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RESEARCH

Thriving in salty environments: *Aspergillus niger's* halotolerance and BTEX biodegradation potential

Kelly Rodrigues[1,2] · Ricardo Salgado[3,4] · Dina Galhanas[3] · Victoria Maura S. Bermudez[1] · Glória Maria Marinho Silva[1] · Ana Maria Álvares Tavares da Mata[3,5] · Luciana Pereira[6,7]

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Abstract
Benzene, toluene, ethylbenzene, and xylene (BTEX) can be found in marine and estuarine waters due to accidental spills of oil and derivatives, as well as in production water and effluents discharged from petrochemical plants. Addressing the bioremediation of these compounds in saline environments and effluents with elevated salinity levels is imperative. In this study, the halotolerance of *Aspergillus niger* was assessed by subjecting it to a stepwise increase in salinity, achieved through progressive addition of NaCl from 2 to 30‰ (v/v). The fungal strain exhibited optimal growth support up to a salinity concentration of 25‰, accompanied by a biomass production rate of (0.93 ± 0.11) g.d^{-1}. The adapted biomass was employed in batch reactors to evaluate the biodegradation of BTEX (1,500 mg.L^{-1}). In the absence of sucrose, the reactors inoculated with fungi demonstrated almost complete BTEX removal within 7 days, with rates ranked as follows: benzene (1.12 d^{-1}) > toluene (0.78 d^{-1}) > ethylbenzene (0.65 d^{-1}) > xylene (0.63 d^{-1}). Enhanced BTEX removal rates were obtained in the presence of sucrose, notably with 2 g.L^{-1}: benzene (3.63 d^{-1}) > toluene (2.10 d^{-1}) > ethylbenzene (1.56 d^{-1}) > xylene (1.50 d^{-1}). Notably, benzene was found to be the sole compound adsorbed onto the fungal mycelium (1.50 ± 0.19) to (13.35 ± 4.72) mg.g^{-1} of biomass.

Keywords Adsorption, *Aspergillus niger* · BTEX · Halotolerant fungi · Salinity

Introduction

Oil and its derivatives, when discharged into the environment, hinder the diffusion of oxygen into water and have the potential to bioaccumulate within biological communities (Pinto et al. 2014). These hydrocarbons have the propensity to assimilate into nature by binding with protein molecules and fatty tissues of organisms, leading to their concentration and transfer throughout the food chain without undergoing structural alterations. Consequently, they pose a threat to ecosystems, increasing the risk of contaminating aquatic sediments and entering the food chain, thereby contributing to public health concerns. Benzene, toluene, ethylbenzene and xylene (BTEX) represent petroleum-derived monoaromatic hydrocarbons that constitute one of the most extensively produced chemical groups, with application in industrial activities such as the production of paint, gasoline, plastics, and various other commercial and industrial applications (Perdana et al. 2019). Recognized as hazardous pollutants (Perdana et al. 2019), they are frequently detected in marine environments, particularly following oil spills. Wang et al. (2018) documented the 592 spills that occurred between 1990 and 2017, characterizing them as the most prevalent accidents worldwide. In 2017, two major (> 700 ton) and four medium-sized (7 to 700 ton) oil spills were recorded, alongside the most severe oil tanker disaster

✉ Luciana Pereira
lucianapereira@ceb.uminho.pt

1 Federal institute of Ceara, PGTGA, Fortaleza, Brazil
2 Federal University of Ceará, PPGERN, Fortaleza, Brazil
3 Polytechnic Institute of Setubal, CINEA-ESTS, Setubal, Portugal
4 Universidade Nova de Lisboa, LAQV/REQUIMTE, Lisboa, Portugal
5 iBB-Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal
6 CEB - Centre of Biological Engineering, University of Minho, Braga 4710-057, Portugal
7 LABBELS – Associate Laboratory, Braga, Portugal

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of the past 35 years, which resulted in the release of over 100,000 tons of ultra-light oil (Ashok et al. 2019). In marine waters, BTEX concentrations have been documented to vary, with, xylene levels, as example, ranging from 0.0021 to 7.7 $\mu g.L^{-1}$ (Duan et al. 2017). The exposure of corals to BTEX, and the resulting environmental are highly variable due to the complex composition of petroleum and its derivatives. This variability is further influenced by differing rates of bio- and photodegradation, as well as the varying solubility of individual compounds (Turner et al. 2021). BTEX also pose chronic toxicity risks to humans and can cause damage to the central nervous system. Among these compounds, benzene stands out as the most toxic, recognized as a carcinogenic substance (Mello et al. 2019).

The presence of BTEX in marine ecosystems and their significant negative impacts are further exacerbated by the fact that these compounds persist for decades without degrading. A potential solution lies in harnessing microorganisms adapted to marine environments for effective bioremediation solution (Birolli et al. 2018). Bacterial species such as *Pseudomonas*, *Marinobacteria* and *Alcanivorax* have been documented for their role in the biodegradation of BTEX in saline waters, while fungi of the genera *Fusarium* and *Drechslera* are known for their ability to biodegrade crude oil (Khalil et al. 2021). Fungi, particularly *Ascomycota*, are frequently encountered in extreme and anthropogenically polluted environments, where other microorganisms may struggle to thrive. So, mycoremediation relying on the ability of fungi to metabolize BTEX as a sole carbon source can be applied (Prenafeta Boldú et al., 2019). However, though fungal bioremediation is widely applied in terrestrial environments, its use in marine ecosystems remains less explored, highlighting the need for further research into the mechanisms underlying the biodegradation of aromatic hydrocarbons and other pollutants (Bovio et al. 2017).

Fungi are effective in pollutant removal through both biosorption and biodegradation (Cheng et al. 2020). For instance, *Aspergillus sydowii* has shown greater efficiency in biodegrading polycyclic aromatic hydrocarbons, while *Aspergillus destruens* achieved pollutant removal via adsorption (Gonzalez-Abrandelo et al., 2019). Biosorption and biodegradation often occur together, with adsorption making pollutants more accessible to enzymatic degradation. Halophilic fungi, including species such as *Cladosporium*, *Aspergillus*, *Penicillium*, and *Wallemia* (Mansour 2017), have garnered interest for their bioremediation potential in high salinity environments, where they play key roles in biodegradation (González-Abrandelo et al. 2019). For example, the study by Birolli et al. (2018) investigated the degradation of anthracene using *Aspergillus sydowii* CBMAI 935, *Penicillium citrinum* CBMAI 1186, *Trichoderma harzianum* CBMAI 1677, and CBMAI 847. Their findings revealed that although these fungi show greater growth in media with lower salt concentrations *Aspergillus sydowii* CBMAI 935 exhibited significantly greater anthracene degradation in a saline environment, likely due to enzyme production stimulated by NaCl-induced stress. In some cases, the addition of a primary carbon source enhances biomass growth and enzyme production, further promoting biodegradation (Zhang et al. 2023). For example, the degradation of aromatic polycarbonates by Fusarium sp. strain ZH-H2 improved significantly with the addition of starch (Zhang et al. 2023).

Aspergillus niger has been isolated from petroleum contaminated sites, demonstrating its capacity to grow in the presence of petroleum hydrocarbons such as BTEX (Usman, 2020). Indeed, reports indicate that *Aspergillus niger* exhibits a high capacity for the bioremediation of various aromatic hydrocarbons derived from petroleum in contaminated water, including surface and groundwater, as well as in wastewater (Rodrigues et al. 2017; Sánchez-Vázquez et al. 2018), Prianka and Lens 2022). However, its efficiency in saline environments remains undocumented in the literature. This study aims to evaluate the potential of *Aspergillus niger* for BTEX removal in saline environments, with significant implications for mycoremediation in marine ecosystems impacted by oil spills.

Materials and methods

Aqueous saline medium

The aqueous medium used had the following composition ($g.L^{-1}$): $NaNO_3$ (2), K_2HPO_4 (2), KCl (0.5), $FeSO_4$ (0.01), $MgSO_4.7H_2O$ (0.5). NaCl was added to provide a salinity ranging from 2 to 30‰ (v/v) to the medium. The pH was adjusted to 5.0 ± 0.1 with 0.1 M HCl.

Aspergillus niger AN 400 cultivation

The strain of *Aspergillus niger* (SISGEN: A6835C3) used in this study was obtained from the Laboratory of Chemistry and Environment at the School of Technology of Setúbal (ESTSetubal-IPS). Subculturing was performed to obtain an inoculum from the supplied strain. Inoculum preparation was made following the methodology described in Rodrigues et al. (2024). Two different culture media were used, both of which previously subjected to autoclaving and then poured into Petri dishes. For the halotolerance test, *Aspergillus niger* was cultivated on Petri dishes containing Saboraud agar culture medium (Silva et al. 2017; Marinho et al. 2019; Rodrigues et al. 2024). For the BTEX degradation

assay, the inoculum underwent cultivation through successive subcultures, every 10 days, on Petri dishes containing Czapec medium, with a gradual addition of NaCl at increasing concentrations of 2, 5, 10, 15, 20, 25, and 30% (v/v) during each subculture. Incubation occurred at 30 °C.

Halotolerance test

For studying the tolerance of *Aspergillus niger* to salt conditions, three 250 mL glass bottles were used as reactors, which were filled with an aqueous medium containing sucrose (30 $g.L^{-1}$). and inoculated with 10 fungal colony mycelium discs of a diameter of 1.3 cm. The reactors incubated at room temperature in a shaker operating at 250 rpm. Every 7 days, the old medium was replaced by fresh medium with incrementally increasing salinity levels: 2, 5, 10, 15, 20, 25, and 30‰ (v/v). The biomass from reactors was also weighed at each medium change, and the wet weight obtained. Thus, with the removal of the old medium, the biomass was taken out, weighed, and then reintroduced into a reactor containing freshly prepared medium with higher salinity. Before weighing, the biomass was subjected to vacuum filtration to remove as much moisture as possible. At the end of the experiment, in addition to the wet weight, the dry weight of the resulting biomass was obtained after subjecting it to vacuum filtration, following by drying in an oven at 105 °C.

BTEX bioremediation assay

To evaluate the efficacy of *Aspergillus niger* in biologically removing BTEX, eight 500 mL batch reactors (designated as R1 - R8) were utilized. R1 and R2 served as the abiotic control (duplicates). Biological reactors, in duplicate, consist of (R3 and R4) with 2 $g.L^{-1}$sucrose, (R5 and R6) with 30 $g.L^{-1}$sucrose, and (R7 and R8) without sucrose. Each reactor received 200 mL of aqueous medium with a salinity of 25‰, containing the following components ($g.L^{-1}$): $NaNO_3$ (2), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4$ (0.01), K_2HPO_4 (1). The reactors were sealed with cotton and gauze plugs and subjected to sterilization through autoclaving at 120 °C for 20 min. After sterilization and returning to room temperature (≈25 °C), sucrose was added at a concentration of 2 $g.L^{-1}$ to R1, R2, R3, and R4, and 30 $g.L^{-1}$ to R5 and R6. The BTEX compounds were added to reactors from a concentrated stock solution (12,000 $mg.L^{-1}$) prepared in 96% ethanol, resulting in an initial concentration of 1,500 $mg.L^{-1}$ of each of the compounds.

ReactorsR3, R4, R5, R6 R7 and R8 were inoculated with *Aspergillus niger*, each receiving 10 discs of 1.3 cm, cut from a colony grown on a Petri dish. The strain used in reactors was previously acclimated to 25‰ salinity. The reactors were operated for 7 days under agitation, 150 rpm, at 25 °C, with liquid samples collected every 24 h to determine the concentration of BTEX. At the end of the experiment, the presence of adsorbed BTEX on the biomass that grew in the fungal reactors was checked. To extract BTEX bound to the mycelium, all the reactor biomass was removed and centrifuged at 2,000 rpm for 2 min, and then transferred to a 150 mL bottle to which 100 mL of n-hexane and glass beads were added. After sealing it with a screw cap, the mixture was vigorously shaken manually for 15 min to release compounds possibly adsorbed on its surface. Following this procedure, 2 mL of the supernatant were withdrawn for BTEX determination.

Sample preservation and analytical methods

All the samples were kept at -0.5ºC until the analysis was carried out. Biomass concentration and pH was determined according to APHA (2014). BTEX were determined by gas chromatography (GC) (Thermo Scientific Trace 1300/1310, Germany) operated with flame ionization detector (FID) (300 Hz), according to USEPA Method 8020B (USEPA, 1996) and Assadi et al. (2010). First, the BTEX were extracted from water samples collected from each reactor using a liquid-liquid extraction (LLE) technique prior to GC-FID injection. For this, 2 mL of each sample, at room temperature, was added to 2 mL of n-Hexane in a 10 mL glass vial equipped with a magnetic stir bar. The vial was then sealed with a screw cap and stirred at 600 rpm for 60 min to facilitate BTEX extraction. Following stirring, 1.5 mL of the supernatant extract was collected and transferred to a 25 mL glass flask fitted with a screw cap and containing molecular sieves to eliminate any trace moisture (15 min). Subsequently, 1 mL of the extract was transferred to vials and injected into the GC-FID for analysis.

The separation was performed on a silica capillary column (HP-5MS, 30 m x 0.25 mm d.i., df: 0.25 mm, Thermo Scientific) at a helium flow of 35 $mL.min^{-1}$, with a purity of 99.99%. The injector temperature was 280ºC, split 1:33 and the detector temperature were kept at 280ºC. The GC gradient temperatures were: 35ºC (1.5 min) 12ºC.min^{-1} to 80ºC, and 25ºC.min^{-1} to a final temperature of 280ºC (1.5 min). The carrier gas was helium (35 $mL.min^{-1}$), with a purity of 99.99%, and an injection volume of 1 µL. Data acquisition and data processing were carried out with the Xcalibur 2.0 software.

Removal kinetic rate constants and half-life calculations

The rate constant (k) for BTEX biodegradation by *Aspergillus niger* in saline medium was appropriately fitted to a first-order reaction, according to Eq. (1):

$$\ln\left(\frac{Ct}{Co}\right) = -k.t \quad (1)$$

Where, t is the biodegradation time, Ct is the residual concentration of BTEX at time t and Co its initial concentration. So, the rate constants for BTEX removal were calculated in the Origin 7.0, applying the first-order equation.
Half-life times ($t_{1/2}$) were calculated by Eq. (2):

$$t_{1/2} = 0.693/k \quad (2)$$

Results And Discussion

Halotolerance of *Aspergillus niger*

Although *Aspergillus niger* is not a marine-origin specie, its tolerance to increase salinity levels was investigated to assess its potential for bioremediation of oil spills containing BTEX in marine environments. The cellular growth rate over the 7 days of batch reactors operation at increasing salinities was measured and the results are presented in Fig. 1a. The daily cellular growth rate increased from salinities of 2 to 10‰ NaCl, reaching a peak of (2.55 ± 0.18) g.d^{-1}. However, beyond this salinity threshold, growth declined, likely due to the stress conditions prevalent in the reactors. Between 15 to 30‰ of NaCl, growth fluctuated, with a maximum rate (0.93 ± 0.11) g.d^{-1} at 25‰ of NaCl. In the studied by Cantrel et al. (2006) *Aspergillus* spp. exhibited halotolerance when growth at salinities of 10, 15, 20, and 25‰ over 10 days. Notable, among the species, *Aspergillus niger* exhibited halotolerant up to 25‰, with a cellular growth rate of (0.90 ± 0.14) g.d^{-1}, a value closely matching the results obtained in the present study.

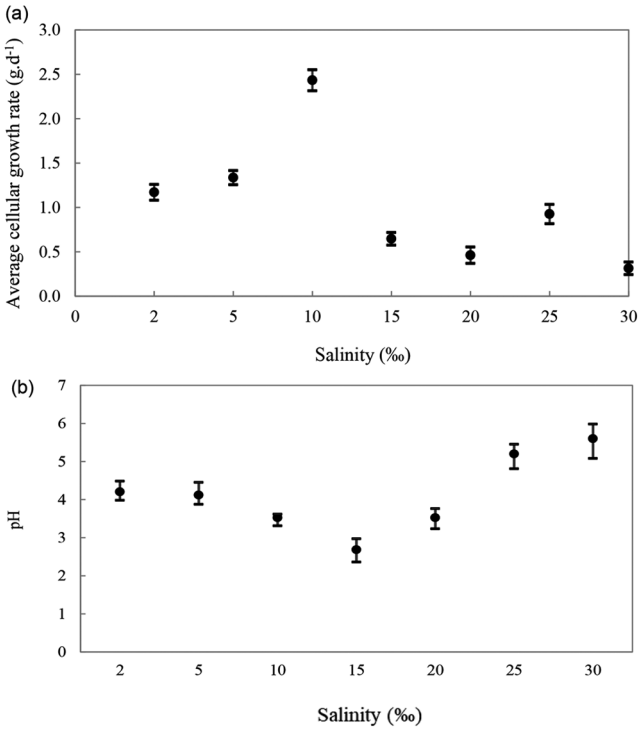


Fig. 1 **(a)** Average cellular growth rates (g.d^{-1}) and **(b)** final pH, of the aqueous medium for salinities of 2, 5, 10, 15, 20, 25 and 30‰. The results are the average of triplicate reactors. Initial mean pH in reactors was (5.02 ± 0.04)

A change in the color of the medium from yellow at 2, 5, and 10‰ NaCl to black at 15, 20, and 30‰ NaCl, was observed, which could be related to the production of melanin, a pigment typically formed after intense cellular growth (Griffin 1994; Venkatachalam et al. 2019). *Aspergillus niger*, and other fungi species of terrestrial and freshwater origins produce melanin and other pigments as a product of their secondary metabolism in saline and hypersaline media (Cantrell et al. 2006). Melanin production endows them with the capability to colonize a wide array of hostile environments. This adaptability has earned them the designation of polyextremophiles, owing to their adeptness at thriving in exceptionally adverse conditions and environments characterized by extremes in pH, temperature, high salinity, as well as those abundant in aromatic hydrocarbons and phenols (Venkatachalam et al. 2019). The black pigmentation of the melanin produced by *Aspergillus* spp. also renders the cell membrane more resistant to high salinity and confers a dark hue, thus serving as a survival strategy in those extreme conditions (Griffin 1994). Nevertheless, it is important to note that secondary metabolites like melanoids may have environmental impacts. One approach to mitigate this is through the immobilization of *Aspergillus niger*, which can help control and/or release the production of these compounds, enhance fungal stability, and improve the overall safety and efficiency of the bioremediation process. Furthermore, the addition of a carbon source, and the corresponding organic charge equilibrium, can help to reduce the formation of these toxic metabolites by ensuring better control of metabolic pathways.

On the final day of the batch (7th day), pH measurements were taken (Fig. 1b). pH is one of the most important parameters and is directly related to microbial metabolism, influencing microbial growth and the production of catabolic enzymes, which are primarily produced in acidic environments, typically between 4 and 6 (Perdana et al. 2019; Elyamine et al. 2021). Under these conditions, *Aspergillus niger* is known to produce key enzymes such as lignino-lytic enzymes such as laccases and peroxidases, as well as hydrolytic enzymes like lipases and esterases, all of which play crucial roles in the degradation of xenobiotics, including hydrocarbons like BTEX (Kumar and Chandra 2020; Elyamine et al. 2021; Alaidaroos 2023; Kannan et al. 2024;

Tesfaye et al. 2025). These enzymes facilitate the breakdown of complex organic compounds by oxidizing or hydrolyzing chemical bonds, making them particularly effective in the bioremediation of persistent pollutants including petroleum hydrocarbons described in this study (Perdana et al. 2019). Common biodegradation pathways utilize those enzymes, in particular laccase, manganese peroxidase and lignin peroxidase, to produce quinones from polycyclic aromatic hydrocarbons (PAHs), facilitating ring cleavage and subsequent breakdown into CO_2. Alternatively, cytochrome P450 enzymes (CYPs) first oxidize the substrate, producing an epoxide that can either spontaneously convert into a phenol or undergo enzymatic conversion to a dihydrodiol (Khan et al. 2023). In addition to lignin and manganese peroxidases, versatile peroxidase (VP) is also involved in these processes. VP, with its broader substrate specificity, can directly oxidize aromatic compounds and other persistent pollutants (Rodriguez et al., 2004; Pozdnyakova et al. 2013). The oxidation of three- and four-ring PAHs, including anthracene, phenanthrene, fluorene, pyrene, chrysene, and fluoranthene, by VP from *Bjerkandera fumosa*, was demonstrated by Pozdnyakova et al. (2013). They identified the oxidation products of these PAHs as being 9,10-anthraquinone, 9,10-phenanthrenequinone, and 9-fluorenone.

In this study, in general, an optimal cellular growth rate was observed in the pH range of 3.5 to 5.2. Lower and higher pH values seemed to decrease the biomass production rate. As can be observed in Fig. 1a, there was a pH decrease from the initial mean pH (5.02 ± 0.04) to (3.47 ± 0.15) for salinities up to 10‰, accompanied by an increase in fungal growth. At salinities of 15, 20, and 30‰ NaCl, the pH on the 7th day was (2.7 ± 0.1), (3.5 ± 0.8), and (6.6 ± 0.1), respectively, and the fungal growth was subdued. Surprisingly, the pH recorded in the medium containing 25‰ NaCl was (5.2 ± 0.3) and fungal growth increased unexpectedly, when compared to exposure to 20‰ NaCl. The decrease in pH in submerged fungal cultures is often attributed to the excretion of H^+ during ATP generation through aerobic respiration and the entry of ammonium (NH_4^+) into the fungal cell, resulting in the release of H^+ to the medium thus contributing to medium acidification (Griffin 1994).

In high-salinity environments, microbial cells are subjected to elevated osmotic pressure, which hinders cellular growth. As salinity increases, osmotic pressure escalates, thereby intensifying the energy expenditure required to maintain osmotic balance. Consequently, the energy that would otherwise be allocated to cellular growth is redirected toward mitigating the adverse effects of osmotic pressure, leading to the synthesis of osmoprotectant substances. These secondary metabolites act to protect cells against cellular dehydration (Venâncio et al. 2017). Elevated salinity can indeed slow fungal growth, disrupt the production of extracellular compounds, and hinder organic matter degradation. However, certain non-marine fungal species exhibit improved bioremediation potential at higher salinities, likely due to the production of fungal metabolites that assist it in coping with the adverse conditions like osmotic stress.

BTEX removal in batch reactors

The *Aspergillus niger* demonstrated tolerance to nearly all studied salinity conditions, exhibiting robust growth up to 25‰, the salt saturation used in the BTEX biological removal assays. BTEX removal occurred in all reactors (Fig. 2; Table 1). Within one day, the abiotic control reactors achieved a BTEX removal of (42.51 ± 10)%, while in the fungal reactors without sucrose achieved (59.8 ± 9.3)%, increasing to (74.0 ± 6.4)% and to (86.1 ± 8.7)% in those containing 30 $g.L^{-1}$ and 2 $g.L^{-1}$ of sucrose, respectively. Following this period, in biological reactors, benzene was no longer detected in the biological reactors R3 and R4 (with 2 $g.L^{-1}$ sucrose), and was present at very low concentrations in the other reactors. With 30 $g.L^{-1}$ of sucrose, benzene was totally degraded within 2 days (Fig. 2a). Indeed, although benzene is known as being highly toxic and causing growth inhibition of organisms in aquatic environments, *Aspergillus niger* grew and developed during the period of operation of the reactors.

For the other compounds, in reactors containing fungi and sucrose, a reduction in the concentrations was observed until day 4, when 100% removal was observed. In the absence of sucrose, BTEX removal by fungi also occurred, though at a slower rate, reaching an average concentration of 9.5 $mg.L^{-1}$ on the 5th day of incubation. By the day 7, near-total removal of the compounds was achieved. In general, xylene exhibited higher persistence, with, after 5 days, an average concentration of (67.0 ± 2.8) $mg.L^{-1}$ in the reactors containing 30 $g.L^{-1}$ sucrose (R5 and R6), (182.5 ± 9.2) $mg.L^{-1}$ in the reactors without sucrose (R7 and R8), and (538 ± 8.6) $mg.L^{-1}$ in the abiotic reactors (Fig. 2d). However, after 7 days of reactors operation, 100% removal of xylene was obtained in the reactors with sucrose (Table 1).

The BTEX removal in saline medium followed a first-order kinetics (Fig. 2). BTEX removal rate (k) and half-life (t1/2) were calculated (Table 1). In the abiotic reactors, with the exception for benzene that was removed at a rate of 0.84 d^{-1}, the removal rate was similar for the other compounds, around 0.5 d^{-1}, taking between 0.8 and 1.4 days, and the final removal degree was circa 85% for benzene, 77.1% for toluene, 73.2% for ethylbenzene and 69.2% for xylene. In the fungal reactors containing active *Aspergillus niger*, there was a consistent trend indicating better removal rates of BTEX (as individual compounds), with benzene exhibiting the highest removal rate followed by toluene,

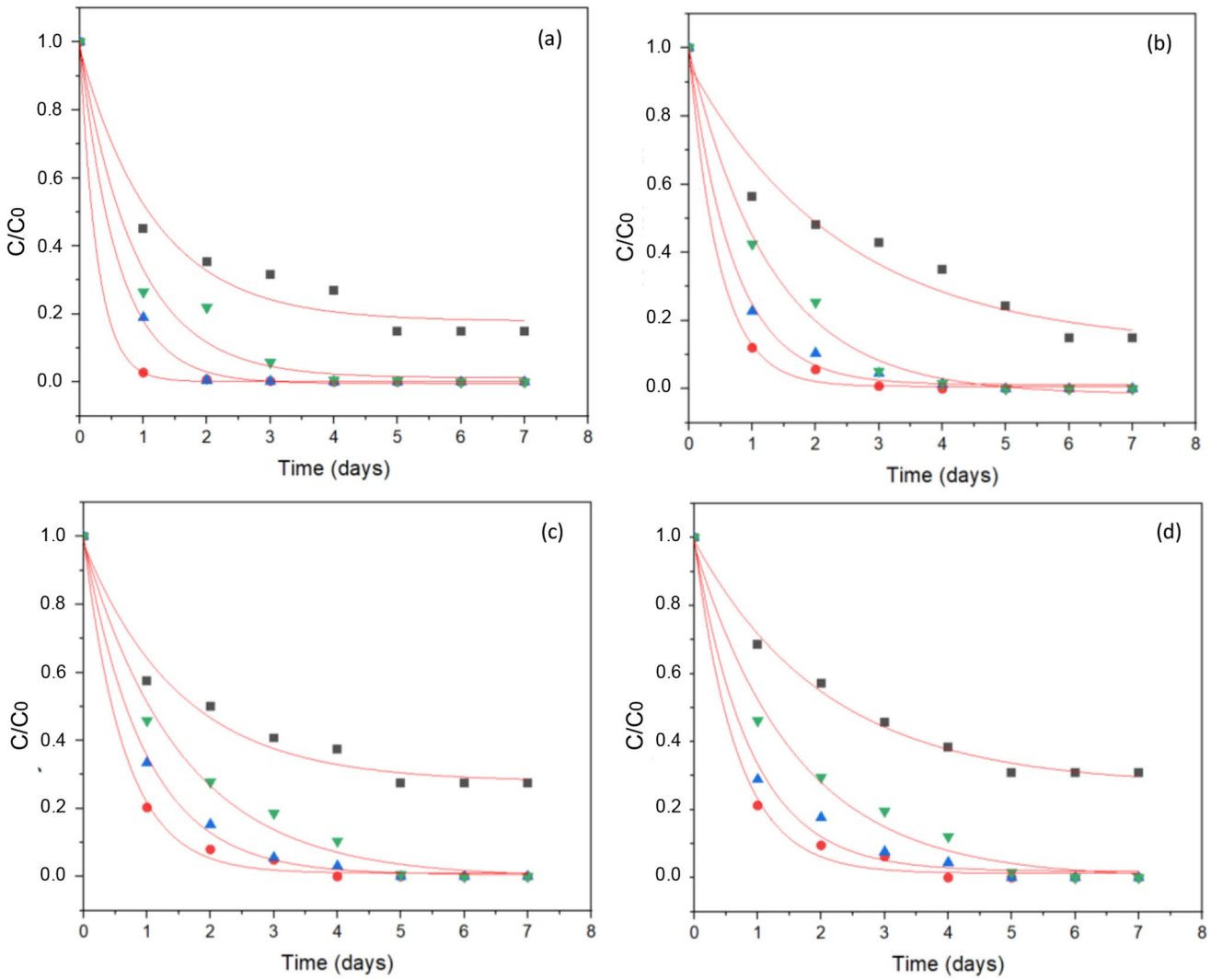


Fig. 2 Decay of BTEX in terms of the final-to-initial concentration ratio (C/C0) in the abiotic reactors R1 and R2 (■), fungal reactors R3 and R4 (2 g.L^{-1} sucrose) (●), fungal reactors R5 and R6 (30 g.L^{-1} sucrose) (▲), fungal reactors R7 and R8 (no sugar) (▼). **(a)** Benzene, **(b)** Toluene, **(c)** Ethylbenzene, and **(d)** Xylene. The initial concentration of BTEX was 1,500 mg.L^{-1}

ethylbenzene, and xylene, observed in both the medium without sucrose and in those supplemented with sucrose (Fig. 2a, b,c, d). The trend of benzene exhibiting the highest removal rate followed by toluene, ethylbenzene, and xylene (Fig. 2b, c and d) is consistent with previous studies. Removal rates ranged from 1.12 to 0.63 d^{-1} in fungal reactors without sucrose, from 3.63 to 1.50 d^{-1} in those with 2 g.L^{-1} sucrose, and from 1.69 to 1.04 d^{-1} in fungal reactors containing 30 g.L^{-1} sucrose (Table 1).

Laccase and hydroxylases are enzymes widely produced by *Aspergillus niger*. They catalyze the oxidation of aromatic compounds, using molecular oxygen as the electron acceptor and generating water as a byproduct (Lubers, 2021).

BTEX removal rates in the biological reactors were higher than in the abiotic ones, with the most significant increase noticed for benzene, which exhibit removal rates 2-, 6- and 3-fold higher, without sucrose, with 2 g.L^{-1}, and with 30 g.L^{-1} sucrose, respectively. The addition of sucrose also enhanced the removal rate of the other BTEX compounds, notably with especially in the presence of 2 g.L^{-1}, where the removal rate was 4-fold higher for toluene, and 3-fold higher for the other two. This indicates, on the one hand, biological degradation of BTEX and, on the other, that the addition of a simple carbon source, even at low concentrations, promotes this biodegradation. In addition, in this study, cellular growth was higher in the presence of sucrose, with final concentration of (27.89 ± 1.09) and (50.05 ± 5.87) g.L^{-1} in reactors receiving 2 and 30 g.L^{-1} of sucrose, respectively. In contrast, when fungi were provided only with BTEX as a source of carbon and energy, cellular growth was approximately 3 to 5 times lower than in reactors supplied with

Table 1 BTEX removal percentages (%), rates (k) and corresponding half-lives ($t_{1/2}$) in the abiotic, and biotic reactors (with 2 $g.L^{-1}$ sucrose, 30 $g.L^{-1}$ sucrose and without sucrose), in saline medium (25‰). The results are the average of duplicated reactors. The standard deviation error was below 5%

Condition	Abiotic reactors (R1, R2)			Biotic reactors							
				2 $g.L^{-1}$ sucrose (R3, R4)			30 $g.L^{-1}$ sucrose (R5, R6)			Without sucrose (R7, R8)		
Compound	**%**	**k (d^{-1}) R^2**	**$t_{1/2}$(d)**	**%**	***k*(d^{-1}) R^2**	**$t_{1/2}$(d)**	**%**	***k*(d^{-1}) R^2**	**$t_{1/2}$(d)**	**%**	***k*(d^{-1}) R^2**	**$t_{1/2}$(d)**
Benzene	85.1	0.84 (0.965)	0.83	100	3.63 (0.999)	0.19	100	1.69 (0.999)	0.41	99.7	1.12 (0.973)	0.62
Toluene	77.1	0.55 (0.982)	1.25	100	2.10 (0.998)	0.33	100	1.43 (0.997)	0.49	100	0.78 (0.994)	0.89
Ethylbenzene	73.2	0.51 (0.970)	1.35	100	1.56 (0.997)	0.44	100	1.13 (0.998)	0.61	99.3	0.65 (0.990)	1.07
Xylene	69.2	0.48 (0.993)	1.44	100	1.50 (0.995)	0.46	100	1.04 (0.991)	0.67	98.4	0.63 (0.988)	1.11

2 and 30 $g.L^{-1}$ of sucrose, respectively. Notable, although greater cell growth with 30 $g.L^{-1}$ of sucrose, BTEX removal was faster in reactors with only 2 $g.L^{-1}$, which corroborates the idea that removal is driven not only by adsorption to the biomass but also to biodegradation.

Fungi likely faced challenges in assimilating complex carbon sources in the high-salinity medium (30‰ NaCl), thereby limiting their growth, as stated previously by Venâncio et al. (2017). The addition of simple sugars like sucrose is cited as beneficial, as it supports primary fungi metabolism, promotes initial growth, and serves as a crucial energy source under high-salinity conditions. This energy is used not only for osmotic pressure regulation but also for enhancing mycelial growth. Sucrose, being an easily assimilated carbon source, also stimulates the production of enzymes involved in the degradation of contaminants such as BTEX, via co-metabolism (Garnier et al. 2000), a phenomenon observed in the fungal-inoculated reactors. Under aerobic conditions, microorganisms typically use mono- and di-oxygenase enzymes to hydroxylate the aromatic rings of the compounds, forming intermediates including catechol, which are subsequently broken down by ortho- or meta-cleavage dioxygenases into Krebs cycle intermediates (Sei and Fathepure 2009). Indeed, numerous studies report that supplementing with a primary carbon and energy source is extremely beneficial for the biodegradation of recalcitrant compounds, by stimulating enzymes production of enzymes such as lignin peroxidase and laccases (Aranda et al. 2017) directly related to the process,. However, this effect can vary. For instance, Cao et al. (2020), found that while glucose supplementation (0.2 $g.L^{-1}$) increased the enzymatic activity of lignin peroxidase (227.5 $U.L^{-1}$) and laccase (108.4 $U.L^{-1}$), it did not enhance benzo(a)pyrene removal. Authors attributed this to competition between glucose and benzo(a) pyrene and the energy expenditure involved in the production of lactate during glucose metabolism via glycolysis.

In our previous study (Rodrigues et al. 2017), using *Aspergillus niger* in a continuous reactor for BTEX removal from petrol-contaminated water, glucose supplementation improved benzene removal efficiency (from 72 to 96%), but negatively affected ethylbenzene removal (from 90 to 72%). This was attributed to the preferential consumption of glucose over ethylbenzene by *Aspergillus niger*. García-Peña et al. (2001), noted that ethylbenzene is generally more resistant to biodegradation than the other hydrocarbons. However, in our study, although not very significantly xylene was more resilient than ethylbenzene.

It is important to note that the concentrations of BTEX in this study were much higher than that reported in estuaries, seas, and oceans, were they range from 0.001 $\mu g.L^{-1}$ to approximately 22.6 $\mu g.L^{-1}$, except near point sources of BTEX, such as discharges from power plants, refinery

outfalls, produced water discharges, and incidents involving oil and derivative spills, where these compounds are found in concentrations between 2,566 and 6,750 $mg.L^{-1}$. BTEX concentrations tend to be even higher in coastal waters than in inland waters (Brooks et al., 1984; Astrahan, 2018).

To ascertain the contribution of adsorption and confirm the role of biodegradation in BTEX removal, the amount of BTEX adsorbed onto fungal biomass was measured at the end of the batch experiment. In the case of benzene, adsorption on biomass within the reactors was as (13.35 ± 4.72), (1.50 ± 0.19) and (2.04 ± 0.10) $mg.g^{-1}$ (wet weight) without sucrose, and with 2 $g.L^{-1}$ and 30 $g.L^{-1}$ of sucrose, respectively, which corresponds to (40.2 ± 4.1), (13.9 ± 1.1) and (33.8 ± 5.8) % of total benzene removal. These findings indicate that, while adsorption contributed to BTEX removal, the majority of removal occurred through biodegradation. Adsorption to biomass enhances the accessibility of the compounds to enzymatic action, thus facilitating both adsorption and biodegradation as complementary mechanisms for BTEX removal.

Although the greatest cell growth occurred in the reactors with fungi and 30 $g.L^{-1}$ of sucrose (R5 and R6), the mass of benzene adsorbed to the mycelium was lower than that recorded in reactors (R7 and R8), where the fungi depended solely on BTEX as a carbon source for their growth. This observation suggests that sucrose not only favored the increase in biomass in these reactors, but also promoted the biodegradation of benzene, thus justifying the lower adsorbed quantity obtained. No adsorption of toluene, ethylbenzene, and xylene onto the mycelium was found, which may be due to complete degradation and assimilation of these compounds by microorganisms. It is worth noting that the biodegradation of benzene, compared to toluene and xylene, is more challenging in an environment where these compounds are simultaneously available to microorganisms, as benzene requires more energy for breaking down the aromatic structure, followed by xylene and toluene (Prenafeta-Boldú et al. 2002; Perdana et al. 2019). For instance, Prenafeta-Boldú et al. (2002) reported that the soil fungus *Cladophialophora* sp. strain T1 could grow on a water-soluble gasoline fraction containing BTEX. While benzene was not metabolized, toluene, ethylbenzene, and xylene compounds were degraded through a combination of assimilation and cometabolism. Toluene and ethylbenzene served as carbon and energy sources, whereas the xylene compounds were cometabolized. Specifically, o-xylene and m-xylene were converted to phthalates, while p-xylene was mineralized only in the presence of toluene. The degradation of toluene, ethylbenzene, and xylenes was attributed to the same monooxygenase enzyme, targeting the side chain. The benzene ring group has strong resonance high resonance due to its electron delocalization, making it highly stable and, therefore, requiring more energy to break down.

Perdana et al. (2019) also observed significant hydrocarbon removals by filamentous fungi. In their study, a mixture of benzene and toluene in a ratio of 1:3 was tested in a non-saline environment, and biodegradation of 25% for benzene and 75% for toluene, was achieved by *P. chrysosporium*, over 5 days at 25 °C. However, *Aspergillus* sp. demonstrated the ability to utilize both monoaromatic hydrocarbons using them as a carbon source, achieving benzene and toluene removals of up to 75% after two weeks in a discontinuous reactor.

The adsorption capacity of the fungal mycelium is also influenced by the interaction between different hydrocarbons present in the medium, which compete for adsorption sites, as well as by factors such as the presence of polar compounds, hydrophobicity, solubility, molecular weight, among other. Additionally, the unique physiological characteristics and active adsorption sites of the mycelium may favor the adsorption of aromatic compounds, particularly due to their abundant conjugated structures, diverse functional groups, and high carbon content (Rama et al. 2019). Melo et al. (2019) observed that xylene exhibited higher adsorption onto microbial biomass compared to toluene and benzene, likely due to xylene higher molecular weight and lower solubility in water. Adsorption is generally favored for compounds with lower solubility and a higher molecular weight. However, in the present study, the presence of ethanol altered BTEX solubility, influencing the dissolution of these compounds. Given that benzene has the lowest dipole moment, it is more prone to being adsorbed onto the fungal biomass. The stability of the benzene ring, combined with its hydrophobicity, confer hydrocarbons a strong tendency to adsorb onto solid surfaces such as clay and organic matter, with higher molecular weight further enhancing adsorption potential.

Volatilization of BTEX can also occur, particularly in the presence of ethanol, a highly polarized molecule with a very high electric dipole moment (1.69 D). Ethanol can temporarily polarize less polar compounds like BTEX, by altering they electronic cloud. Ethanol high solubility in water, facilitated by dipole-dipole interactions established between water and ethanol molecules (hydrogen bonds), enhances the dissolution of BTEX in aqueous phase, making them more accessible to microorganisms (Rama et al. 2019). In the current study, similarly to the findings by Perdana et al. (2019), the highest removal of BTEX occurred on the first day, with biological reactors achieving removal rates ranging from around 60% (xylene and ethylbenzene) to 100% (benzene), and abiotic reactors showing removals from 30% (ethylbenzene) to 57% (benzene). Ethanol influenced the improved dissolution of these compounds in the medium, especially

benzene. The higher electric dipole moment of ethylbenzene (0.64 D), toluene (0.37 D) and xylene (0.59 D) favor their solubility using a small initial fraction of ethanol when compared to benzene (0 D). This suggests that benzene is much less affected by ethanol in terms of solubilization and more prone to volatilization. Notwithstanding, the experimental design was aimed at minimizing volatilization, and the observed BTEX removal is likely due to biodegradation. Previous studies on BTEX-contaminated groundwater similarly attributed concentration decay primarily to biodegradation with adsorption and volatilization having minimal contributions (Wiedemeier, 1995). In groundwater, a higher ethanol-to-gasoline ratio leads to a lower BTEX removal because ethanol is preferentially degraded, depleting oxygen and electron acceptors, thus delaying the biodegradation of BTEX compounds without the necessary nutrients for natural attenuation processes or significantly delaying the degradation process (Rama et al. 2019). In groundwater systems, a higher ethanol-to-gasoline ratio can lower BTEX removal rates because ethanol is preferentially degraded, depleting oxygen and electron acceptors, thus delaying the biodegradation of BTEX (Rama et al. 2019).

Conclusions

Aspergillus niger showed significant growth and efficient BTEX removal in environments with salinity levels up to 25‰, particularly when supplemented with sucrose as a carbon source. The demonstrated halotolerance, highlight its potential for bioremediation in saline environments. Xylene proved to be the most persistent hydrocarbon in all reactors, but complete BTEX removal was achieved after 7 days in biological reactors with sucrose, and nearly 100% removal was observed even without sucrose supplementation. BTEX were removed through both adsorption and biodegradation, with final adsorption percentages of around 14%, 34% and 40% in the reactors with 2 $g.L^{-1}$ of sucrose, 30 $g.L^{-1}$ of sucrose and no sucrose, respectively.

The removal rates observed in the Fungal reactors supplemented with sucrose exhibited significantly higher removal rates, Fungal reactors supplemented with sucrose exhibited significantly higher removal rates, yet *A. niger* was able to eliminate BTEX even in the absence of sucrose at levels surpassing those found in polluted marine environments. This suggests that sucrose supplementation may not be necessary in marine settings, where *A. niger* can effectively address BTEX contamination. These results highlight the potential of fungal bioprocesses for remediation in high-salinity areas, such as mangroves, coastal regions, and petrochemical sites. Notwithstanding, the potential applicability of the proposed technology can be further optimized through further studies involving immobilized biomass. This approach would allow the use of microorganisms attached to floating support materials, which may facilitate the introduction and sustained activity of the studied species in marine and/or estuarine waters for effective environmental decontamination. Such an application is particularly relevant for addressing surface-layer contamination, where these compounds tend to accumulate, forming plumes.

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Declarations

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