Potential of edible fungi to degrade antibiotics present in different solid wastes

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Abstract

The evolution of resistant bacterial strains is a natural phenomenon that occurs when mutant microorganisms reproduce between them generating new resistant bacterial strains. The incorrect and excessive use of antibiotics is accelerating this process, since those pharmaceuticals are not completely metabolized, and they are excreted in the medium as the original compound. Antibiotics reach the environment through the current inadequate cleaning process in wastewater treatment plants and the use of livestock manure and sewage sludge as organic amendment applied directly on soils and croplands. Some edible spent mushroom substrates holding active fungal mycelia could be used to remove antibiotics from composts and organic wastes before applying since they have a powerful enzymatic system with oxidative activity, such as cytochrome P450 and extracellular peroxidases capable of diffusing in the matrix and oxidize antibiotics (manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase). The aim of this work is to promote antibiotic removal, sulfonamides and tetracyclines which have been detected at low concentrations in organic wastes from different origins, sewage sludges, manures, and composts through the inoculation of four edible fungi (*P. ostreatus, A. bisporus, P. eryngii and P. edodes)*. Firstly, twelve organic amendments were analysed to detect the presence of antibiotics, secondly a survival assay was done to assess ligninolytic fungal growth and finally an antibiotic removal assay was performed with *A. bisporus* which showed the greatest growth and the highest extracellular peroxidases activity. Results were really promising since *A. bisporus* reached some Sulfonamides and Tetracyclines removal rates up to 90% after 21 days growing on organic wastes of different origin.

Keywords

antibiotics, ligninolytic fungi, bioremediation, agricultural wastes, sewage sludge

Introduction

Emerging contaminants (EC) are a wide group of compounds of different origin and chemical structure that are released to natural ecosystems in low concentrations but continuously, which make bacteria resistant to them and subsequently evolve producing new resistant bacterial strains [1]. Those pharmaceuticals residues are not currently under regulation, so that they are released into the environment without any control, moreover their removal is not mandatory. They stand for a real threat to human health due to the increase of resistant bacteria and the toxic effects produced to ecosystem, although their environmental impact is not thoroughly known.

Antibiotics are included in EC group. They are especially dangerous because of the effect on the bacterial resistance, since all king of bacteria, pathogen or not, are exposed continuously to sub-lethal doses. In the last few decades antibiotics consumption has been exponentially increasing, not only by humans but

livestock and aquiculture. So that they are excreted in the medium as the original compound since they are not completely metabolized [2]. In addition, the increase in human population has made increase the sewage sludge production, a final residue in Wastewater treatment plants (WWTP), which must be eliminated since this organic material accumulate a lot of contaminants, not only antibiotics but any other personal care and pharmaceutical substance. The application of sludge in agriculture as soil amendment is one of the most useful solutions for its disposal, taking advantage of its organic matter content [3]. Sewage sludge is normally composted together with other organic residues before being applied to soils, but even composted it kept a lot of active pharmaceuticals, including antibiotics.

Thus, antibiotics reach natural ecosystems through WWTPs where the traditional processes do not clean properly wastewater and they are released in the effluent water as well as in the sewage sludge. In addition, other composted organic materials, such as cattle manure or crops residues which hold organic pollutants as well, are also applied on soils to enhance their organic matter content [4]. Besides antibiotics could affect the bacteria involved in biogeochemical cycles and the ones responsible for supporting biological processes in water and soil [5]. So, those useful organic materials are dangerous at the same time due to the content in antibiotics and other EC, which must be removed before being applied to soils and farmland, since resistant coliforms have been reported in soil and on vegetables at harvest [6].

Sulfonamides (SAs) and Tetracyclines (TCs) are worldwide prescribed for livestock so their residues are widely detected in all kind of organic wastes such as compost, cattle manure or sewage sludge [7].

Ligninolytic fungi are well known to be able to oxidize a lot of organic compounds thanks to their powerful ligninolytic enzymatic system. They have been reported to degrade organic contaminants such as synthetic dyers, polycyclic aromatic hydrocarbons (PAHs) [8-10]; [11], heavy oils, or pharmaceutical compounds including antibiotics[12];[13]. Some of these fungi are widely cultivated for human consumption co-generating a huge amounts of spent mushroom substrate [14]; [15]. The usefulness of spent mushroom substrate to degrade organic pollutants has already been proved, as well the key role of the fungal mycelium which remain active inside the spent substrate, in the process [16]; [9]; [17].

The main goal of this work is to assess the capability to grow and remove the SAs and TCs present in organic wastes materials of different origin of four edible ligninolytic fungi which were directly inoculated or held on wheat straw: *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus eryngii* and *Lentinula edodes*. All of them produce extracellular peroxidases able to oxidise organic compounds such as antibiotics. Extracellular peroxidases, such as laccase and manganese peroxidase (MnP) are secreted by their mycelia to oxidize different organic substrates such as lignin or cellulose and they can diffuse in the solid growth medium and oxidize antibiotics. *A. bisporus* resulted to show the higher growth and ligninolytic enzymes production; thus, it was selected to carry out the antibiotic removal assay.

Materials and methods

Chemical and reagents

Antibiotics were purchased from Sigma Aldrich (St. Louis, MO, USA), Demeclocycline hydrochloride hydrate, Sulfathiazole -$^{13}C_6$ and Sulfamethoxypyridazine-d_3. 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), HCl 37% and Trichloroacetic acid 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). Specific culture media MacConkey was acquired from Scharlaub (Barcelona, Spain).

Organic amendments and fungal strains

Six organic wastes were selected from twelve that were previously analyzed, two sewage sludges which were obtained from two WWTPs, SS1 in Almeria (Spain) and SS2 in Madrid (Spain), two livestock manures (MS1 from sheep and MS2 from cattle, hen and goat), and two composts from vegetable wastes (VC1 and VC2).

Four ligninolytic edible fungi were used, *P. ostreatus*, which was isolated from spent mushroom substrate collected in a commercial crop placed in Quintanar del Rey (Cuenca, Spain). *A. bisporus, P. eryngii and P. edodes* were obtained from *c*ommercial strains purchased from Gurelan Mycelium located in Huarte (Navarra, Spain). Fungi were inoculated both directly to the organic wastes from malt extract agar (MEA) and using wheat straw as fungal carrier. The inoculation was in all cases done in a laminar flow hoods to prevent biological contamination.

Determination of ligninolytic enzymes

Ligninolytic enzymes (laccase and MnP) were determined in the enzymatic extract according to Garcia-Delgado et al.[9]. Laccase and MnP were extracted from 1,5 g of organic material samples using 30 mL of Tris-HCl 0,1 M buffer at pH 7,5. The mixture was then shaken at 160 rpm for 1h at 4 ^{o}C in an iced water bath. Then, the aqueous suspension was centrifuged (8000xg, 10 min) and the supernatant was paper filtered and assayed for ligninolytic activities.

Laccase activity was spectrophotometrically determined following the oxidation of 2 mM 2,6-dimethoxy phenol in 50 mM sodium acetate pH 5.0 at 477 nm (ϵ = 14600 M^{-1} cm^{-1}).

MnP activity was assayed by Mn^{3+}-malate complex formation in 1 mM $MnSO_4$ in 50 mM malic acid/sodium malate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2 at 270 nm (ϵ = 11590 M^{-1} cm^{-1}).

One unit of enzyme activity (IU) is defined as the amount of enzyme which produces 1 μmol of product per minute under the assay conditions.

Quantification of antibiotics

All the materials were previously analysed to assess the presence of antibiotics to perform the degradation assay.

Antibiotics were 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. Demeclocycline hydrochloride hydrate, Sulfathiazole -$^{13}C_6$ and Sulfamethoxypyridazine-d_3 were used as internal standards (IS)

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

Elution gradient for Sulfonamides			
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

TCs residues concentration in sampled materials were much higher than SAs when the origin of the solid waste was manure or sewage sludge, while vegetable wastes showed higher SAs content. SMX was the most abundant antibiotic of the SAs family, its concentration ranged between 10 and 1500 ng kg^{-1}, meanwhile TCs were ranged of μg kg^{-1}. The highest concentration of TC and OTC were found in sewage sludge, where TC concentration was 10 μg kg^{-1} and OTC 88 μg kg^{-1}.

Fungal survival assay by direct inoculation

Three pieces from MEA culture of the four fungi were directly inoculated on six organic wastes, SS1 and SS2 (sewage sludges), MS1 and MS2 (manure composts) and VC1 and VC2 (vegetable wastes composts) and incubated for 14 days days at 28 ^{o}C in darkness. Fungal growth was checked every day to verify their survival on these media.

Fungal survival assay using wheat straw as a fungal carrier

Three mycelial pieces of 5 mm from MEA culture of each fungi were inoculated and grown in glass containers on straw-based substrate for 20 days before the inoculum on the materials. Then, approximately 5 g of straw carrying the fungi were laid on the six materials in Petri dishes and incubated for 14 days at 28 ^{o}C in darkness. Every day fungal growth was checked.

Antibiotics removal assay on two selected materials

A.bisporus was selected to follow the removal assay since it showed higher growth and extracellular peroxidases production in the preliminary survival assay with a fungal carrier of wheat straw. As it was able to grow in every tested material, two of those materials of different origin with higher antibiotics (SAs and TCs) content were selected to carry out the degradation assay, one sewage sludge (SS1) and one manure compost (MS1).

Glass containers were filled with 80 gr of wheat straw based spent substrate and then sterilized in autoclave at 121ºC for 20 min before inoculation. Three mycelial pieces of *A. bisporus* of 5 mm from MEA culture were inoculated and grown in the glass containers for 20 days. Then, an aliquot of 5 g of straw holding the mycelium was placed on the selected materials in Petri plates. Antibiotic analysis and MnP and laccase activities measures were done every seven days for 3 weeks.

Antibiotics were extracted from 3 g of each material with TCA 0,1%, then shaken for 10 min. centrifugated (9000xg, 5 min) and paper filtered. Antibiotics were concentrated and purified by a SPE procedure using lipophilic/hydrophilic balanced Oasis HLB cartridges. Analytes were eluted with ethyl acetate, then evaporated to dryness under 12 psi N_2 flow and 42 ºC, and finally reconstituted in 0.25 mL of methanol/water (15/85) to be analysed by UHPLC-MS.

Ligninolytic enzymes activity was analysed just after the extraction of the aliquots.

Results and discussion

Fungal survival assay by direct inoculation

After the direct inoculation of the four fungi (*P. ostreatus, P. eryngii, A. bisporus and L. edodes*), fungal growth was monitored daily for 14 days. All the four fungi had grown on VC1 at day 7 of while in SS1 only *A. bisporus* and *P. eryngii* were able to grow and develop mycelium. No growth was shown in manures (MS1 and MS2) nor vegetable compost VC2, probably because of the physicochemical characteristics or humidity levels. The sources of carbon in those materials might not be easy enough for the fungi to degrade which could prevent the fungal growth.

Fungal survival assay using wheat straw as a fungal carrier

When fungi were inoculated with wheat straw as a carrier in SS1, *P. ostreatus, A. bisporus, P. eryngii* showed a significantly higher growth and development after only 3 days. *A. bisporus* showed a vigorous growth after 7 days in SS1, VC1 and VC2. Meanwhile *P. ostreatus, P. eryngii* and *L. edodes* grew only in VC1. At day 14 *A. bisporus* had grown in all materials except from SS2. In SS1 the mycelium was very active, producing even a change of color in the sludge which could mean a great degradation of different

compounds of the material. The rest of the fungi only kept present in VC1 and SS1, but they hardly grew. So, *A. bisporus* was selected to follow the antibiotic removal assay.

Wheat straw was a suitable carbon source for all the fungi which allow them to develop and perform the antibiotics degradation in contrast with the lack of growth that happened when the fungi were inoculated directly on the organic wastes. In addition, since lignocellulosic substrates enhance ligninolytic fungi to grow, other easier biodegradable carbon sources could enhance bacterial growth considering that those organic wastes probably have a great bacterial load which might interfere with the performance of the fungi [18].

Enzymatic activities determination in Fungal survival assay using wheat straw as a fungal carrier

Since SS1 presented higher fungal growth using wheat straw as fungal carrier, it was selected to assess the ligninolytic enzymatic activity. MnP and laccase activity were measured at day 14; MnP was detected in the three fungi, although *A. bisporus* showed the highest activity, 1203 U kg^{-1}. *P.ostreatus* and *P.eryngii* secreted much less MnP, 42 and 57 U kg^{-1} respectively. Meanwhile, laccase was only produced by *A. bisporus* at a huge level, 10700 U kg^{-1}. This high laccase production correlated with the vigorous growth of this fungus, since according to some researchers, extracellular laccase production can be used as a mycelial growth marker when *A.bisporus* grows in compost [19].

A.bisporus is a litter-decomposing fungus which has been proved by other researchers to grow and produce elevated levels of extracellular enzymes on straw-based substrates [20], which correlate with the results of this assay. So that, *A. bisporus* seemed to be the best choice to perform the degradation assay due to its growth and peroxidases production.

Antibiotic removal assay of A. bisporus on two selected organic wastes

The extracellular enzymatic expression of *A.bisporus* resulted to be different depending on the material where the fungus grew. MnP and laccase were measured once a week for 3 weeks in the two selected materials (SS1 and MS1), following the described procedures.

Laccase was only secreted in SS1 and MnP in both materials. When *A. bisporus* grew on sewage sludge (SS1) the main expressed enzyme was laccase which showed a great activity at day 7 (1173 U kg^{-1}) showing the highest at day 14 (3210 U kg^{-1}), then laccase activity decreased with levels of 2145 U kg^{-1} at day 21, meanwhile MnP showed its highest level at day 21 (1449 U kg^{-1}) despite showing only 3 U kg^{-1} at day 7. (Fig 1). Meanwhile, *A.bisporus* displayed other enzymatic pattern when the fungus grew on manure (MS1), being MnP the main extracellular enzyme secreted since laccase was not detected along the 3 weeks of the assay (Fig.2). In addition, the fungus did not reach such an elevated level occurred when it grew on sewage sludge. MnP in MS1 reached the highest level at day 14, while in SS1 the peak of MnP happened at day 21. In any case, ligninolytic enzymes were much more produced by *A. bisporus* on sewage sludge than on manure compost.

According to those findings the growth medium had an important influence on extracellular peroxidases expression. Some other researcher had obtained comparable results with other ligninolytic fungi growing in different media [21]. This is an interesting researching topic to achieve the best conditions to obtain high peroxidases levels from *A.bisporus*, which has a high potential and awesome perspectives to be used in bioremediation of polluted materials.

A.bisporus was tested to remove antibiotics from the two selected organic wastes which previously were analysed and resulted to have both families of antibiotics, SAs and TCs which are known to have different chemical structure and consequently a different behaviour.

The results were absolutely promising, since A.bisporus was able to remove both SAs and TCs from the two of the organic materials, SS1 and MS1. Regarding SS1, the removal rates of SAs were higher than 90% at day 21 apart from SP, which was a 77%. (Fig.3). It is remarkable the fact that all the five SAs which were detected in this sludge (SDZ, STZ, SP, SMZ and SMX) showed a removal rate above a 67% in the first week, reaching even that a 90% of STZ removed and 98% of SMX at day 7. So, most of SAs contents had been removed around day 7, even SP which suffer a fast removal during the first week, just like the other four although its removal rate was hardly increased along the rest of the weeks the assay lasted. The case of the two TCs present in the sludge (TC ad OTC) was like SAs, despite the much higher

content of them SS1 had that ranged in µg kg^{-1}. Both TC and OTC were completely removed along the assay and what is more at day 14 the 98% of both TCs had already been removed.

Antibiotics removal correlated with laccase production, which showed at day 14 an activity 3 folds higher than at day 7, so that laccase seemed to be responsible for TC removal, specially the first week when MnP was almost inexistent (7 U kg^{-1}) compared to laccase (1173 U kg^{-1}) (Fig.1). Those findings agreed with previous assays using immobilized and free laccase from genus *Cerrena*, which could be correlated with TC removal [22] or Rodriguez-Rodriguez at al. (2012) who could linked laccase from *T.versicolor* with SAs [23]. Actually, MnP was also present in much lower concentration though, therefore it might be involved in antibiotic removal since in early works it has been correlated to TCs degradation [24] and to other organic pollutants [25].

Despite having a lot less MnP expression as well as an inexistent laccase production when *A. bisporus* grew on manure compost (MS1), both TCs and SAs were removed. However, this material had initial antibiotic concentrations much lower than SS1, except from SMX which was much higher. the SAs detected in manure compost were SMX, SDZ, STZ and SMZ and while the TCs were TC and OTC.

Most of TCs were removed after 7 days knowing that TC initial concentration was 272 ng kg^{-1} and OTC 829 ng kg^{-1}. TC was completely removed the first week while OTC was in two weeks. Whereas SAs were removed at a lower rate along the assay, despite having lower initial concentrations apart from SMX, whose concentration was similar to TC. SAs were removed above 90% at day 21 except for SMX which even at the end was removed around a 70% of the initial concentration.

Therefore, MnP could be related to TCs and SAs removal since laccase was not present.

The removal pathway must have been different since in this material *A.bisporus* did not show any laccase activity, so that MnP could be responsible for TCs and SAs removal although other enzymes might have been involved in antibiotic degradation such as CYP 450 or other oxidases like aryl-alcohol oxidase, aryl-alcohol dehydrogenase, quinone reductase or glyoxal oxidase since they were not measured or taken into account and some other researchers have previously related some of those enzimes to pharmaceuticals degradation [25, 26].

Comparing the results obtained from the fungal behavior growing on these two different substrates, it was very interesting the fact that the same fungus had two patterns of ligninolytic enzymes expression and therefore two different paths of antibiotics removal, since in both cases antibiotics were efficiently removed. MnP seemed to be better TCs oxidizer than laccase since it was secreted and was present in both materials and the removal rate was the same knowing that both TCs content and MnP level were much higher in SS1 than MS1 (Figs. 1 and 2). Meanwhile, some SAs removal, like SMX, was more efficient when laccase was present although SS1 held less SMX than MS1 (Figs 3 and 4). On the other hand SP was not completely removed from SS1 (Fig. 3) despite the huge levels of laccase and MnP secreted.

All those findings are very promising since *A.bisporus* is worldwide cultivated for human consumption and the amount of its spent substrate generated is enormous, so the possible use of this residue to bioremediate organic amendments which hold antibiotics or any other organic pollutants before applying on soils could be an excellent choice; and it opens an interesting researching topic as well as its enzymatic behaviour depending on the substrate it grows.

The use of spent mushroom substrates can be an effective and environmentally friendly way to clean solid wastes and valorise organic residues obtained from the commercial cultivation of the fungi.

Conclusions

A.bisporus was able to remove both SAs and TCs from two different solid organic wastes, such as sewage sludge and sheep manure compost which held those antibiotics in different concentrations.

A.bisporus was able to grow on different organic wastes, develop an active mycelium and secreted elevated levels of extracellular peroxidases.

MnP could be correlated to TCs and SAs removal since *A. bisporus* only secreted MnP when grew on sheep manure compost and the antibiotics were removed.

Spent mushroom substrate residues holding active mycelia may be an effective and environmentally friendly way to clean organic amendments before being applied on soil or croplands avoiding antibiotics release in the environment.

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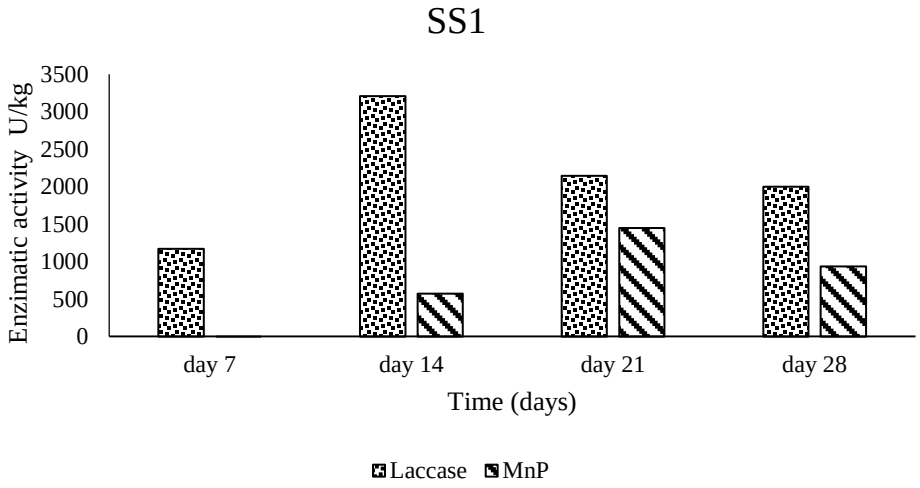

Fig. 1. *A.bisporus* laccase and MnP activities growing on sewage sludge (SS1) during the removal assay

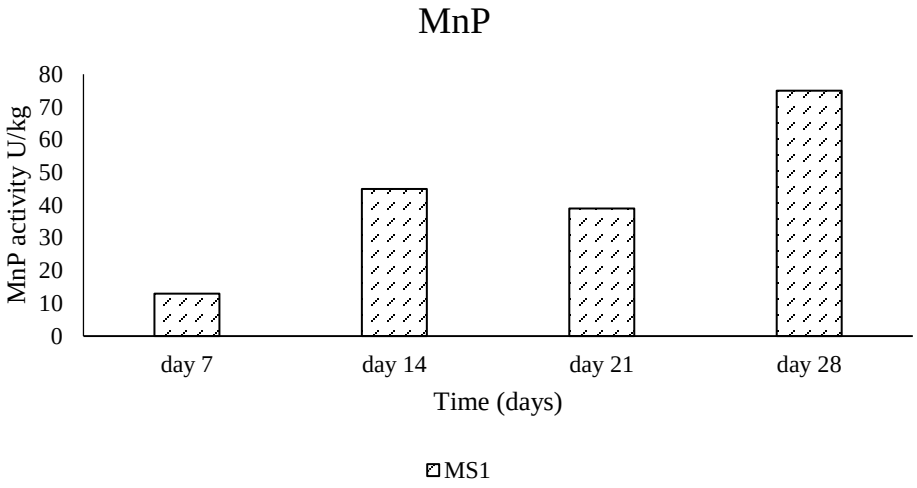

Fig. 2. *A. bisporus* MnP activity growing on manure compost (MS1)

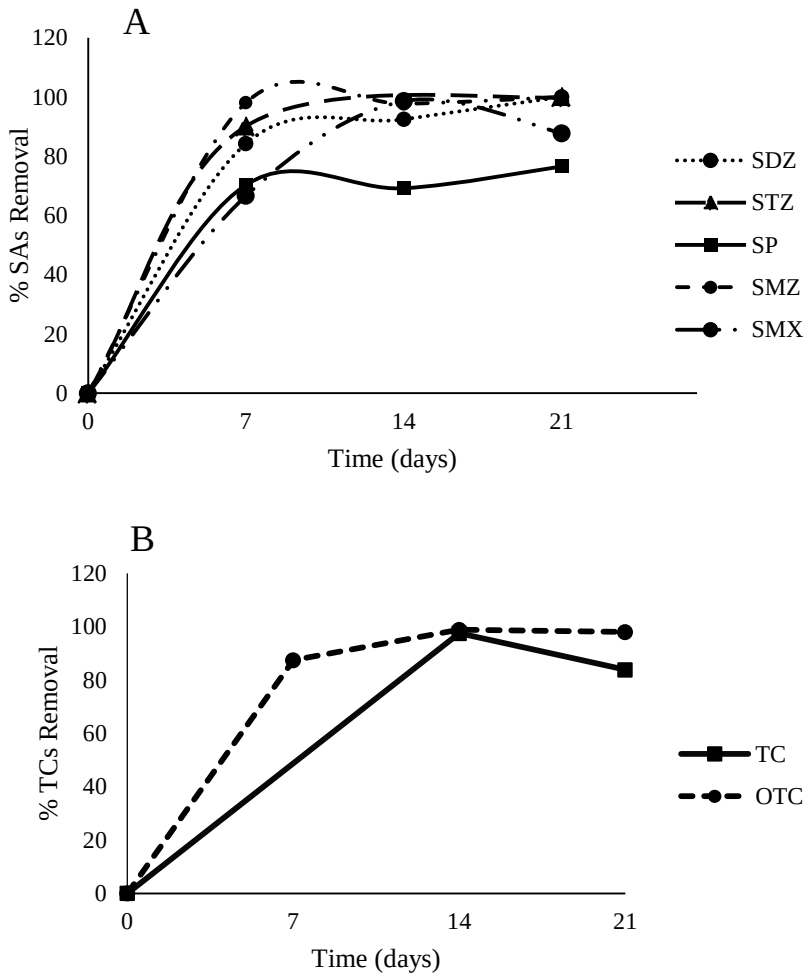

Fig. 3. Percentage removal of A) Sulfonamides; B) Tetracyclines in SS1

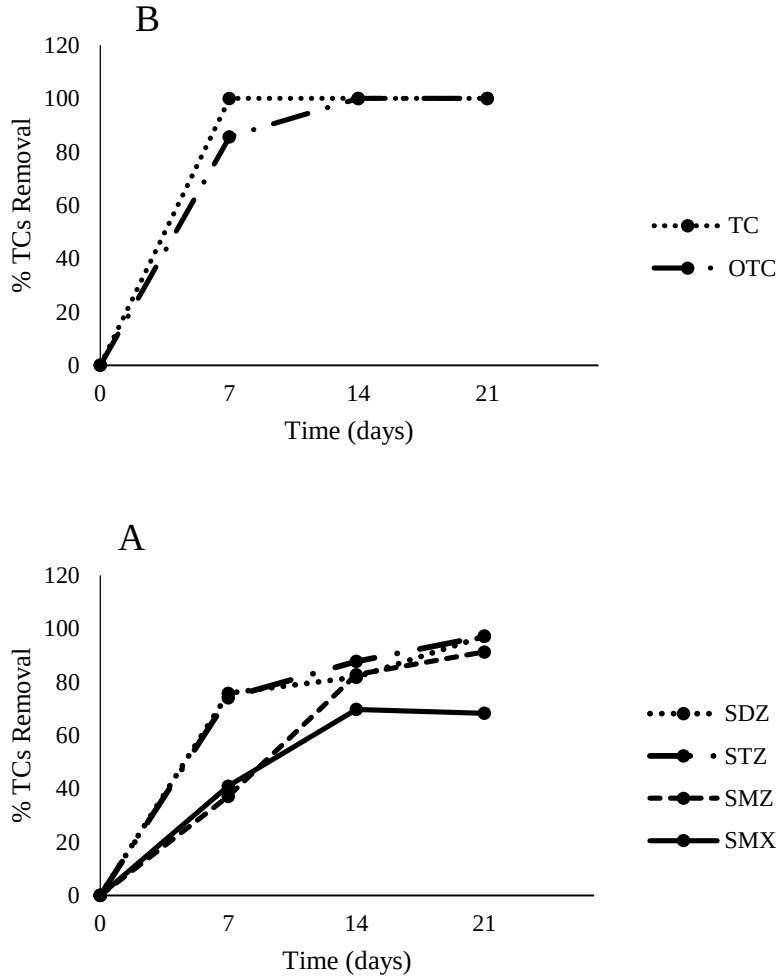

Fig. 4. Removal percentage of A) Sulfonamides; B) Tetracyclines in MS1