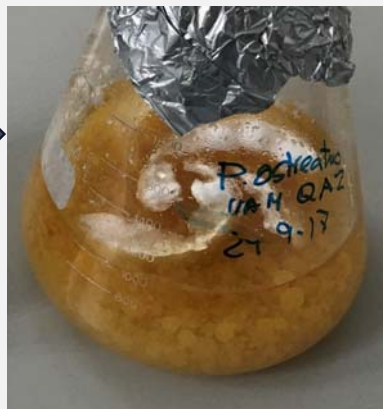
1 mL



- 3% malt extract
- 5 SAs 0.1 mM
- agitation and darkness



3 pieces of culture medium

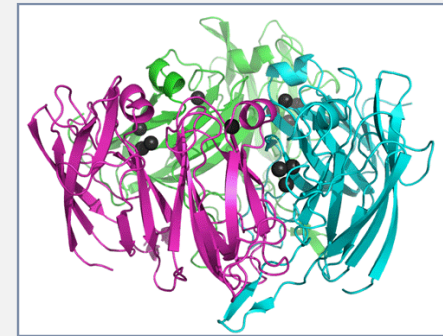


1 mL



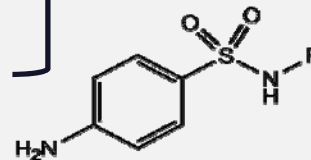
1mL daily for 17 days

Enzymatic activity

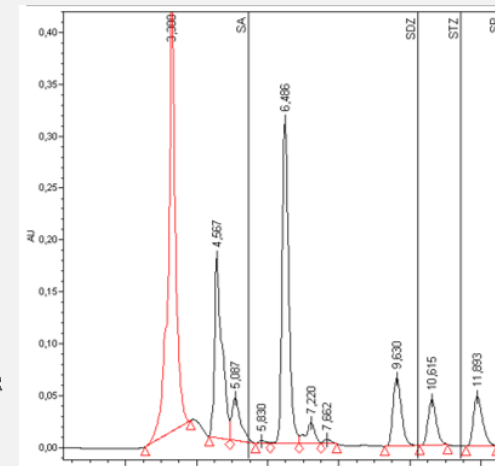


Laccase and MnP activity

SAs analysis



Sulfonamides general structure



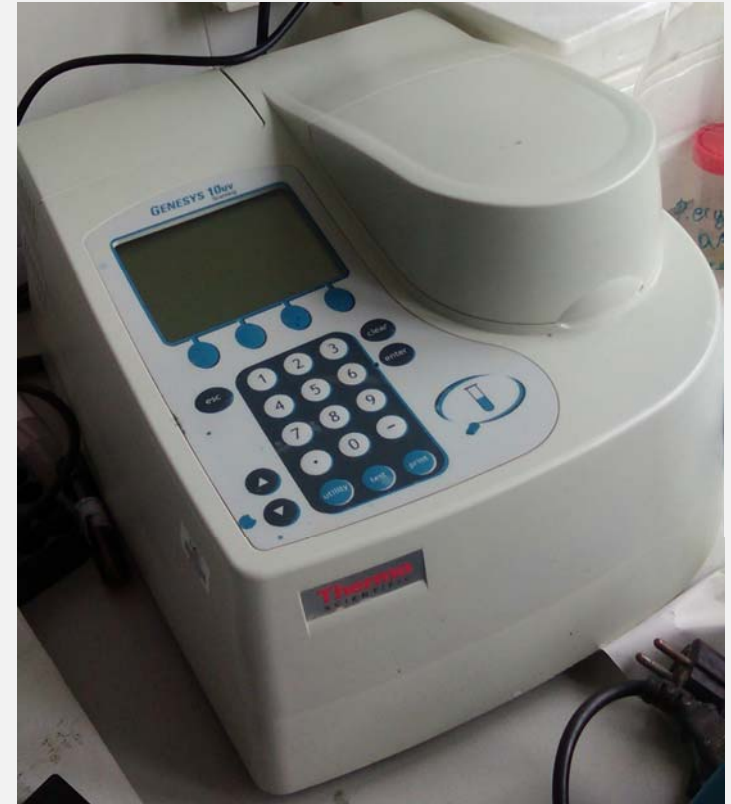
HPLC-PDA

QUANTIFICATION OF SAs

- **Sulfonamides:** Sulfadiazine (SDZ), Sulfathiazole (STZ), Sulfapyridine (SP), Sulfamethazine (SMZ) and Sulfathiazole (SMX).
- **HPLC system:** Separation module coupled with a photodiode array detector (PDA), (Waters)
- **Chromatographic separation of SAs:**
 - Luna C18 (250 mm × 4.6 mm: 5 μm) column
 - Gradient elution program with 20 mM ammonium acetate with acetic acid and ACN:MeOH (1:1)
 - Flow rate of 0.9 mL min⁻¹.
 - 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.

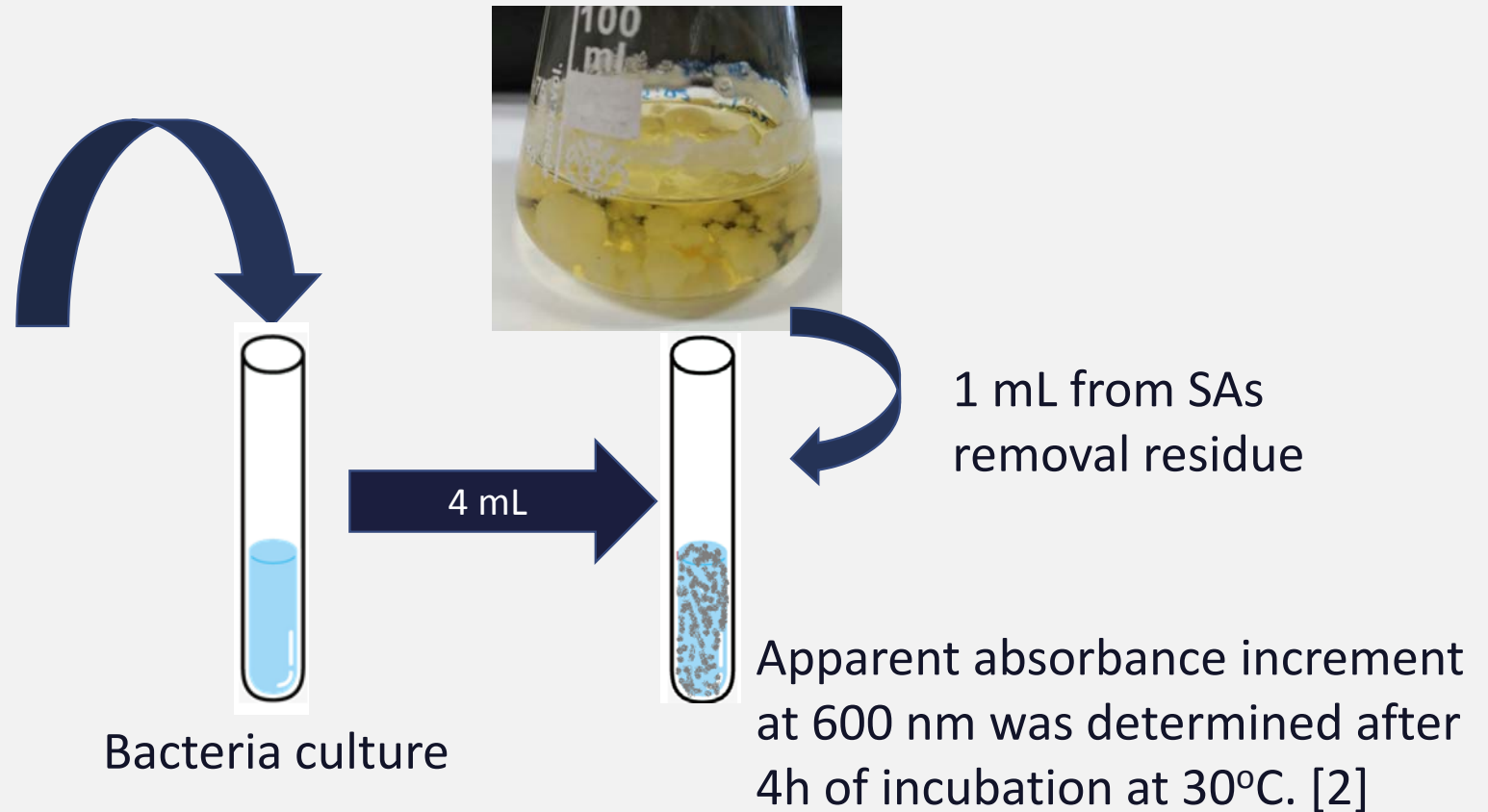
ENZYMATIC ACTIVITIES DETERMINATION

- **Laccase** and **MnP** were spectrophotometrically determined
- **Laccase activity** by oxidation of 2 mM 2,6-dimethoxy phenol [1].
- **MnP activity** by Mn^{3+} - malate complex formation in 1 mM MnSO_4 [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.



Spectrophotometer Genesis Thermo Scientific

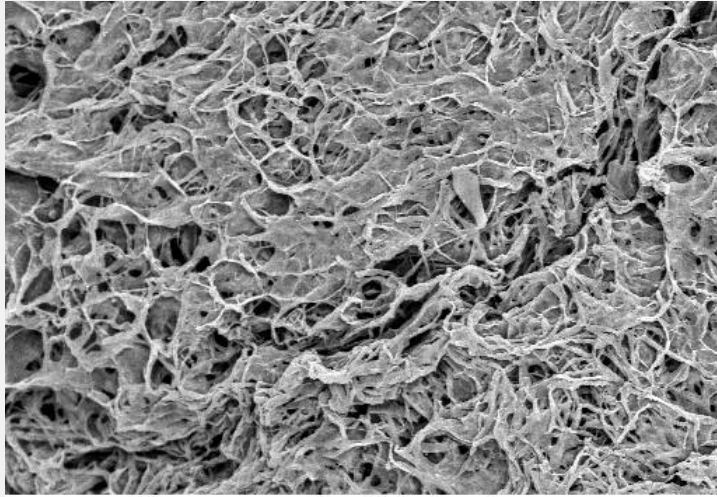
RESIDUAL ANTIBACTERIAL ACTIVITY ASSAY



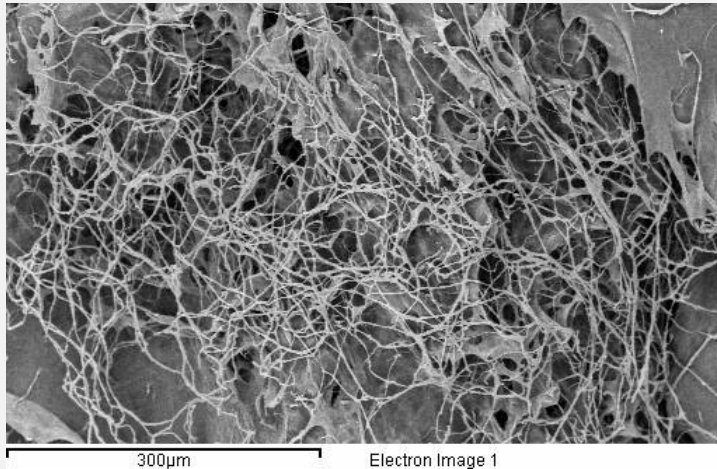
- Controls without antibiotics and with 0.1 mM of SAs were performed in parallel.

2. Ashrafi, S.D., Rezaei, S., Forootanfar, H., Mahvi, A.H. y Faramarzi, M.A. (2013). The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*. *International Biodeterioration and Biodegradation*, 85: 173-181. doi: 10.1016/j.ibiod.2013.07.006

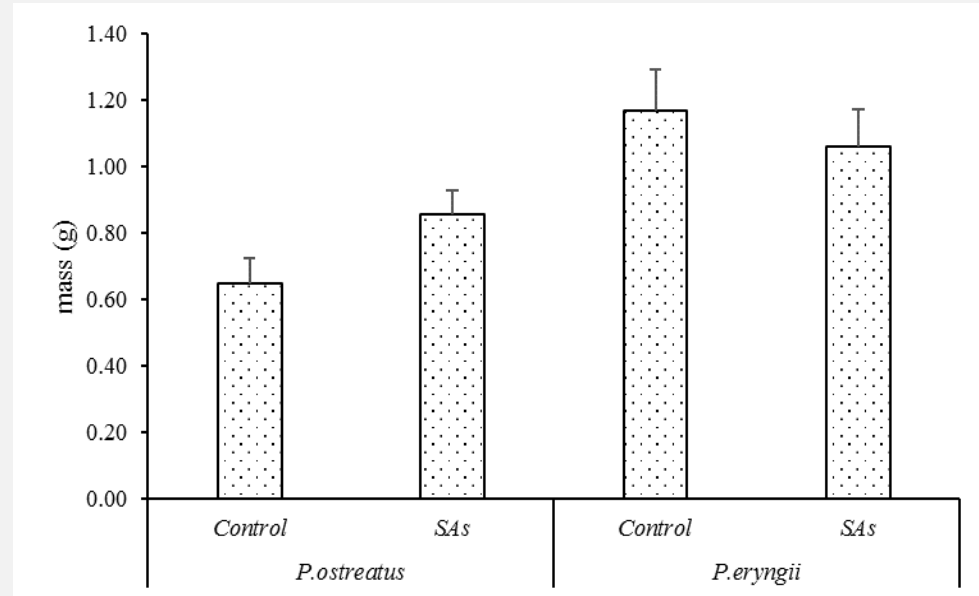
RESULTS : Fungal Growth



SEM micrograph of *P. ostreatus* mycelia Control



SEM micrograph of *P. ostreatus* mycelia SAs sample ($1,3 \mu\text{g L}^{-1}$)



- There were no significant difference between controls and antibiotic samples
- A different way of growing was appreciated by Scanning Electron Microscope (SEM).

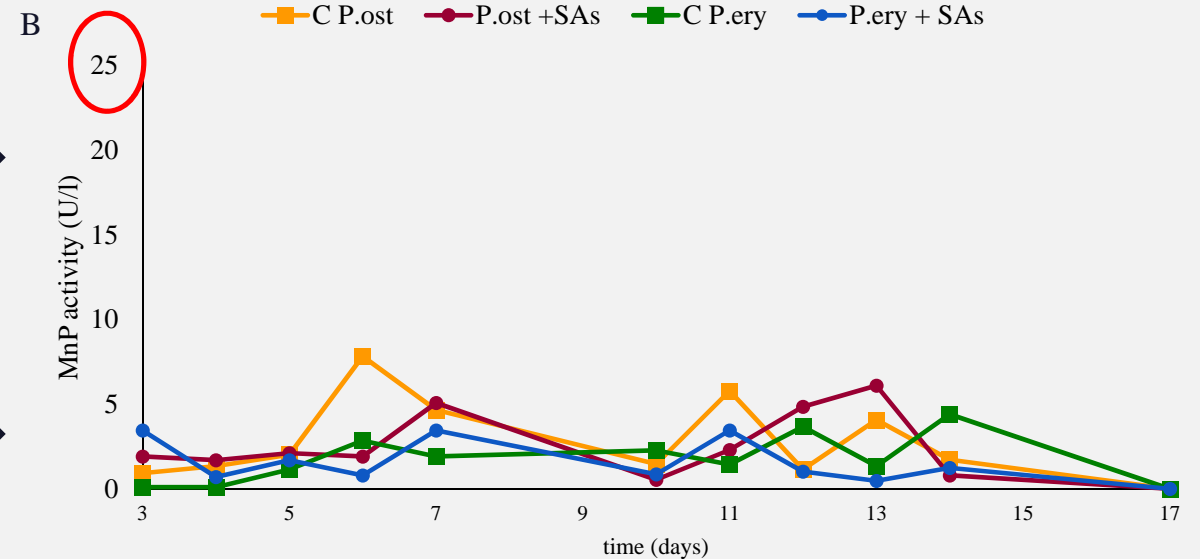
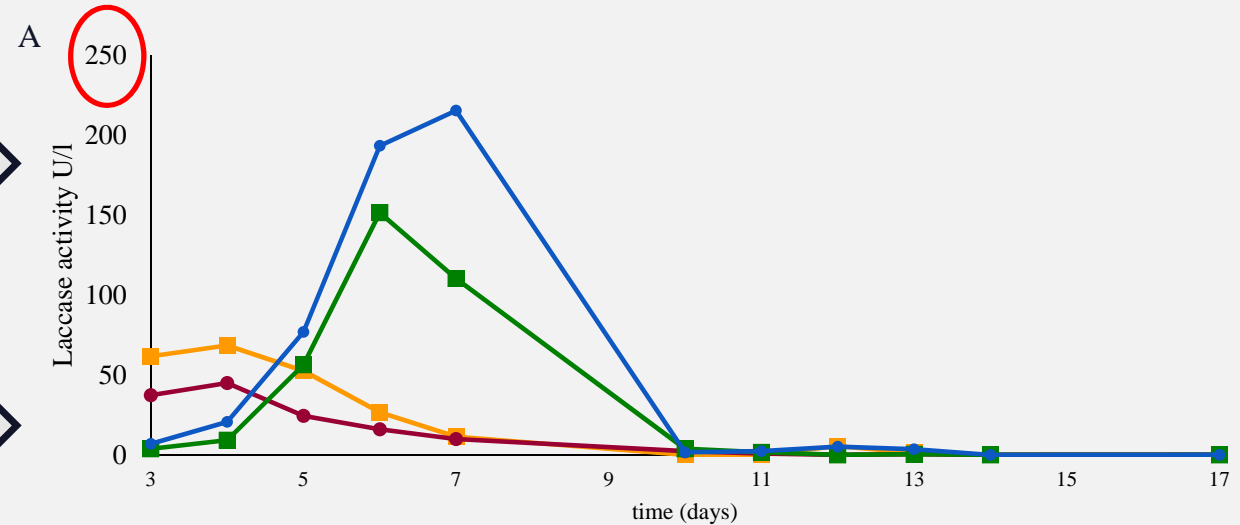
RESULTS : Enzymatic activity

■ Lacasse was much higher produced than MnP by both fungi.

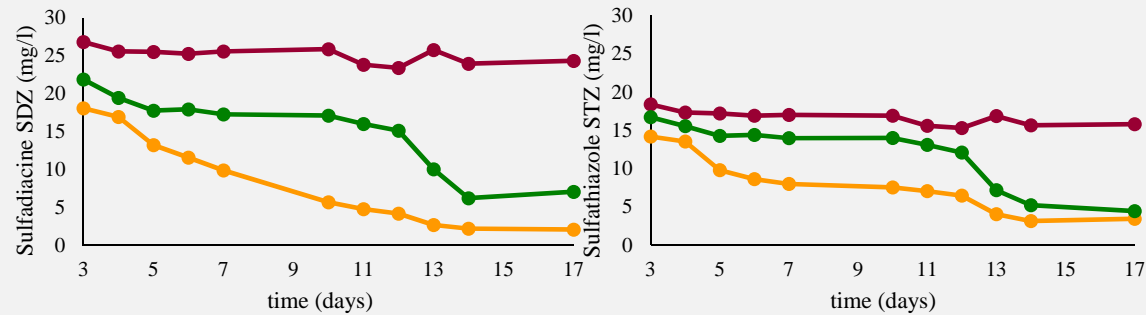
■ *P. ostreatus* showed higher laccase levels in control (68 UL⁻¹)

■ *P. eryngii* showed higher activity in presence of SAs reaching 215 UL⁻¹ at day 7.

■ MnP was hardly produced in addition to its irregular expression in both fungi



RESULTS: SAs removal assay

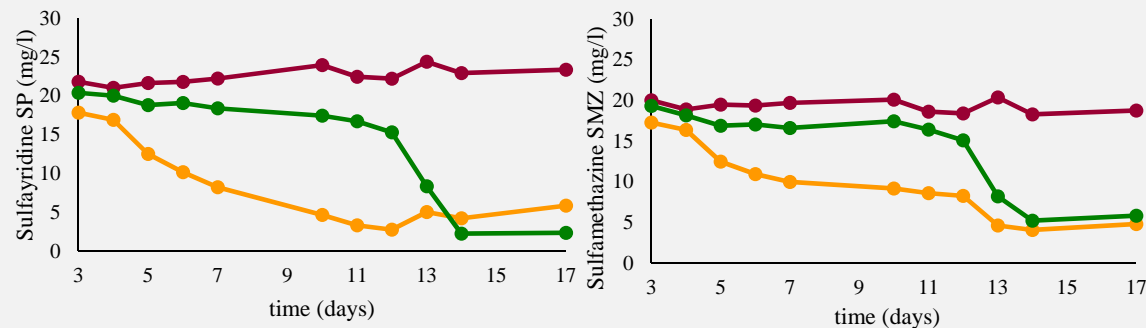


➤ *P. ostreatus*

- Removed SAs very efficiently a rate higher than 70% at day 10 (SP, SDZ)

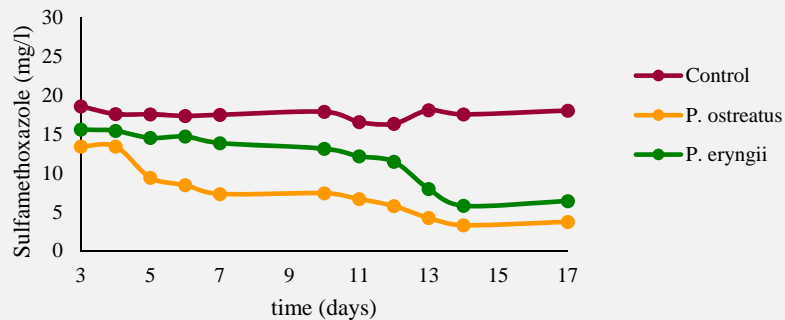
➤ *P. eryngii*

- Removed SAs slower than *P. ostreatus* reaching at day 14 a 89% of removal (SP)



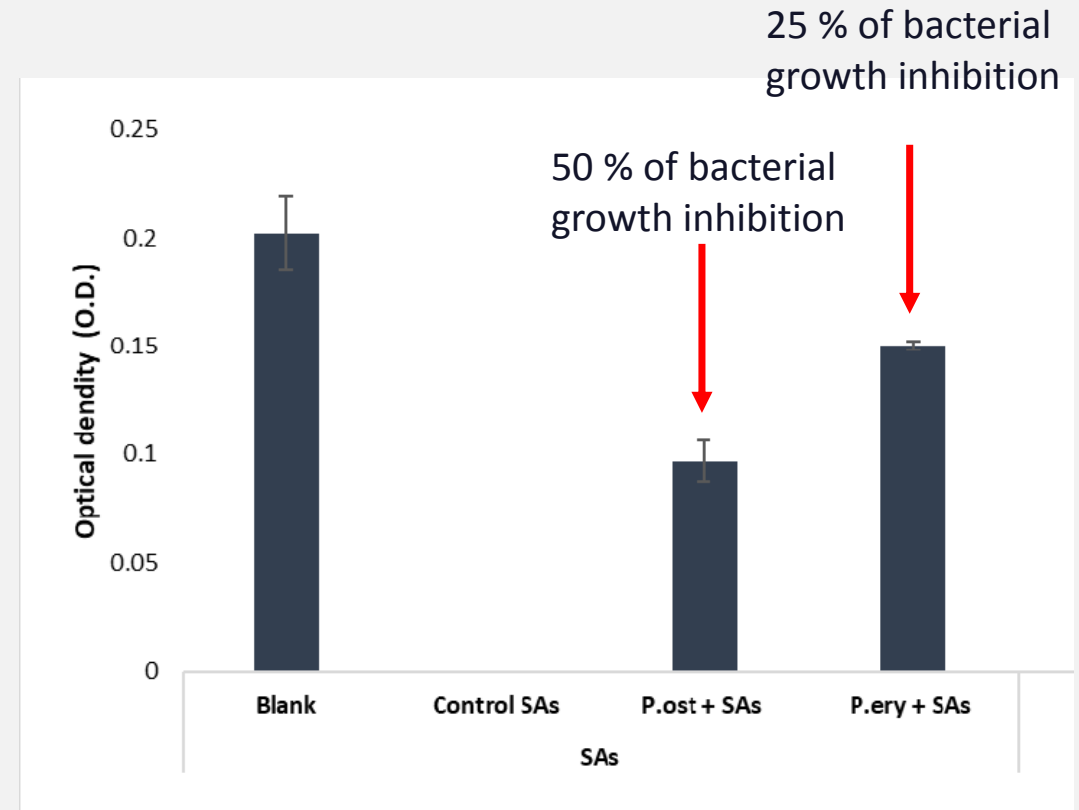
➤ Laccase was involved in SAs removal

➤ MnP could not be linked due to the low levels and irregular behavior



RESULTS: Residual antibacterial activity

- *P. eryngii* residues inhibited bacterial growth less than *P. ostreatus* compared to control.
- *P. eryngii* seemed to be more efficient degradant regarding bacterial growth inhibition since 75 % of the antibiotic activity disappeared



BIOFILTER: REMOVAL OF SAs FROM A WWTP



The sampled water was analysed to know if there were any SAs by UHPLC-MS-MS



SAs concentration in the sampled water ranged between 20 and 400 ng L⁻¹



Effluent water of a real WWTP was sampled

- **UHPLC/MS/MS:** UHPLC module coupled with a TQD triple quadrupole detector, Waters (Mildford, MA, USA)
- MRM mode in positive electrospray.
- Column: BEH C18 (100x2.1 mm 1,7 μ m) at 45°C.
- Sulfathiazole ⁻¹³C₆ and Sulfamethoxypyridazine-d₃ were used as internal standards.

BIOFILTER: REMOVAL OF SAs FROM A WWTP

Materials and Methods

Five fungal pellets were taken from malt extract culture

Fungi were grown in Teflon containers filled with their spent substrate and 250 mL of malt-agar for 3 days

400 mL the effluent of a urban WWTP were introduced by a peristaltic pump at a flow of 8 mL min^{-1} .

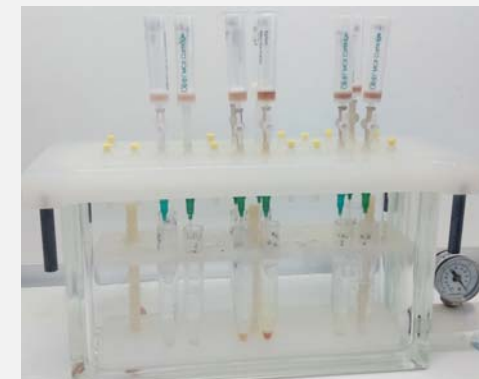
Aliquots of 30 mL were taken to be analysed



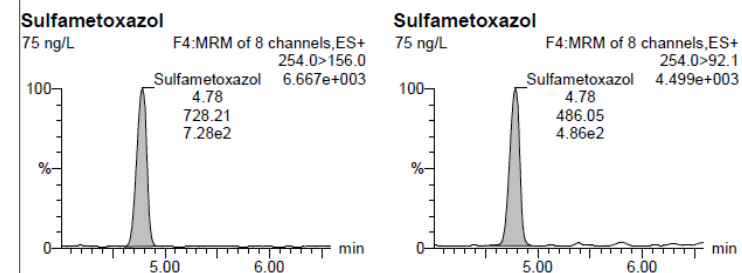
BIOFILTER: REMOVAL OF SAs FROM A WWTP

Materials and Methods

Antibiotics were then concentrated and purified by a SPE procedure using AEDT-McIlvaine buffer (50/50).

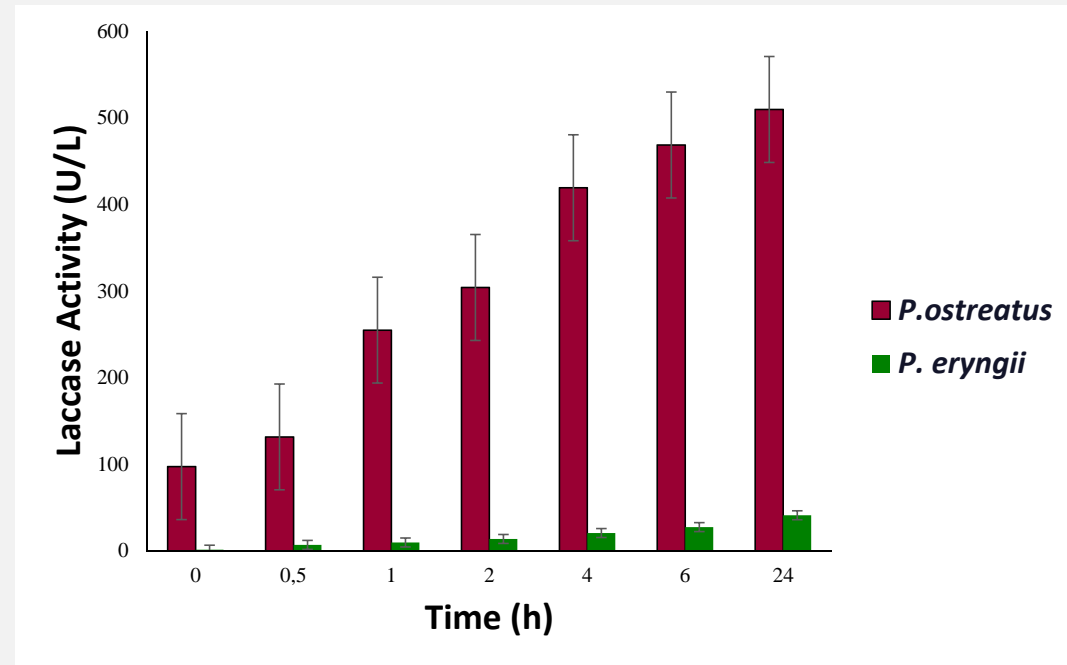


Analytes were eluted with ethyl acetate, evaporated to dryness, and reconstituted in methanol/water (15/85) to be analysed by UHPLC-MS-MS.

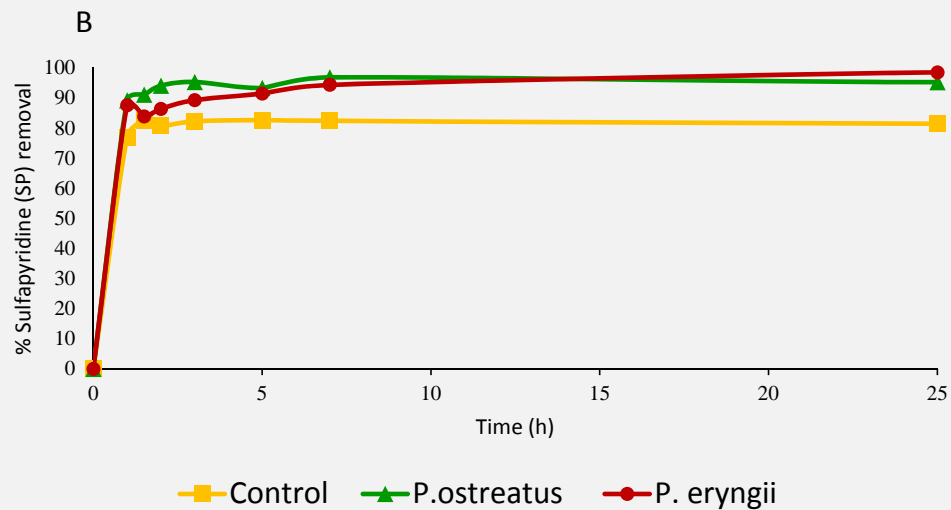
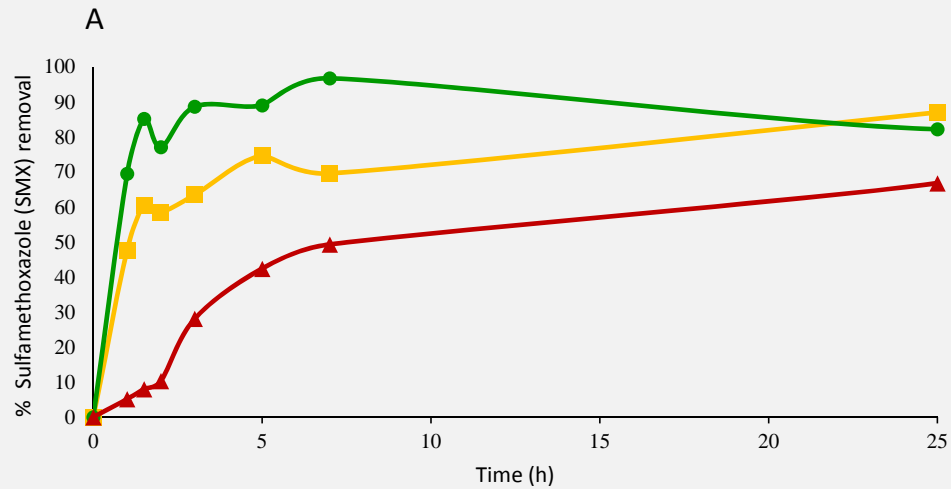


RESULTS: Enzymatic activity

- *P.ostreatus* showed much higher laccase expression in 24 h (510 UL^{-1}) than in the previous assay due to the semi-solid substrate straw based.
- *P.eryngii* had much lower laccase levels than *P.ostreatus* despite its efficient SAs removal while in the previous assay occurred just the opposite.
- Laccase showed a key role in SAs removal since MnP was hardly segregate by both fungi.



RESULTS: SAs removal



- *P.ostreatus* removed 93% of SMX in 24 h from a initial concentration of 424 ng L⁻¹
- 79 % of SP was removed in 24 h from an initial concentration of 21 ng L⁻¹.
- *P.eryngii* had a SMX removal rate of 67 % in 24 h.
- Meanwhile 94 % of SP was removed in 24 h.
- *P.eryngii* seemed to remove SAs slower than *P.ostreatus* in SMX case which was in higher concentration.

CONCLUSIONS

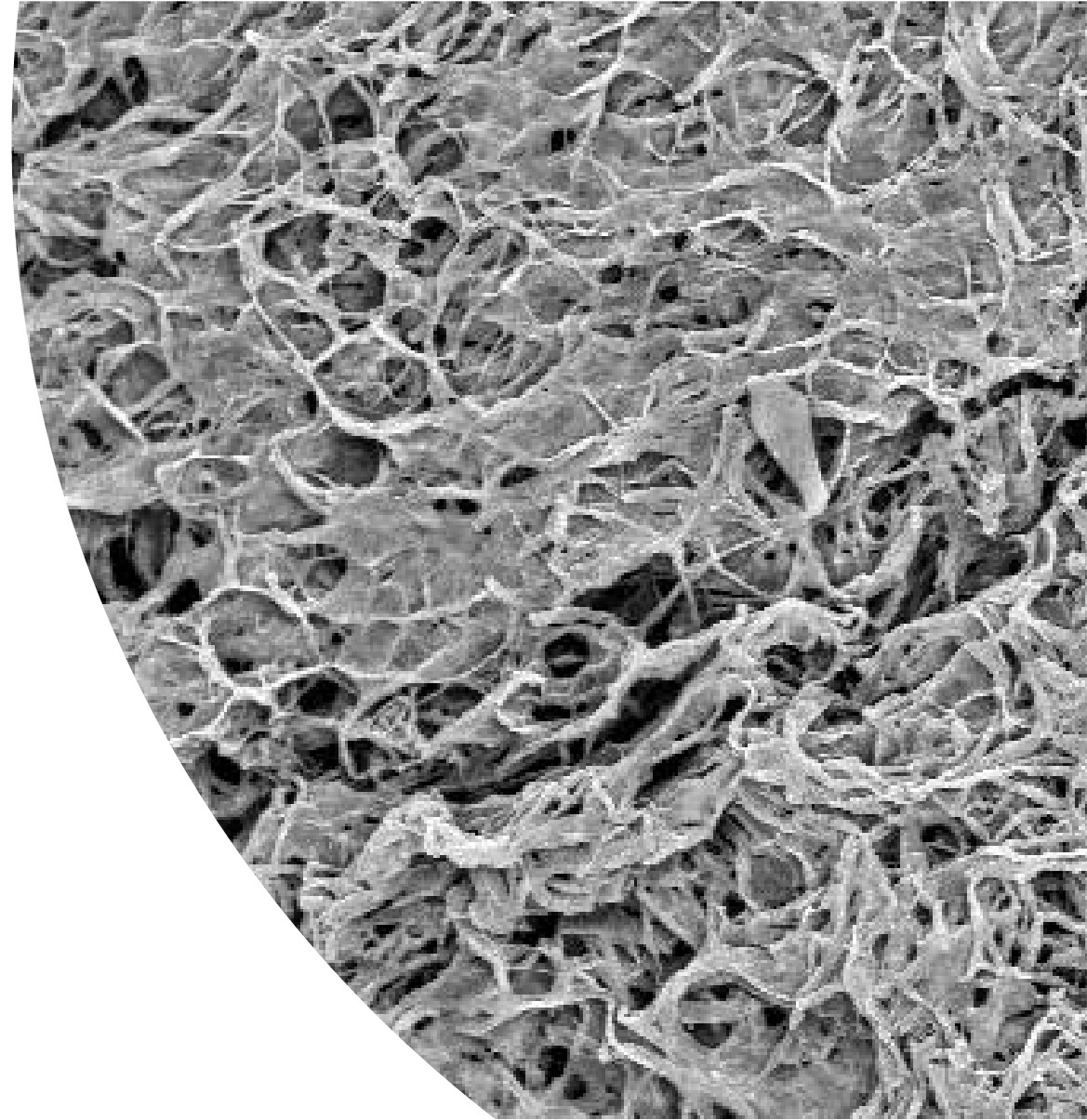
P. ostreatus removed most of SAs found in the effluent water of a WWTP in 24 h.

P.ostreatus appeared to be the most powerful fungus for SAs removal from the effluent water of a WWTP, at high and low concentrations as well as in the liquid medium assay

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.

Laccase could be linked to SAs removal both in liquid medium and biofilters with the effluent of a WWTP

Thank you very
much for your
attention!!!!



Electron Image 1