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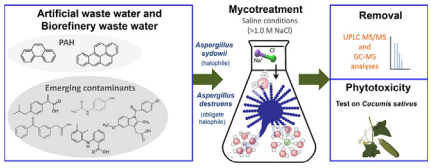
# First demonstration that ascomycetous halophilic fungi (*Aspergillus sydowii* and *Aspergillus destruens*) are useful in xenobiotic mycoremediation under high salinity conditions

Deborah González-Abradelo[a,1], Yordanis Pérez-Llano[a,1], Heidy Peidro-Guzmán[a,1], María del Rayo Sánchez-Carbente[b], Jorge Luis Folch-Mallol[b], Elisabet Aranda[c], Vinoth Kumar Vaidyanathan[d], Hubert Cabana[e], Nina Gunde-Cimerman[f], Ramón Alberto Batista-García[a,f,*]

[a] Centro de Investigación en Dinámica Celular, Instituto de Investigaciones en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos (UAEM), Ave. Universidad 1001, Col. Chamilpa, CP. 62209 Cuernavaca, Morelos, Mexico
[b] Centro de Investigaciones en Biotecnología, UAEM, Ave. Universidad 1001, Col. Chamilpa, CP. 62209 Cuernavaca, Morelos, Mexico
[c] Instituto Universitario de Investigación del Agua, Universidad de Granada, Calle Núñez Blanca 1, CP. 18003 Granada, Spain
[d] Department of Biotechnology, SRM Institute of Science and Technology, Mahatma Gandhi Rd, Potheri, SRM Nagar, Kattankulathur 603203, Tamil Nadu, India
[e] Faculté de Genié, Université de Sherbrooke, Sherbrooke, 2500 Boulevard de l Université, J1K 2R1 Sherbrooke, Quebec, Canada
[f] Faculty of Biology, University of Ljubljana, Kongresni trg 12, 1000 Ljubljana, Slovenia

## GRAPHICAL ABSTRACT

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## ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) and pharmaceutical compounds (PhC) are xenobiotics present in many saline wastewaters. Although fungi are known for their ability to remove xenobiotics, the potential of halophilic fungi to degrade highly persistent pollutants was not yet investigated. The use of two halophilic fungi, *Aspergillus sydowii* and *Aspergillus destruens,* for the elimination of PAH and PhC at saline conditions was studied. In saline synthetic medium both fungi used benzo-α-pyrene and phenanthrene as sole carbon source and removed over 90% of both PAH, *A. sydowii* due to biodegradation and *A. destruens* to bioadsorption. They removed 100% of a mixture of fifteen PAH in saline biorefinery wastewater. Test using *Cucumis sativus* demonstrated that wastewater treated with the two fungi lowered considerably the phytotoxicity. This study is the first demonstration that ascomycetous halophilic fungi, in contrast to other fungi (and in particular basidiomycetes) can be used for mycotreatments under salinity conditions.

* Corresponding author at: Centro de Investigación en Dinámica Celular, Instituto de Investigaciones en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos (UAEM), Ave. Universidad 1001, Col. Chamilpa, CP. 62209 Cuernavaca, Morelos, Mexico.
*E-mail address:* rabg@uaem.mx (R.A. Batista-García).
[1] Authors contributed equally to this work.

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## 1. Introduction

Anthropogenic activities have significantly impacted the health of different ecosystems with negative environmental consequences in terms of pollution. The generation and dissemination of xenobiotic compounds have characterized the industrial development of the last century. Xenobiotics are highly persistent in the environment with robust ecotoxic properties. There is growing interest in using biological approaches to remediate polluted environments in water bodies and waste waters contaminated with xenobiotics (Liu et al., 2017). Several biological treatments such as rhizoremediation, phytoremediation, bioattenuation, bioaugmentation, biostimulation and enzymatic degradation have similar or higher efficiency in removing contaminants than physico-chemical methods (Bisht et al., 2015). Therefore bioremediation is currently regarded as an approach with a high potential for industrial scale applications.

Contaminated environments pose different restrictions on microorganisms intended for industrial cleanup processes. Thus they can be threatened by extreme pH and temperature values, limited oxygen availability and toxic concentrations of persistent chemicals like heavy metals or polycyclic aromatic hydrocarbons (PAH) (He et al., 2013; Huang et al., 2015). An additional problem is the high salinity of waste discharges of industries that use salt for physico-chemical treatments, such as oil extraction sites and wastewaters from the petrochemical, agro-food, textile and leather industries as well as from the production of fertilizers, pesticides, herbicides, pharmaceuticals and dyes (Castillo-Carvajal et al., 2014). NaCl concentrations in such waste waters can range from 10 to 120 g/L (Lefebvre and Moletta, 2006). Thus, it is necessary to perform new biotechnological screening to characterize new microbial strains with the ability to remove xenobiotics under extreme conditions such as high salinity.

Although halophilic microorganisms have been investigated as promising agents for biotechnology (Mokashe et al., 2018) and to degrade pollutants at high salt concentrations since the 1990s, their bioremediation potential is unexploited (Castillo-Carvajal et al. 2014). In recent years some studies have shown progress in the use of halotolerant or halophilic bacteria to degrade xenobiotics such as formaldehyde (Veenagayathri and Vasudevan, 2010). Halophilic fungi, recognized only in 2000 (Gunde-Cimerman et al., 2000), have not even been screened for this potential. On the other hand, the use of fungi for bioremediation and degradation of recalcitrant compounds in non-saline conditions has gained great interest in recent years, given their outstanding metabolic diversity and versatility. However, to our knowledge, there is no study to date about the ability of halophilic fungi to degrade highly persistent pollutants in saline conditions.

PAH are a heterogeneous and ubiquitous group of hydrocarbons with two or more fused aromatic rings. These compounds are produced by incomplete degradation of organic matter in nature, and from anthropogenic activities such as spillage of petroleum products, waste incinerators, domestic heaters and burning of carbon, oil, gas or wood (Kadri et al., 2017). PAH are among the priority pollutants of EPA (Environmental Protection Agency, United States). The second class of contaminants, pharmaceutical compounds (PhC), have gained in importance in recent years due to their persistence and ubiquity in surface water bodies and the low efficiency of wastewater treatment plants in removing them (Haroune et al., 2014). PhC cause aquatic-life toxicity, development of microbial resistance, and disturbance of endocrine function (Olicón-Hernández et al., 2017). Since PAH and most PhC, dominant pollutants in some waste waters, are characterized by a high persistency and toxicity (Liu et al., 2017; Teijon et al., 2010), multiple investigations have focused on the microbial biodegradation of PAH and PhC, especially by fungi (Cruz et al., 2017; Haroune et al., 2014; Kadri et al., 2017; Olicón-Hernández et al., 2017; Pozdnyakova et al., 2018).

For this study we selected two *Aspergillus* species: the moderate halophile *Aspergillus sydowii* (EXF-12860) (referred to as *A. caesiellus* in Batista-García et al., 2014) and the obligate halophile *Aspergillus destruens* (EXF-10411) (Sklenář et al., 2017). Both are able to degrade biomass by ligninolytic and other enzymes (Batista-García et al., 2014; Sklenář et al., 2017). To assess the potential of these strains in bioremediation of saline wastewaters, we *1)* characterized the removal of selected persistent and toxic PAH and PhC from saline synthetic media and from waste water from a biorefinery; *2)* determined extracellular esterase, laccase and peroxidase activities and screened for their biodegradation potential under saline conditions; and *3)* evaluated phytotoxicity using *Cucumis sativus* as indicator and measuring the effect of aqueous extracts treated with the two fungal species on germinating capacity and plant growth.

This study is the first report demonstrating that halophilic fungi, in contrast to mesophilic fungi (in particular within phylum *Basidiomycota*) can be successfully used for processing of recalcitrant industrial waste under high salinity conditions.

## 2. Materials and methods

All the reagents were analytical grade and obtained from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Saint-Louis, Missouri, United States).

### 2.1. Strains and culture conditions

*A. sydowii* (EXF-12860) is a moderate halophilic filamentous fungus isolated from solid fermentation of sugarcane bagasse (Batista-García et al., 2014), while *A. destruens* (EXF-10411) is an obligate halophile that was isolated from the canvass of an oil painting in Slovenia (Sklenář et al., 2017). Both strains were conserved in 20% glycerol at −80 °C and deposited in the culture collection of the Laboratory of Extremophile Microorganism (Autonomous University of the State of Morelos, Mexico) and the Ex Culture Collection of Extremophilic Fungi at the Infrastructural Centre Mycosmo, at the Biotechnical Faculty, University of Ljubljana, Slovenia. Spores and mycelia obtained from fresh cultures of both aspergilli on Malt Extract Agar (MEA) were used in all experiments. Different NaCl concentrations were tested in order to test the applicability of both strains in real wastewater treatments (data not shown). Optimal growth of *A. sydowii* and *A. destruens* was at 1.0 M NaCl and 1.9 M NaCl, respectively (Batista-García et al., 2014; Sklenář et al., 2017). These NaCl concentrations were used for each strain in all biodegradation experiments.

### 2.2. Growth rate under saline conditions in presence of benzo-α-pyrene and phenanthrene

Growth rates of both fungi in the presence of two model PAH were determined by inoculating a 7 mm diameter plug of fresh culture into a synthetic saline medium (SM) and MEA agar plates with added NaCl (MEA-SM) and benzo-α-pyrene and phenanthrene (1:1) at final combined concentrations of 60, 120, 200 and 240 ppm. In the case of the cultures in synthetic SM, benzo-α-pyrene and phenanthrene were added as unique carbon source. The composition of the SM medium was: 7.8 mg/L $CuSO_4{\cdot}5H_2O$, 18 mg/L $FeSO_4{\cdot}7H_2O$, 500 mg/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $ZnSO_4{\cdot}7H_2O$, 50 mg/L KCl, 1 g/L $K_2HPO_4$, 2 g/L $NH_4NO_3$, 2 g/L $KH_2PO_4$, 100 mg/L $CaCl_2$, 5 mg/L $MnSO_4$, 0.1 mg/L $H_3BO_3$, 0.1 mg/L $NaMoO_4{\cdot}2H_2O$ and 1 mg/L $CoCl_2$. PAH were dissolved in acetone before addition to the SM and MEA-SM media. Plates were incubated for 15 days at 28 °C. The radial growth (diameter) of the colony was measured in triplicate every 24 h.

As positive control, both fungi were inoculated on MEA-SM, that contained the same volume of acetone as used to dissolve the PAH, and incubated at the same conditions. The PAH tolerance rate (TR), expressed as percentage of mycelial growth inhibition, was calculated for three culture replicates [see Eq. (1)] (Lee et al., 2014):

$$TR\ (\%) = FGR\ (mm)/GRC\ (mm) \times 100\%, \quad (1)$$

where FGR is the fungal growth rate obtained from the plates containing PAH and GRC is the growth rate of the control culture (cultures without PAH). When fungi were cultivated on SM, 2% of glucose was added as carbon source.

### 2.3. Polycyclic aromatic hydrocarbons and pharmaceuticals removal from synthetic media at saline conditions

A fresh inoculum of both aspergilli was obtained on Malt Extract Broth (MEB) supplemented with the optimal NaCl concentration for each species. Flasks with 50 mL of MEB were inoculated with two 7 mm diameter fungal plugs and were incubated on a rotary shaker for 4 days at 28 °C and 150 rpm. Biomass was separated by centrifugation at 10,000*g* during 20 min and the inoculum was washed consecutively three times with 0.1% saline solution to remove any trace of MEB.

Two hundred and fifty milliliter Erlenmeyer flasks containing 50 mL of SM with 100 ppm (final concentration) of benzo-α-pyrene:phenanthrene (1:1 w:w) were inoculated with 1.5 g of fresh, washed biomass from each fungus and incubated on a rotary shaker at 28 °C and 150 rpm. To determine the efficiency of fungal PAH removal, both, supernatants and mycelium, were harvested after 0, 4, 8 and 12 days. Bioadsorption was considered as the amount of benzo-α-pyrene and phenanthrene determined in the mycelium fraction. Biodegradation was calculated as the amount of benzo-α-pyrene and phenanthrene not present in mycelium or supernatant fractions, taking non-inoculated culture as controls.

As fungal-based biological treatments for elimination of PhC have received a lot of attention in recent years (Olicón-Hernández et al., 2017), we also investigated the potential of *A. sydowii* and *A. destruens* to degrade - under saline conditions - a mixture of five PhC (1:1:1:1:1, w:w): acetaminophen, mefenamic acid, ketoprofen, indomethacin and ibuprofen. In order to evaluate PhC removal a synthetic medium was used, simulating real conditions. Two hundred and fifty mL Erlenmeyer flasks containing 50 mL of a mixture of five PhC dissolved in SM were inoculated with 1.5 g of fresh and washed mycelium of each fungus. The mixture contained the five mentioned PhC, with a final concentration of 100 ng/L of each PhC. Flasks were incubated on a rotary shaker for 7 days, at 28 °C and 150 rpm, then supernatant and mycelium from triplicate cultures were harvested and the five PhC were quantified. Non-inoculated flasks were included as controls in all experiments.

For the evaluation of passive adsorption of PAH and PhC onto the mycelium, media were inoculated with mycelium inactivated either by heat (autoclaving 45 min at 121 °C) or a biocide treatment (10 mM sodium azide).

### 2.4. Removal of polycyclic aromatic hydrocarbons from biorefinery wastewaters under high salinity

A non-sterile saline biorefinery wastewater (~0.5 M total salt and pH = 8.4) collected from a lignocellulosic biofuel company in Quebec (Canada) was used to evaluate the bioremediation potential of both fungi to remove PAH. The salinity of the wastewater was increased up to 1.0 M and 1.9 M NaCl for *A. sydowii* and *A. destruens*, respectively. Fresh inoculum as mycelium (1.5 g) was added to 50 mL of wastewater in 250 mL Erlenmeyer flasks and incubated on a rotary shaker for 12 days at 150 rpm and 28 °C. Cultures were harvested after 0, 4, 8 and 12 days. Non-inoculated flasks as well as flasks inoculated with inactive mycelium were included as controls.

### 2.5. Extraction and quantification of polycyclic aromatic hydrocarbons and pharmaceuticals

Organic solvent extractions using 2 mL of dichloromethane were performed for the benzo-α-pyrene and phenanthrene determination in 2 mL of both supernatant and 1.5 g of wet mycelium. Three serial extractions were performed for each sample. The recovered dichloromethane (final volume ~ 6 mL) was concentrated six times using a vacuum rotatory evaporator. Gas chromatograph - mass spectrometer (GC–MS) was used to determine the PAH concentration according to (Lee et al., 2010, 2014). GC–MS analysis was performed on a G1800A GC (Hewlett-Packard Company, Palo Alto, California, United States) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m × 0.25 mm i.d., 0.25 mm film thickness) (Hewlett-Packard Company, Palo Alto, California, United States). PAH determinations were conducted using three technical replicates for all samples.

For the analysis of biorefinery wastewaters, 15 PAH were determined using GC–MS: acenaphthene, anthracene, benzo-α-pyrene, benzo*(b)*fluoranthene, benzo*(j)*fluoranthene, benzo*[k]*fluoranthene, chrysene, fluorene, indenol(1,2,3-*cd*)pyrene, naphthalene, dibenzo*(a,h)*-anthracene, fluoranthene, pyrene, phenanthrene and benzo*(a)* anthracene. PAH determinations were also conducted using three technical replicates for all samples.

Analysis of PhC were performed using Ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) on an Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, Massachusetts, United States) equipped with a HSS-T3 column (100 mm × 2.1 mm, 1.8 µm) according to (Hachi et al., 2017; Haroune et al., 2014). The MS analysis was performed using a positive electrospray ionization (ESI+) (Waters Corporation, Milford, Massachusetts, United States) source in multi-reaction-monitoring mode. Three technical replicates were quantified for each sample.

In all cases, the identification of PAH and PhC was based in the determinations of standard compounds with a purity grade higher than 98%. Moreover, the identification for each compound was confirmed using libraries obtained from mass spectrometry.

### 2.6. Determination of enzymatic activities

Esterase, peroxidase and laccase activities were determined in supernatants obtained from cultures containing PAH and PhC, as well as from biorefinery wastewater. Briefly, laccase activity was determined by the oxidation of 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (1 mM) in citrate buffer (0.01 M, pH 5) (Floch et al., 2007). Peroxidase activity was also measured by oxidation of ABTS but including 0.005% hydrogen peroxide as substrate. ABTS oxidation was monitored by its absorbance at 420 nm for 30 min with 2 min intervals (Floch et al., 2007). Esterase activity was determined by the degradation of 2-naphthyl-acetate to naphtol (130 µM naphthyl-acetate, 0.01% Fast Garnet, 0.1% Triton X-100, 100 mM phosphate buffer, pH 7.0) and was monitored at 538 nm during 30 min with 2 min intervals (Dary et al., 1990; Van Asperen, 1962). Enzyme determinations were performed in triplicate for each sample. One international unit (UI) of enzymatic activity was defined as the amount of enzyme catalyzing the oxidation of 1 mmol of substrate per minute.

### 2.7. Toxicological tests

A germination test using cucumber (*Cucumis sativus*) seeds was performed to evaluate the residual toxicity after fungal treatment (Hussain et al., 2015; Luo et al., 2018). Supernatants obtained after 12 days of culturing the two fungal strains in synthetic media and biorefinery wastewater were desalted by organic solvent extraction. NaCl removal was needed because germination of cucumber seeds is inhibited by salinity. Residual PAH and PhC, as well as intermediary metabolites present in the supernatants were recovered through liquid-liquid extractions using hexane and dichloromethane. The use of these two solvents allowed recovering intermediary metabolites in a greater polarity range. The organic extracts were evaporated and re-dissolved in 1 mL of acetone.

Cotton balls of 2 g were soaked with a 1:20 dilution of the extracts

in tap water (total volume of 20 mL). Five randomly picked seeds of cucumber were sown on the cotton and incubated at 25 °C for 7 days in the dark. Controls using tap water, a solution of 19 mL of water and 1 mL of acetone, non-inoculated synthetic media containing PAH or PhC, and biorefinery wastewater (with 1 mL of acetone added) were included in the experiment. The seeds were considered as germinated when they projected a radical extension greater than 2 mm. The germinating capacity was reported according to the total number of germinated seeds in each condition. In parallel, a batch of germinated seeds was used to evaluate the effect of mycotreatments on the plant growth. With this aim, seeds were first germinated using tap water and later, ~3 mm length plants were exposed to the supernatants obtained after fungal bioremediation. After 7 days, the length of the plants was measured by scalimetry, and expressed as relative growth in percentage. Three independent experiments in triplicate were performed.

### 2.8. Data analysis

One-way ANOVA (Analysis of variance) test was conducted to determine statistically significant differences. Finally, a *post hoc* multiple comparison analysis through a Tukey or Sidak tests (accordingly) was performed. Significance levels are always expressed as a value of $p < 0.05$. Data analysis software GraphPad Prism version 6.01 (GraphPad Software Inc., United States) was used.

## 3. Results and discussion

### 3.1. A. sydowii and A. destruens grow in the presence of benzo-α-pyrene and phenanthrene under saline conditions

When growth rates of *A. sydowii* and *A. destruens* strains were determined on solid media in the presence of benzo-α-pyrene and phenanthrene (1:1 w:w) in concentrations from 60 to 240 ppm of total PAH, growth rates were not affected by increasing concentrations of the PAH (Table 1). There was an evident toxic effect of PAH on both strains shown as growth rate reduction. *A. sydowii* was more tolerant to PAH than *A. destruens,* showing up to five-fold faster growth than *A. destruens* in their presence. While *A. destruens* did not grow in the presence of benzo-α-pyrene and phenanthrene as sole carbon source, *A. sydowii* grew in the presence of up to 60 ppm of PAH, indicating that neither fungus could use the PAH as sole carbon source in solid media supplemented with NaCl.

We did not measure growth kinetics in liquid media, but one can infer from the degradation of PAH (Fig. 1) that both strains were able to use PAH as carbon source, possibly because the pellet structure protected inner cells from the toxic effect of PAH, and allowed longer adaptation time. Previous work from our group (Batista-García et al., 2017) established that *A. sydowii* had between 50 and 70% tolerance rate at a final concentration of 10 ppm of each of the two PAH individually. During this study, higher concentrations were tested. However similar tolerance was determined, suggesting that the toxic effect of PAH is induced already from 10 ppm on.

### 3.2. Polycyclic aromatic hydrocarbons removal under saline conditions

With the intention of analyzing the dynamics of PAH removal by *A. sydowii* and *A. destruens* over time, we measured bioadsorption and biodegradation, as well as production of certain enzymes at 4, 8 and 12 days after the mycelium inoculation. While both strains almost completely removed both benzo-α-pyrene and phenanthrene, their kinetics and mechanisms were very different. On the fourth day both fungi showed markedly different removal rates, with close to 80% removal of PAH by *A. sydowii* and 60% by *A. destruens* (Fig. 1A and B). At the eighth day both strains attained over 90% removal. While *A. sydowii* almost completely degraded the PAH, *A. destruens* achieved this only partially (Inserts in Fig. 1A and B). After 12 days, *A. sydowii* degraded both PAH (99% benzo-α-pyrene and 97% phenanthrene), while *A. destruens* almost completely degraded phenanthrene (97%) but only partially benzo-α-pyrene (~55%) (Fig. 1C). Even with incomplete degradation of PAH, bioadsorption played an important role in *A. destruens*, according to the results obtained from the extraction of PAH form the mycelia (Fig. 1C). In general, we concluded that in *A. sydowii* the removal of PAH was mostly related to biodegradation, whereas in *A. destruens* bioadsorption was more efficient. Since the use of halotolerant and halophilic fungi to eliminate xenobiotics in salty conditions is underexploited, our results for both fungi on synthetic media are highly relevant for downstream applications.

Historically, basidiomycetous fungi have been used for removal of PAH, due to their ability to degrade lignocellulose and efficiently remove xenobiotics. For PAH elimination, salt sensitive basidiomycetes *Trametes versicolor, Pleurotus ostreatus, Tyromyces palustris, Coniophora puteana, Gloeophyllum trabeum, Bjerkandera* sp., *Phlebia radiata* and *Ceriporiopsis subvermispora* were best studied (Aranda, 2016). However, there is a growing interest in ascomycetes for removal of xenobiotics, because on one hand they do not require lignin for induction of the extracellular enzymes involved in PAH degradation (Aranda, 2016), and on the other hand they can better adapt to lowered water activity (de Hoog et al., 2005). In comparison to reported fungal treatments, 97% biodegradation of PAH in concentrations used in our study is high. Wu and collaborators (Wu et al., 2009) degraded 60% of benzo-α-pyrene after 12 days of incubation with an *Aspergillus* strain (BAP14) isolated from marine sediments, while Passarini and collaborators (Passarini et al., 2011) reported that *A. sclerotiorum* in 16 days degraded up to 76.6% benzo-α-pyrene and up to 99.7% pyrene. Other studies showed that *Trichoderma viride* degraded 50% of 10 ppm benzo-α-pyrene in 10 days (Argumedo-Delira et al., 2012), while *T. harzianum* removed 69.1% of PAH present in the culture medium (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluroantene, pyrene, anthracene, chrysene, fluoranthene, pyrene, dibenzo anthracene, pyrene) (Zafra and Cortes-Espinosa, 2015). The basidiomycete

**Table 1**
Growth of *A. sydowii* and *A. destruens* on solid medium in the presence of benzo-α-pyrene and phenanthrene at saline conditions.

| Species | Parameter | | Control | BaP:Phe (1:1) Concentration (ppm) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 60 | 120 | 200 | 240 |
| *A. sydowii* | GR in SM | (mm/day) | 1.4 ± 0.2$^{a}$ | 0.1 ± 0.01$^{d}$ | NG | NG | NG |
| | GR in MEA-SM | | 1.3 ± 0.2$^{a}$ | 0.9 ± 0.1$^{b}$ | 1.0 ± 0.2$^{b}$ | 1.0 ± 0.2$^{b}$ | 0.8 ± 0.1$^{b}$ |
| | Tolerance | (%) | 100$^{a}$ | 69.2 ± 3.1$^{b}$ | 76.9 ± 3.3$^{b}$ | 76.9 ± 5.8$^{b}$ | 61.5 ± 0.2.6$^{c}$ |
| *A. destruens* | GR in SM | (mm/day) | 0.6 ± 0.1$^{c}$ | NG | NG | NG | NG |
| | GR in MEA-SM | | 0.7 ± 0.1$^{bc}$ | 0.3 ± 0.01$^{d}$ | 0.2 ± 0.01$^{d}$ | 0.2 ± 0.01$^{d}$ | 0.2 ± 0.01$^{d}$ |
| | Tolerance | (%) | 100$^{a}$ | 42.9 ± 0.4$^{d}$ | 28.6 ± 1.3$^{e}$ | 28.6 ± 1.1$^{e}$ | 28.6 ± 0.3$^{e}$ |

GR: Growth rate; SM: Saline medium; MEA-SM: Malt Extract Agar with NaCl; BaP: Benzo-α-pyrene; Phe: Phenanthrene; NG: no growth. Different letters indicate different statistical orders.

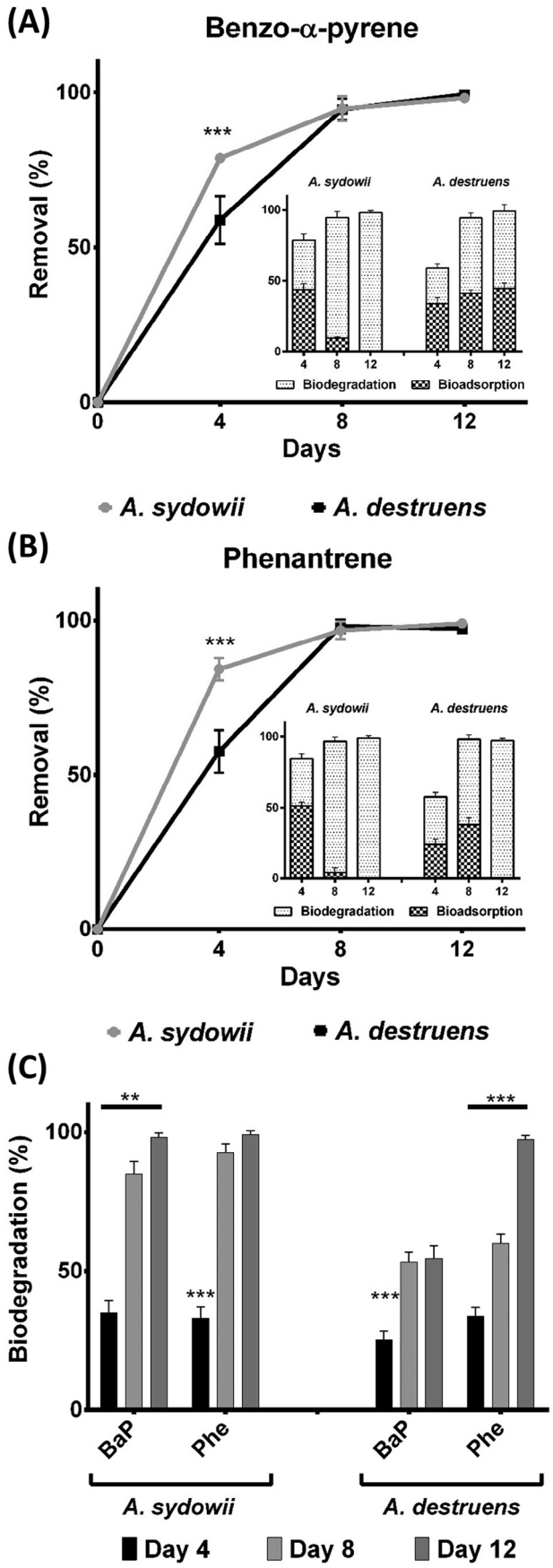



**Fig. 1.** Removal of benzo-α-pyrene **(A)** and phenantrene **(B)** from saline synthetic media, and biodegradation of benzo-α-pyrene (BaP) and phenanthrene (Phe) from saline wastewater **(C).** Asterisks mean differences statistically significant.

*Phanerochatete chrysosporium* was, in comparison a faster degrader, since it removed ~21.3 ppm of benzo-α-pyrene in three days (Liao et al., 1997) and up to 44% of phenanthrene in 60 h (Zheng and Obbard, 2002). Only *A. sydowii*, *A. destruens* and *Fusarium* sp. F092 (Hidayat et al., 2012) have been tested for degradation of PAH under saline conditions. However, *Fusarium* sp. F092 is not a halophilic fungus. In our study A. *sydowii* and *A. destruens* obtained higher removal in saline conditions (Fig. 2), thus increasing the suitability of industrial use of both species.

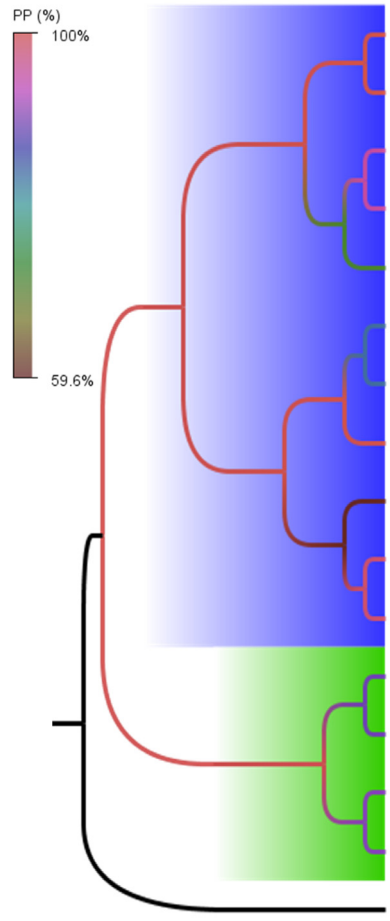
After having determined that *A. sydowii* and *A. destruens* can remove PAH from synthetic media under saline conditions, their potential was tested on wastewater from a wood-processing biorefinery. The waste water included the following PAH in ppm: naphtalene 11.8; acenaphtene 11.9; fluorine 18.4; anthracene 13.7; phenanthrene 212.0; fluoranthene 121.4; benzo-α-anthracene 18.8; chrysene 9.4; pyrene 86.8; benzo-α-fluoranthene 8.4; benzo*[k]*fluoranthene 2.9; benzo*(j)* fluoranthene 3.2; benzo-α-pyrene 3.3. The presence of dibenzo*(a,h)*-anthracene and indenol*(1,2,3-cd)*pyrene in the initial wastewater was also analyzed, but they were not detected.

Both fungi were able in 12 days, under saline conditions, to remove 100% of each of the individual PAH, as determined by GC–MS. In total, they eliminated 498.5 ppm of aromatic hydrocarbons, including PAH containing up to five condensed aromatic rings in either linear or angular arrangements. Thus both species had the capacity to remove PAH in the presence of ≥1 M NaCl in a complex mixture of pollutants with unknown interactive effects. This is the first report on successful PAH removal from a complex, saline wastewater using halophilic fungi, suggesting that both *A. sydowii* and *A. destruens* have a great potential for downstream biotechnological applications.

From previous reports it is known that ligninolytic enzymes play an important role in the biodegradation of PAH. Although higher extracellular ligninolytic enzymes were expected in *A. sydowii* due to its isolation from sugarcane bagasse, the enzymatic activities of *A. destruens* were significantly higher (Fig. 3). Both fungi showed higher esterase activity than laccase and peroxidase activities. This is interesting, because esterases were reported to be active in the hydrolysis and mineralization of PAH (Martínez-Martínez et al., 2014; Sharma et al., 2016). Esterase activity in *A. destruens* spiked at day 4 in synthetic media with PAH reaching approximately 14 UI/mL of enzyme activity. In previous reports the esterase activity during PAH removal was induced by the addition of lignocellulosic material (Sharma et al., 2016) or the use of PAH esters (Martínez-Martínez et al., 2014). Interestingly, in the cultures of *A. sydowii* and *A. destruens* in SM and PAH the most probable inducers are PAH oxidation intermediates, as PAH themselves are not substrates of esterases. This reinforces the notion that esterases might participate in the mineralization of PAH, a topic that is not settled in the current literature.

The apparent contradiction between the biodegradation levels and the extracellular enzymatic activities suggests that these enzymes (from our two species) are less significant for the biotransformation of PAH. Thus, it seems that at least in case of *A. sydowii*, biodegradation was taking place intracellularly. Therefore it is interesting that cytochrome P450 monooxygenases can effectively degrade complex PAH in other ascomycetous fungi (Aranda, 2016; Hernández-López et al., 2016; Uribe-Alvarez et al., 2011).

There are few reports on enzymatic activities of ascomycetes degrading PAH as the only carbon source. The ability of *A. sydowii* and *A. destruens* to produce ligninolytic enzymes (laccases and peroxidases) with PAH as the only source of carbon, without the presence of inducers such as lignin, and importantly at high salinity, gives them an advantage over basidiomycetes (Aranda, 2016). Interestingly, the enzymatic activities of *A. destruens* were lower than of *A. sydowii* in the biorefinery wastewaters (Fig. 3), probably due to inhibitory effect of some compounds. However, it should be noted that *A. destruens* is the first obligately halophilic fungus with characterized production of enzymatic activities (laccases, peroxidases and esterases) in the presence




| Strains | Tolerance (NaCl) [a] | Enzymatic activity [b] | | | | Total removal | | | References [c] |
|---|---|---|---|---|---|---|---|---|---|
| | | Lacc. | Per. | Est. | Other | PAH | Initial conc. | (%) | |
| *Aspergillus versicolor* | 0.5 M | | | | | Pyr | 500 ppm | 24 | Petit et al., 2013 |
| | | | | | | Fluoranthene | 500 ppm | 11 | |
| | | | | | | Anthracene | 500 ppm | 5 | |
| *Aspergillus sydowii* H1 | 2.0 M | + | + | + | | Phen | 100 ppm | 100 | This work and Batista-García et al., 2017 |
| | | | | | | BaP | 100 ppm | 100 | |
| | | | | | | Mix (11 PAHs) | 100 ppm | 100 | |
| *Aspergillus destruens* | 1.89 M | + | + | + | | Phen | 100 ppm | 100 | This work |
| | | | | | | BaP | 100 ppm | 100 | |
| | | | | | | Mix (11 PAHs) | 100 ppm | 100 | |
| *Aspergillus fumigatus* | 0.2 M | | + | | | Anthracene | 10 ppm | 60 | Ye et al., 2009 |
| *Aspergillus sclerotiorum* | 0.5 M | + | + | | | Pyr | 6.0 to 240 ppm | 85 | Passarini et al., 2011 |
| | | | | | | BaP | 10 to 240 ppm | 61 | |
| *Trichoderma asperellum* | 1.4 M | + | + | | | Phen | 333 000 ppm | 78 | Reviewed in Zafra and Cortés-Espinosa, 2015 |
| | | | | | | Pyr | 333 000 ppm | 63 | |
| | | | | | | BaP | 333 000 ppm | 81 | |
| *Trichoderma viride* | 0.25 M | + | | | | BaP | 0.4 mM | 39 | Verdin et al., 2004 |
| *Trichoderma harzianum* | 0.2 M | + | + | | Ring-cleavage dioxygenases | Crude oil | 30 ppm | 40 | Reviewed in Zafra and Cortés-Espinosa, 2015 |
| | | | | | | Anthracene | 400 000 ppm | <10 | |
| | | | | | | Pyr | 10 000 ppm | 25 | |
| *Fusarium solani* | 0.2 M | + | | | | BaP | 0.4 mM | 17 | Verdin et al., 2004 |
| *Fusarium* sp. F092 | 0.6 M | | | | Dioxygenase | Chrysene | 1.0 mM | 48 | Hidayat et al.; 2012 |
| *Fusarium oxysporum* | 0.2 M | + | | | | BaP | 0.4 mM | 8 | Verdin et al., 2004 |
| *Pleurotus ostreatus* sp. | 0.4 M | + | + | | | Crude oil | 80 mg/mL | 98 | Torres et al., 2016 |
| *Pleurotus dryinus* | 0.03 M | + | + | | Aryl alcohol oxidase | Fluoranthene | 121.4 ppb | 90 | Ariste et al., 2018 |
| | | | | | | Pyr | 86.8 ppb | 70 | |
| | | | | | | Phen | 212.0 ppb | 90 | |
| *Phanerochaete chrysosporium* | 0.7 M | | + | | | BaP | 0.21 - 2.41 mg/L | 96 | Zheng and Obbard, 2002 |
| | | | | | | Phe | 1.73 - 17.41 mg/L | 93 | |
| | | | | | | Pyr | 1.23 - 14.88 mg/L | 90 | |
| *Trametes hirsuta* | 0.05 M | + | + | | Aryl alcohol oxidase | Fluoranthene | 121.4 ppb | 90 | Ariste et al., 2018 |
| | | | | | | Pyr | 86.8 ppb | 70 | |
| | | | | | | Phen | 212.0 ppb | 90 | |
| *Candida viswanathii* | 0.02 M | | | | | Naftalene | 1.79 ppm | 90 | Hesham et al., 2009 |
| | | | | | | Phen | 1.89 ppm | 77 | |
| | | | | | | Pyr | 1.96 ppm | 61 | |
| | | | | | | BaP | 1.98 ppm | 56 | |

**Fig. 2.** Comparison of the PAH removal using different fungal strains. **(a)** The tolerance to NaCl reported for the same strain or other member of the specie is shown for illustrative purpose. **(b)** Enzyme activities evidenced in cultures with PAH. **(c)** Only the references related to PAH removal are shown. The phylogram of the fungal strains (showed in the left) was obtained from the Internal Transcribed Spacer rDNA (ITS) marker alignment and colored according to posterior probabilities (PP) calculated from Bootstrap analysis. Ascomycete and basidiomycete strains are shaded in blue and green, respectively. Lacc: laccase, Est: esterase, Per: peroxidase, BaP: benzo-α-pyrene, Phe: phenanthrene, Pyr: Pyrene, ppm: parts per million, ppb: parts per billion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of PAH.

### 3.3. Removal of pharmaceutical compounds

Fungi use overlapping molecular mechanisms to degrade PAH and PhC (Olicón-Hernández et al., 2017). The latter are multi-class emergent contaminants that are important because of the relatively poor efficiency in their removal from wastewater treatment plants (Haroune et al., 2014). To the best of our knowledge this is the first report related to PhC removal by halophilic fungi at saline conditions. Although both fungi showed potential to remove PhC (Fig. 4A), *A. destruens* had a higher removal capacity. *Aspergillus sydowii* degraded in 7 days ~100 ng/L of PhC out of 500 ng/L with varying degrees of the individual compounds, while *A. destruens* degraded ~270 ng/L out of 500 ng/L of the pharmaceutical mixture. However, the total removal of PhC was higher in *A. sydowii*, with ~480 ng/L *vs* 400 ng/L of PhC removed by *A. destruens*. It seems that in particular the adsorption played a more important role. Specifically, *A. sydowii* mycelium adsorbed ~400 ng/L of PhC, while in *A. destruens* adsorption had less impact. The aromatic rings in the structure of these PhC (Fig. 4B) might influence the adsorption to the fungal cell wall (Olicón-Hernández et al., 2017).

Ibuprofen and mefenamic acids were totally removed by both fungi (Fig. 4A). The removal was higher than 93 ± 7% for each PhC using *A. sydowii*. However, removal percentages between 57 ± 5 and 100% were observed for this class of PhC using *A. destruens*. Indomethacin and acetaminophen were the most recalcitrant PhC in both mycotreatments with the lowest removal percentages, 93 ± 7 and 60 ± 5% for *A. sydowii* and *A. destruens*, respectively. *A. sydowii* displayed biodegradation between 5 ± 1 and 19 ± 5% for all PhC, while *A. destruens* catabolized these compounds between 53 ± 4 and 99 ± 4%. In general terms, acetaminophen, ketoprofen and indometacin were the least biodegradable PhC. *A. destruens* was not able to degrade ibuprofen. In conclusion, both *A. destruens* and *A. sydowii* can grow with PhC as a sole carbon source, and, can remove pharmaceuticals in saline aqueous solutions.

Esterase, peroxidase and laccase activities were measured during PhC removal (Fig. 4C). In both fungi, esterase activity was predominant. In *A. sydowii* it peaked already on the first day, while in *A. destruens* it slowly increased until the 7th day, when it was twice as high as in *A. sydowii*. Maximum values for peroxidase activity were observed on 4th day for both fungi, while laccase activity peaked after 7 and 4 days for *A. destruens* and *A. sydowii*, respectively. Esterases hydrolyze esters into an acid and an alcohol, thus they can attack ester bonds of indometacin. However, laccase and peroxidase are non-selective enzymes able to oxidase aromatic compounds. The removal of PhC was

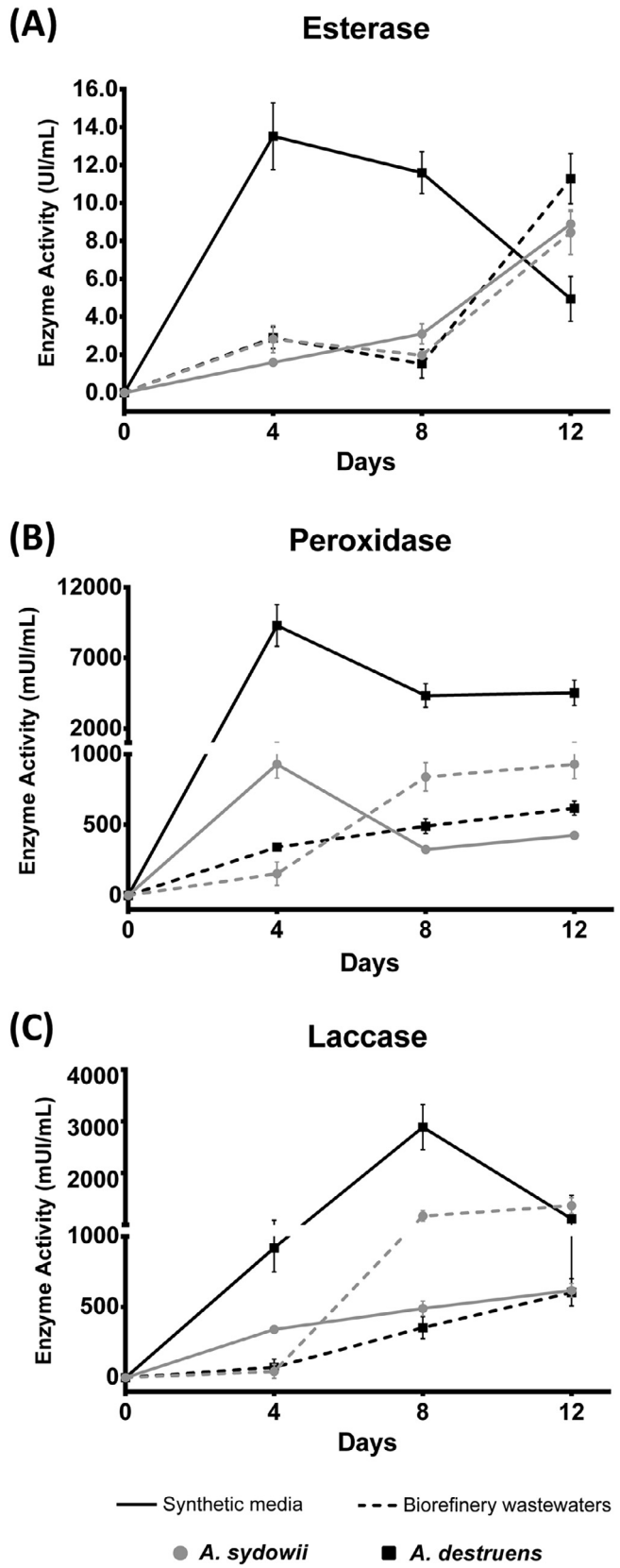



**Fig. 3.** Ligninolytic enzyme activities in synthetic media and wastewater with PAH. Esterase (**A**), peroxidase (**B**) and laccase (**C**).

concomitant to peroxidases and laccases activity, potentially indicating their main role in the degradation of these compounds. However, intracellular mechanisms, described in ascomycetous fungi, should not be disregarded (Aranda, 2016). Removal percentages of PhC were similar to those exhibited by some white-rot fungi such as *Trametes hirsuta* (Haroune et al., 2014) and *T. versicolor* (Marco-Urrea et al., 2010) and with the participation of laccase activity in non saline conditions. In the case of *T. versicolor* a combination of bioadsorption and enzymatic processes have also been described during removal of different micropollutants under non saline conditions (Nguyen et al., 2014). Our results confirm that, as in other fungi, bioadsorption plays a key role in removal of several PhC even at conditions of high salinity (Haroune et al., 2014).

### *3.4. Phytotoxicity in Cucumis sativus: influence of mycotreatments on germination and plant growth*

#### *3.4.1. Polycyclic aromatic hydrocarbons as phytotoxic compounds*

Twenty seeds of *C. sativus* were employed in the tests and, overall 18 ± 2 germinated when tap water was used in germination assays (Fig. 5A). Synthetic media containing PAH and un-treated desalted biorefinery wastewater negatively affected the germination of *C. sativus*, with only 8 ± 1 and 4 ± 1 germinated seeds, respectively. Thus, both untreated systems were toxic and inhibiting the germination process of *C. sativus*. Samples obtained after treatments with either fungal species revealed a decrease of this toxicity. After 12 days, aqueous extracts from synthetic media and wastewater treated with *A. sydowii* equally reduced the inhibition of germination to 16 ± 1 and 15 ± 1 seeds, respectively. However, differences were observed when synthetic media and wastewater were treated with *A. destruens*. No differences in germination were found between control (germination supported with tap water) and synthetic media after treatment of PAH with either fungal species (Fig. 5A). In general, mycotreatments of PAH in both synthetic media and wastewaters by both species improved the germinating capacity of *C. sativus*.

The synthetic medium supplemented with PAH inhibited the *C. sativus* growth (after germination) to 56%, while the untreated wastewater allowed a higher plant growth of 71%, compared to the control. In general, mycotreatments positively influenced plant development when the cucumbers grew in the presence of aqueous extracts from treated systems. After mycotreatment of synthetic media with *A. sydowii* and *A. destruens*, a better plant growth was obtained (105.5 and 70%, respectively). When *A. sydowii* removed PAH from biorefinery wastewater, plant growth was 40% higher than the positive control. Possibly, the fungus produced some metabolites with plant growth promotion properties. *A. destruens* also decreased the phytotoxicity of the untreated synthetic medium and wastewater since after treatments plant growth was stimulated by 14 and 20%, respectively. In conclusion, it was observed that toxicity on plant growth was markedly decreased after treatments with both fungi.

The phytotoxicity of PAH has been largely demonstrated in many plants as legumes and grasses. Particularly, the negative influence of PAH on germinating capacity has been documented for several species (Kaur et al., 2017). Germination tests have been defined as promising tool to examine ecotoxicity of pollutants such as PAH (Banks and Schultz, 2005; Kirk et al., 2002; Maila and Cloete, 2005). PAH also negatively influence plant growth because they affect water absorption, early root development, cell expansion and have also been postulated to cause mechanical disruption in the cytoplasmic membrane of new cells (Kaur et al., 2017). Also, PAH influence the production and action of certain hormones (i.e. auxins) and inhibit cell organelle metabolism (Kaur et al., 2017). *C. sativus* has been recommended by the Food and Drug Administration (Washington DC) and United States EPA, respectively, as a useful test for plant ecotoxicity (Wang and Freemark, 1995). It has also been reported that *C. sativus* seeds can accumulate phenanthrene and benzo-α-pyrene causing a decrease in its germinating capacity (Rončević et al., 2016).

Our study confirms that PAH are phytotoxic with an impact on germination and growth of *C. sativus*. Hence, after removal PAH,

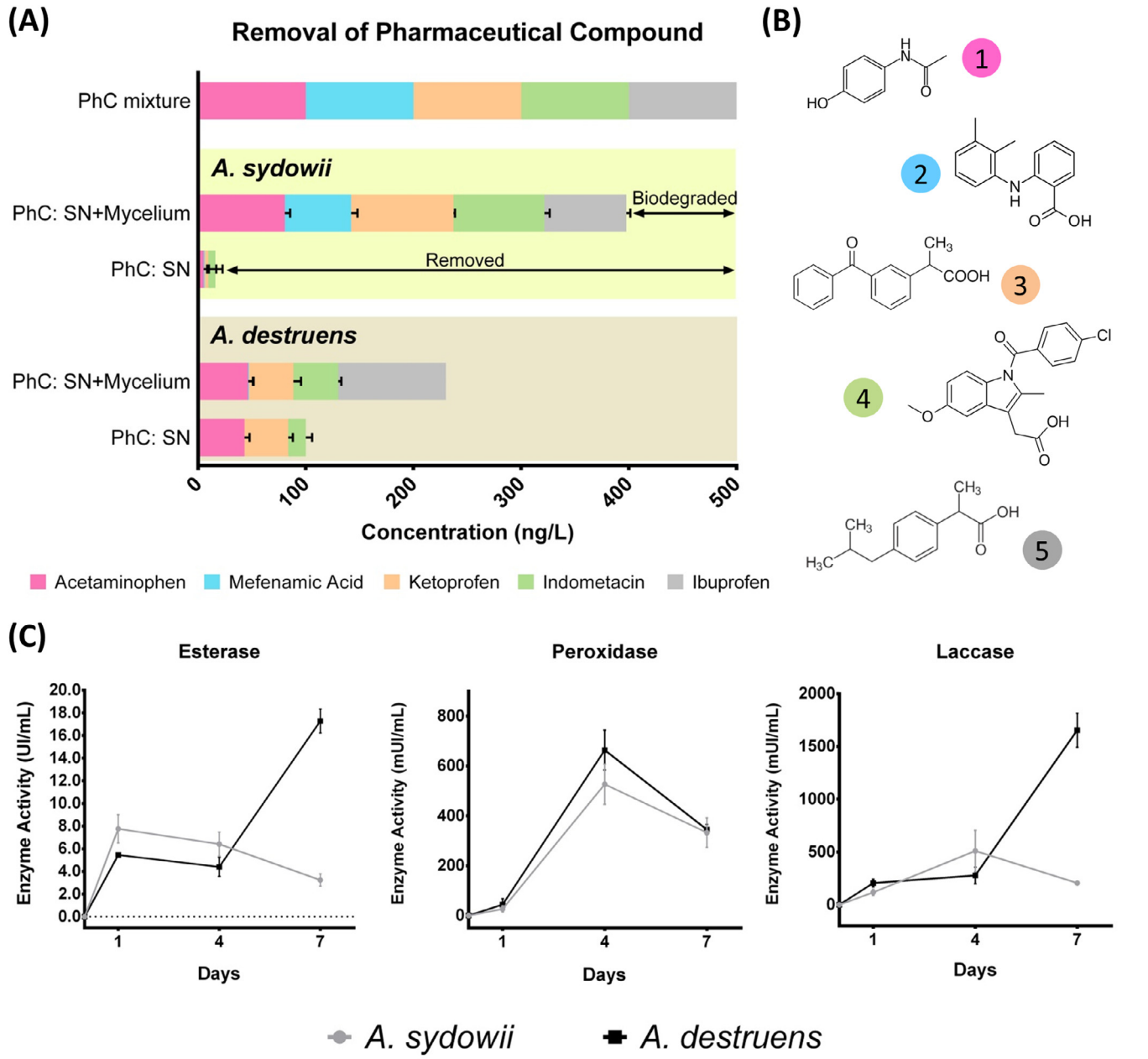



**Fig. 4.** Bioremediation potential for elimination of PhC. PhC mixture: abiotic control, PhC: Pharmaceutical compounds, SN: Supernatant **(A).** Removal of PhC in synthetic media. **(B)** Structures of the used PhC. **(C)** Ligninolytic enzyme activities during mycotreatment.

synthetic media and wastewater would be less phytotoxic. As treated wastewaters are often destined for agricultural irrigation, it is important to demonstrate that they have no ecotoxic effect on relevant crops before discharging them. Our study shows that treatments with two halophilic fungal species can have a beneficial effect and hence support the biotechnological value of both strains to reduce the ecotoxicity of PAH.

### 3.4.2. Pharmaceutical compounds removal and phytotoxicity

Despite the fact that these PhC are emergent pollutants with a demonstrated effect on animal health, their phytotoxicity has been scarcely studied. Some reports propose that they could be phytotoxic but depending on the plant species and exhibition time. It is not known in depth how their negative effects in plants occur (Carvalho et al., 2014). However, there is no information about *C. sativus* related with phytotoxicity induced by these analgesic compounds.

The same two tests were performed to examine the influence of PhC on germinating capacity and plant growth of *C. sativus*. There were no differences between negative and positive controls, i.e. the seeds treated with PhC at 500 ng/L had the same germination percentage and plant growth rate than those treated with tap water alone (data not shown). Since it is known from other studies (Carvalho et al., 2014) that the PhC studied here indeed have phytotoxic effects, our results indicate that germination tests in *C. sativus* are not able to detect any toxicity exerted by the selected pharmaceuticals at the tested concentrations.

## 4. Conclusions

The halophilic *A. sydowii* and *A. destruens* may be useful for biotechnological downstream processing of various industrial wastewaters, in particular with regard to reducing the amount of toxic PAH and PhC under salty conditions (> 1 M NaCl). Both fungi eliminated 100% of PAH at a concentration of 500 ppm of complex fifteen PAH in

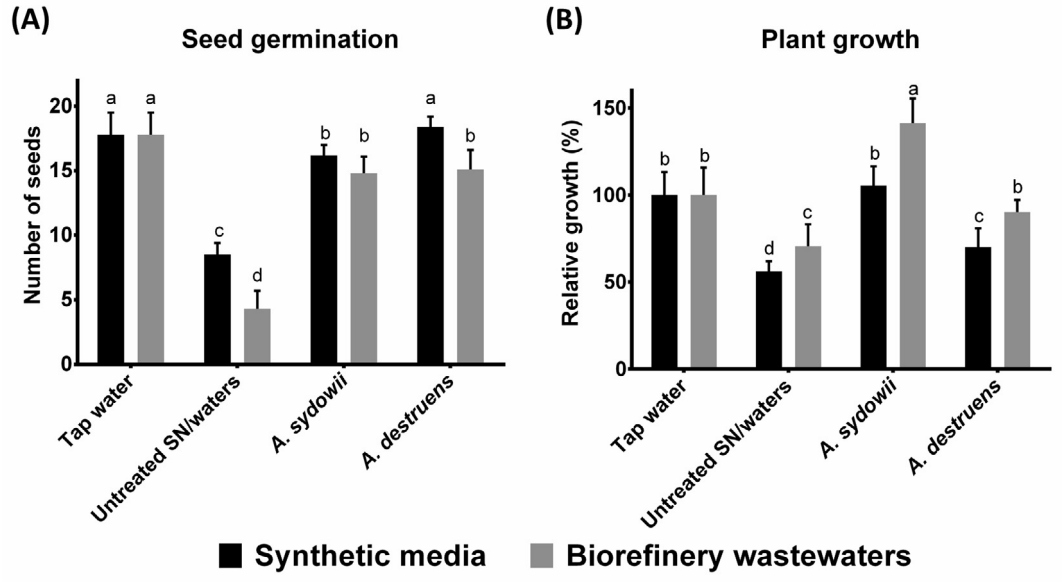



**Fig. 5.** Effect of mycotreatments on phytotoxicity. Toxicity of synthetic media with PAH and biorefinery wastewater after mycotreatment was assessed by measuring seed germination **(A)** and seedling growth **(B)**. Positive (tap water) and negative (untreated supernatant (SN)/waters) controls were used. Different letters indicate statistically significant differences.

wastewater from a biorefinery at high salt concentrations. We also found that the two fungi had some capacity to eliminate PhC that are known to have ecotoxic effects. This is the first report that underlines the capacities of halophilic fungi for xenobiotic biodegradation under low-water activity.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.02.002.

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