Stability of fungal biomass continuously fed with tannic acid in a non-sterile moving-packed bed reactor

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Abstract

A number of bacteria and fungi are known to degrade tannins. In this study, the efficiency of the white-rot fungus, *Bjerkandera adusta* MUT 2295, was evaluated for the treatment of a synthetic solution prepared with tannic acid. Tests were performed in continuously fed, bench-scale, packed-bed reactors, operated under non-sterile conditions with biomass immobilized within PolyUrethane Foam cubes (PUFs). The main parameters monitored to evaluate the process efficiency were: soluble Chemical Oxygen Demand (sCOD), Total Organic Carbon (TOC) removal, and activities. of Tannase and Lignin Peroxidase. At the end of the process, additional parameters were evaluated, including the increase of fungal dry weight and the presence of ergosterol. The reactor was operative for 210 days, with maximum sCOD and TOC removal of 81% and 73%, respectively. The reduction of sCOD and TOC were positively correlated with the detection of Tannase and Lignin Peroxidase (LiP) activities. Increases in biomass within the PUF cubes was associated with increases in ergosterol concentrations. This study proved that the fungal-based system tested was efficient for the degradation of tannic acid over a period of time, and under non-sterile conditions.

List of abbreviations

AS	Activated Sludge	MnP	Manganese-dependent Peroxidase
AGS	Aerobic Granular Sludge	MUT	*Mycotheca Universitatis Taurinensis*
COD	Chemical Oxygen Demand;	pCOD	particulate Chemical Oxygen Demand;
DP	Degradation Percentage	PAHs	Polycyclic Aromatic Hydrocarbons
GLY	GLucose and Yeast liquid solution	PUFs	PolyUrethane Foam
HPLC	High Performance Liquid Chromatography	RE	Removal Efficiency
HRT	Hydraulic Retention Time;	sCOD	soluble Chemical Oxygen Demand;
LiP	Lignin Peroxidase	SMEW	Standard Method for the Examination of Water and Wastewater
LMEs	Lignin-Modifying Enzymes	TA	Tannic Acid
MEA	Malt Extract Agar	TOC	Total Organic Carbon
MI	Mitotic Index	WRF	White-Rot Fungi

1. Introduction

Tannins are water-soluble polyphenolic compounds, that can be grouped in two major categories: hydrolizable tannins and condensed tannins (Spennati et al., 2019; Sharma et al., 2017). They are widely distributed in plants (Chávez-González et al., 2018) and play a role in protecting plants from herbivores and pathogens (Sharma et al., 2017). Due to their abundance in the natural environment, these compounds are also a major component of organic matter in highly polluted wastewaters, such as landfill leachate and, above all, industrial tannery effluents (Klein et al., 2017; Polizzi et al., 2017). Among the diverse types of compounds used in tanning processes, vegetable tannins are commonly used because of their unique ability to precipitate proteins (Schrank et al., 2017).

Tannins can be toxic and inhibit the growth of a number of microorganisms. They are also resistant to microbial attack, and consequently are recalcitrant to biodegradation (Giaccherini et al., 2017; Mannucci et al., 2010). Therefore, a number of chemical and physico-chemical technologies have been applied to remove the recalcitrant fraction from tannery and tannin-rich wastewaters, including chemical oxidative degradation, electrochemical decomposition, coagulation, flocculation, and adsorption (Elabbas et al., 2016). Although remarkable results have been achieved by chemical and physico-chemical technologies (Natarajan and Manivasagan, 2017), these processes are characterized by high costs, limited applicability, and can be harmful to the environment (Baccar et al., 2011). In contrast, biological approaches, are more cost effective and environmentally sustainable (Baccar et al., 2011). Hence, considerable attention is now being given to the identification of innovative approaches to complement these chemical and physico-chemical methods, or to find less expensive and more environmentally friendly alternatives to them.

Some microorganisms are not affected by the antimicrobial properties of tannins and are able to grow on the surface of tannery pits or wastes (Prigione et al., 2018). In particular, several fungal strains have been reported to both grow on tannery wastewaters, and to degrade tannin compounds (Spennati et al., 2019). Fungal degradation of tannins is closely associated with the presence of Tannase, an enzyme that catalyses the breakdown of hydrolisable tannins and gallic acid esters. Tannase has several industrial and commercial applications, including the food industry and animal feeds, as well as applications in the cosmetic, pharmaceutical, chemical, and leather industries (Farag et al., 2018). In addition to Tannase, Peroxidases and Laccases may also be involved in tannin biodegradation (Tilli et al., 2010).

In this context, the exploitation of White-Rot Fungi (WRF), which secrete extracellular Lignin-Modifying Enzymes (LMEs), could represent a valuable tool for the treatment of tannery and tannins-rich wastewaters. WRF have been shown to be effective in degrading a wide-range of xenobiotic compounds and effluents (Rodríguez-Couto, 2016), such as Polycyclic Aromatic Hydrocarbons (PAHs) (Di Gregorio et al., 2016), synthetic dyes (Zhang et al., 2018), pharmaceutical wastewater (Lucas et al., 2016; Vasiliadou et al., 2016), olive mill wastewater (Ntougias et al., 2015), bleaching wastewater from pulp paper industries (Costa et al., 2017), textile wastewater (Rodriguez-Couto, 2013), and petrochemical wastewater (Palli et al., 2016).

Several studies are available in literature related to the use of fungi in tannery wastewater including experiments on synthetic components of tannery wastewater (i.e. dyes) (Baccar et al., 2011), on natural tannins (Spennati et al., 2019) and with real effluents (Natarajan and Manivasagan, 2017). However, at present, only few studies on fungal degradation have been carried out under continuous conditions (Gullotto et al., 2015). Bacterial contamination and limited knowledge of the optimal conditions for fungal degradation have been reported among the main obstacles to the scale-up of fungal-based applications (Palli et al., 2016).

Fungi can be cultured in suspended form, using pellets, or attached to, or immobilized within, natural or synthetic supports (Spina et al., 2012). Among diverse types of synthetic supports, PolyUrethane Foam cubes (PUFs) have been proved to be low cost and easy-to-handle tools for fungal growth, and can be reused for several batches (Spina et al., 2012; Saetang and Babel, 2010). In addition, higher levels of extracellular enzyme activities have been detected using PUFs as support media (Spina et al., 2012).

Landfill leachate usually contains tannins (Spina et al., 2018; Bardi et al., 2017a,b). In previous studies, the ability of *B. adusta* MUT 2295 to remove recalcitrant molecules from difficult wastewaters, including a TA-containing solution, was tested in both batch and continuous processes.

The strain was selected since multiple batch-tests demonstrated its ability to decolourise landfill leachate (Bardi et al., 2016) and to reduce TA Chemical Oxygen Demand (COD) (Bardi et al., 2017b). In addition, continuous fed-batch bioreactors inoculated with this strain, operated for more than two months under non-sterile conditions, provided remarkable results in the removal of the recalcitrant fraction of landfill leachate (Bardi et al., 2017a). The objective of the current study is to further evaluate the ability of *B. adusta* to grow in a continuous, batch-fed reactor, using TA as a primary carbon source under non-sterile conditions. The feasibility of the fungal-based system would enlarge the current knowledge about continuous treatment with fungi, without co-substrate addition, representing the first step for the scale-up of such processes to substitute or complement current technologies for recalcitrant compounds removal.

2. Material and methods

2.1. Fungal strain and inoculum preparation

The white-rot fungus used in this study, *Bjerkandera adusta* MUT 2295, was obtained from the *Mycotheca Universitatis Taurinensis* (MUT), and was cultured on Malt Extract Agar (MEA, 20 g/L glucose, 20 g/L malt extract, 20 g/L agar, 2 g/L peptone) at 25 °C for one week and maintained at 4 °C. After the cultivation of *B. adusta* on MEA, fungal colonies were cut in squares (approximately 1 cm × 1 cm) and homogenized under sterile conditions, with sterile saline (9 g/L NaCl). The homogenate was inoculated into 2 L flasks containing glucose and yeast extract liquid media (GLY, 5 g/L glucose; 1.9 g/L yeast extract) and 2 cm^3 PUFs (specific surface 600 m^2 m^{-3}, density 25 kg m^{-3}, Pores/Volume ratio: 0.97). Fungal homogenate was added with the ratio 1.5 mL/cube. Flasks were incubated in agitation (150 rpm) at room temperature (23 ± 2 °C) for one week in order to enable the immobilization of the fungus into the cubes. After 7 days, 60 cubes were removed and added into a polyethylene cage for the inoculation inside the reactor. The cage was previously tyndallized at 60 °C for three cycles of 30 min each. All chemicals used in this study were of analytical grade and purchased from VWR Canada.

2.2. Tannic acid solution

The synthetic solution, used in this study, was prepared according to the recipe proposed by UK Environmental agency (2009) to simulate leachate from a conventional non-hazardous landfill. The solution contained tannic acid (1.0 g/L), ammonium chloride (2.0 g/L), sodium chloride (2.0 g/L) and sodium bicarbonate (4.0 g/L). The solution was prepared by adding the chemicals directly into deionized water, without sterilization. All the reagents, used for the preparation of the solution, were of analytical grade and purchased from WVR Canada.

2.3. Reactors design

As shown in Fig. 1, a 5 L packed-bed bench-scale reactor (R1) was set-up to perform continuous tests. The reactor was fed with a working volume of 4.5 L. The pH was monitored through a pH probe and kept controlled using a pH controller, which was set at 6.0 in accordance to the optimal pH of many enzymes (Bardi et al., 2017b). The adjustments were performed with sulfuric acid (10%). The air diffuser, located at the bottom of the reactor and connected to an air pump, provided air continuously with a flow of 2.0 L/min. PUFs cubes, embedded with *B. adusta,* were added to a polyethylene cage and fixed to a rotating shaft (about 5.0 rpm/minute).

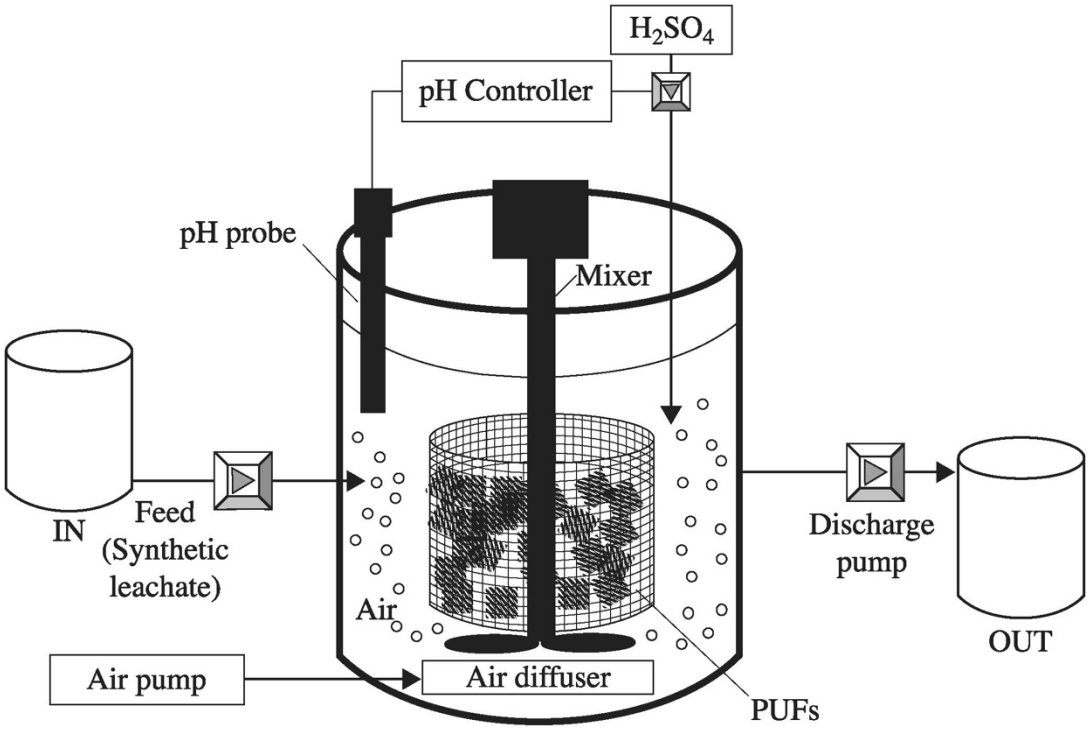

Fig. 1. Representation of the packed-bed bioreactor design.

Simulated leachate was pumped in and discharged using a peristaltic pump (ISMATEC Reglo ICC, Digital Peristaltic Pump). New feed was prepared weekly and kept in the inlet tank, whereas the treated leachate was discharged in a outlet tank before sampling. The hydraulic retention time (HRT) was 72 h (h). The pumps cycle was 6 h, with a pump-flow of 6.9 mL/min. In every cycle, the reactor was fed with 416 mL of influent during 1 h; the same amount was discharged during the outlet that lasted also 1 h. A lag time of 4 h occurred between the end of the discharge and the beginning of the following feeding. Influent and effluent times were controlled using timer switches. The reactor was kept in a room with controlled temperature (23 ± 2 C°).

2.4. Experiments with bench-scale reactor: parameters for the evaluation of treatments efficiency

Grab samples were collected from the outlet tank daily and from the inlet tank in correspondence of feed preparation that occurred weekly. A panel of parameters was measured to evaluate the efficiency of the treatment. In particular, COD, soluble COD (sCOD), Total Organic Carbon (TOC), and enzymatic activities were measured during the fungal treatment. COD and sCOD were measured through the dichromate method, according to Standard Methods for the Examination of Water and Wastewater (SMEW, 21st Edition, APHA, 2005). Soluble COD, sCOD, was measured after samples filtration, using Whatman filter papers grade 1. Particulate COD (pCOD) was given as the difference between COD and sCOD. TOC was measured using the TOC Analyzer (TOC Fusion Teklink). COD, sCOD and TOC Removal Efficiency (RE) was calculated as the percentage (%) removed respect the corresponding influent value.

Ammonium nitrogen (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) were measured via a flow injection analyzer (Quick Chem 8500, LACHAT Instruments). Samples for ammonium nitrogen, nitrite and nitrate were previously filtered using Whatman filter papers grade 1.

2.5. Batch tests on tannic acid degradation using aerobic granular sludge

As a control, COD and sCOD removal from a TA synthetic solution was tested using Aerobic Granular Sludge (AGS), through batch tests. The tests were performed in triplicate with different concentrations of AGS, i.e.: 1.2, 60, 120, and 240 g/L. Control tests without granules were conducted in parallel with the treated tests for 10 days using 500 mL flasks under shaking conditions (150 rpm) and at room temperature (23 ± 2 C°). In each flask, 250 mL of TA solution were added. COD and sCOD were measured during the experiment, as described in section 2.4.

2.6. Enzymatic activities

2.6.1. Enzymatic activities inside the 5 L packed-bed bench-scale reactor (R1)

Enzymatic activities of Manganese-dependent Peroxidase (MnP), Tannase, and Lignin Peroxidase (LiP) were measured at regular intervals during the treatment. MnP activity was determined spectrophotometrically (Biotek Powerwave Xs Microplate Spectrophotometer) by measuring at 590 nm dimethyl amino benzoic acid/3-methyl-2-benzothiazolinone hydrazone hydrochloride (DMAB/MBTH), in 0.1 M succinate lactate buffer, pH 4.5, at 25 °C (Vyas et al., 1994). The enzymatic activity was calculated in international Units (U), where 1 unit is defined as the amount of enzyme that oxidases 1 μmole of substrate per minute.

Lignin Peroxidases (LiP) was measured with veratryl alcohol (2 mM) at 21 °C. The reaction mixture contained 100 mM sodium tartrate buffer (pH 3.0), veratryl alcohol (2 mM), H_2O_2 (0.4 mM) and sample. The total volume was 500 μL. The reaction was started by adding H_2O_2, and A_{310} was monitored for 5 min, using the procedure modified from Vares et al. (1995). Tannase was quantified according to Miller colorimetric method with 3, 5-dinitrosalicylic acid reagent, measuring the glucose produced through the enzymatic breaking of tannic acid in gallic acid and glucose (Miller, 1959).

2.6.2. Tannase expression outside R1

The capability of *B. adusta* MUT 2295 of producing Tannase was assayed also on agar plates and on microplates. Tannase expression on agar plates was assayed according to Bavendamm procedure, using Malt Agar plates added with 0.5% TA (Bavendamm, 1928). Tannase production was also monitored through the ability to degrade TA (Panno et al., 2013; Tilli et al., 2010). Fungal inocula consisted of mycelium disks (Ø 3 mm) inoculated in 48 well sterile polystyrene flat-bottom microtitre plates, containing 800 mL of TA solution (TA 10 g; malt extract 20 g; peptone 2 g; H_2O 1L). Plates were incubated in the dark at 24 °C for seven days. The Degradation Percentage (DP) was expressed as: $DP = 100\ (Abs_0\text{-}Abs_7)/Abs_0$, where Abs_0 is the absorbance at time 0, immediately after inoculation, and Abs7 is the absorbance after seven days, measured at 260, 280, 310 nm.

2.7. Dry weight measurement

The dry weight of fungal biomass, inside the cubes, was measured before starting continuous tests in R1, after the immobilization in GLY, and at the end of continuous experiment. The procedure consisted in weighting a colonized PUF after overnight standing at 65 °C (Anastasi et al., 2012). To discriminate the weight of fungal biomass inside a cube and the weight of the cube, from each measurement the dry weight of a single empty cube was subtracted. Empty cube dry weight was calculated as the average among three replicates. To establish fungal growth during the treatment, dry weight increase was calculated as the difference between the dry weight of fungal biomass at the end of the experiment and at the end of the immobilization

phase. At the end of the immobilization phase, the dry weight was measured on 3 PUFs and the same number of PUFs was weighted at the end of the experiment, after opening the cage.

2.8. Ergosterol measurement

Ergosterol concentration was measured at the end of the immobilization phase and at the end of the continuous experiments. The analyses were performed using High Performance Liquid Chromatography (HPLC) system (Waters Alliance e2695) with auto-sampler and UV detection, measuring at 282 nm. The column was RPC18 (LiChrosorb RP 18 5 μm 100 Å 15 cm × 4.6 mm) chromatography column, at room temperature, with 100% methanol as mobile phase. The protocol for ergosterol measurement was modified from Beni et al. (2014). The principal modification to the method were: 1) 4 g of biomass (humid weight) were employed for each measurement; 2) ultrasonic shaking was reduced to 3 min and 3) hexane extraction was carried out prior saponification. To calculate ergosterol concentration in the samples before and after the treatment, a calibration curve was plotted. Ergosterol concentration was measured in four stock solutions containing 1, 5, 10, 15 and 20 ppm of ergosterol and a calibration curve was plotted. The peaks area obtained with HPLC increased linearly with ergosterol concentration with $R^2 = 0.9939$ and a slope of 15213.

2.9. Toxicity assays

The toxicity was assayed using the seeds of *Vicia faba minor*, which is considered a sensitive bioindicator of clastogenic effects of different environmental pollutants (Ruffini Castiglione et al., 2016). The test was performed by taking into account three different endpoints: 1) phytotoxicity as root elongation measure; 2) mitotic index; and 3) micronuclei frequency as an index of genotoxicity. The assay was performed following the procedure described by Giorgetti et al. (2011). Briefly, *Vicia faba minor* seeds were germinated at 24 °C for 72 a) in the influent and b) in the effluent at after different times of the treatment (control = water; T_0 = influent from the initial tannic acid solution; Tx, different time of treatment). The Phytotoxicity test was scored by measuring the root length (cm) of each sample (in triplicate) in relation to the length of the roots of seeds germinated in deionized water (control). Root-tips for each treatment and control were catted and stored in EtOH 70% overnight. The 70% EtOH was then replaced with a solution of ethanol/glacial acetic acid (3:1 *v*/*v*) for the analysis of the subsequent endpoints. Root-tips were squashes and stained following Feulgen technique as described in Venora et al. (2002). At least 1000 nuclei, randomly selected for each slide, were analysed by means of light microscope for the estimation of the mitotic activity and the micronuclei frequency. Mitotic activity, expressed as mitotic index MI (number of mitoses per 1000 nuclei), indicated the levels of cytotoxicity of the matrices. Micronucleus frequency assay (MNC test, number of micronuclei per 1000 nuclei) were determined for the evaluation of the genotoxicity of the matrices.

3. Results and discussion

3.1. R1 process performance and tannic acid degradation tests using aerobic granular sludge

The reactor was operated for 210 days. The values of COD measured in influent and effluent during the treatment are indicated in Fig. 2A, while sCOD trend is not shown due to the high similarity with COD pattern (sCOD trend is reported in Supplementary Material Fig. S1). COD removal efficiency is also reported in Fig. 2A. The average COD removal toward influent was 51%, with a maximum removal of 82% after 32 days of treatment, corresponding to 420 mg/L of residual COD (Table 1). The average sCOD removal was 52% with a maximum of 81% after 19 days, corresponding to 319 mg/L of residual sCOD (Table 2).

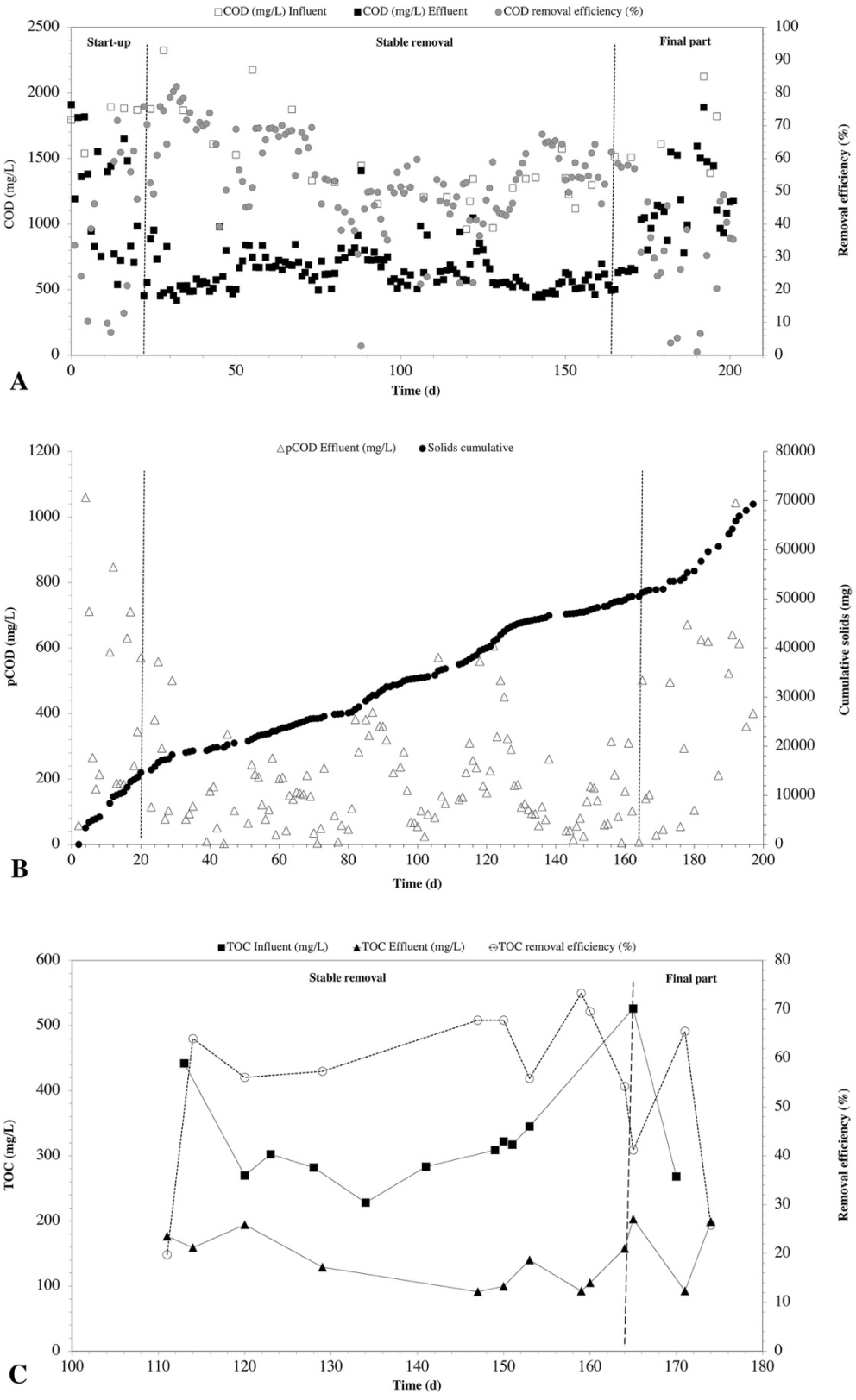

Fig. 2. A) COD (mg/L) in the influent, effluent, and COD removal (dots) during the treatment with *B. adusta*. B) pCOD (mg/L) in the effluent and cumulative solids in the effluent during the treatment with *B. adusta*. C) TOC (mg/L) in the influent, effluent and TOC removal (dots) during the treatment with *B. adusta*. In all the graphs vertical lines separate the different stages of the treatment (Settling, Stable removal and Final part).

The whole operating time can be divided into three stages according to the removal rates, including i) a start up time; ii) an intermediate stage of stable removal, and iii) a final part with lower removal efficiency. In the first stage, which lasted approximately up to day 20 of the treatment, the observed removal efficiency fluctuations could be probably related to a progressive acclimation to the new environment and growth of fungi. During the second leg of the treatment, COD and sCOD removal increased with average removal of 56 ± 14% and 56 ± 13%, for COD and sCOD, corresponding to average reductions of 870 ± 405 and 670 ± 230 mg/L for COD and sCOD, respectively. The second part of the treatment lasted approximately up to day 164. In the last part of the treatment, starting from day 165, removal efficiency decreased up to the end of the process, with an average removal of 34 ± 17 and 33 ± 15 of COD and sCOD, corresponding to average reductions of 722 ± 473 and 356 ± 189 mg/L for COD and sCOD, respectively.

The stability of the pattern was confirmed also by the results of pCOD, whose cumulative trend was increasing approximately up to day 150 (Fig. 2B). During the stable part of the process, approximately 50 g of pCOD was released in the effluent. The presence of this pCOD could be attributed to mycelial growth in suspended form (Borràs et al., 2008) or, alternatively, biomass detachment from PUFs during the treatment (Spennati et al., 2019; Spina et al., 2012). Results of TOC removal are shown in Fig. 2C. The average reduction was 55% and a maximum removal was equal to 73% after 159 days, corresponding to a residual TOC of 92 mg/L (Table 2). While COD and sCOD maximum removal percentages have been achieved in the first part of the experiment, TOC maximum removal percentage have been achieved at the very end of the stable phase (day 159).

The lack of correspondence between the maximum removal percentages achieved in COD and sCOD trends with TOC pattern could be explained as a consequence of possible changes in the microbial community during the treatment. It is possible to hypothesize that at the beginning of the experiment the fungal strain inoculated, *B. adusta*, was the predominant species of the system and that, with the stabilization of the process, a community change occurred leading to higher bacteria abundance, which could explain the efficiency loss after day 164. According to this hypothesis, the community would have evolved starting from organisms able to degrade tannic acid towards organisms that could generate complex by-products during tannic acid degradation. Indeed, efficiency loss seems to be compatible with contamination from other fungi or bacteria that affected the performance of *B. adusta* (Spennati et al., 2019).

On the other hand, pH fluctuations may also explain the efficiency loss that occurred in the last leg of the treatment (Fig. S2). Influent pH was stable along the treatment with an average value of 7.6 ± 0.1, while effluent showed higher variability, with an average value of 5.3 ± 0.4. In several cases, such as day 16, 146-147 and from day 164–166, those values were higher than 6, indicating a failure of pH-controller system, which could have negatively affected the system performance.

Table 1
Average and maximum removal achieved during the treatment with *B. adusta* MUT 2295 considering 1) COD, 2) sCOD, 3) TOC.

Parameter	Average Removal (%)	SD	Average In (mg/L)	SD	Average Out (mg/L)	SD	Maximum Removal (%) (day)	Value of Max. Removal (mg/L)
COD	51	17	1518	333	770	318	82 (32)	420
sCOD	53	16	1152	281	573	228	81 (19)	352
TOC	55	17	293	81	142	43	73 (159)	92

The present study revealed the ability of the system to degrade greater percentages (82%,

residual COD of 420 mg/L), over longer periods of time, and without co-substrate addition compared to previous batch tests, in which *B. adusta* reduced a maximum of only 61% (approximately 1604 mg/L) of tannic acid COD (Bardi et al., 2017b). From the pattern achieved, it is reasonable to assume that *B. adusta* was able to exploit tannic acid as primary carbon source and that the degradative process observed was not due to a co-metabolic pathway, which is particularly important since the great majority of the studies of bioremediation using basidiomycetes required additional carbon sources (Bardi et al., 2017a). According to our knowledge, this is the first report about tannic acid degradation performed by this strain without additional carbon sources. In addition, tannic acid degradation experiments in continuous using fungi have not been reported in literature.

Table 2
Average values and removal achieved during the three stages of the treatment with *B. adusta* MUT 2295.

Days	COD (mg/L)	SD	sCOD (mg/L)	SD	COD (mg/L)	SD	sCOD (mg/L)	SD	% COD Removal	SD	% sCOD Removal	SD
	Influent		Influent		Effluent		Effluent					
1-20	1755	166	1752	159	1205	429	796	413	37	22	58	20
20-164	1430	343	1077	195	638	149	495	116	56	14	56	13
165-200	1661	269	1076	141	1073	336	816	230	34	17	33	15

The data for removal of sCOD during the 10 days of the experiments using different concentrations of Aerobic Granular Sludge (AGS) are shown in Fig. 3. Considering the similarities between COD and sCOD pattern, only the sCOD results are shown. From the results of sCOD removal percentages, it is possible to observe an initial increase of sCOD values (120 h) that could be explained as a partial granules disruption in several trials. Although the exact causes for disintegration of aerobic granules are still unknown (Kocaturk and Erguder, 2016), it is reasonable to hypothesize that it could have occurred as a consequence of COD removal by microbial granules (Kang and Yuan, 2017). At the end of the batch test (240 h) the sCOD removal trend was increasing in association with the increase of granules concentration up to 120 g/L. Indeed, sCOD maximum removal was achieved with an AGS concentration of 120 g/L, reaching 44% of sCOD reduction, corresponding to a residual sCOD of 535 mg/L. The reduction in the unseeded control was 10% for both sCOD and COD (Fig. 3). When comparing these results with those achieved through batch tests with *B. adusta*, it is possible to confirm that, in both experiments, only a small percentage of COD and sCOD reduction has been achieved in the unseeded trials, providing evidence of the role of fungi in increasing the efficiency in the degradation of the synthetic solution.

Indeed, during previous batch tests, in the trials inoculated with *B. adusta* and glucose as co-substrate, higher removal has been achieved compared to the use of AGS, reaching 61 ± 2% of removal on tannic acid 1.3 g/L, corresponding to a reduction of 1604 ± 27 mg/L of COD (Bardi et al., 2017b). These results, together with those achieved through continuous tests, showed that the use of the selected fungal lead to higher performances in the degradation of tannic acid compared to the use of AGS. Continuous tests on tannic acid removal using AGS were performed by other authors, providing further evidence to the results achieved in the present study. Indeed, Ren et al. (2017) set-up two reactors, operated respectively with AGS and Activated Sludge (AS), to treat in continuous a synthetic old leachate, prepared with tannic acid (TA 200 mg/L, initial COD ranging from 458 to 654 mg/L). These authors reported 1) COD removals of 73 ± 8% during the stable phase, using AGS and 2) COD removals of 62 ± 8% during the stable phase, using AS. As indicated above, the use of *B. adusta* resulted in greater percentages of COD removal (up to 82%), using a higher concentration of tannic acid (1 g/L).

3.2. Enzymatic activities inside R1

Although Manganese Peroxidase (MnP) was described for the first time in *B. adusta* by Heinfling et al. (1998) and has been widely reported subsequently in *B. adusta* spp. (Spina et al., 2012) MnP activity was not detected in the tested conditions. Several reasons could explain the absence of MnP activities when scaling-up from batch tests to continuous bench-scale reactors. For example, it is possible that the peroxidases enzymes were inactivated rapidly, as reported by Anastasi et al. (2010). Alternatively, extracellular enzymes washing out of the system could have occurred, as described by Badia-Fabregat et al. (2017). Moreover, continuous production of extracellular enzymes is not considered to be mandatory for the maintenance of continuous fungal systems (Badia-Fabregat et al., 2015, 2017). None-the-less, intracellular enzymes have also been reported to play a key role in the degradation of recalcitrant molecules (Kües, 2015; Yang et al., 2013) and could have been involved in the degradative process observed in R1.

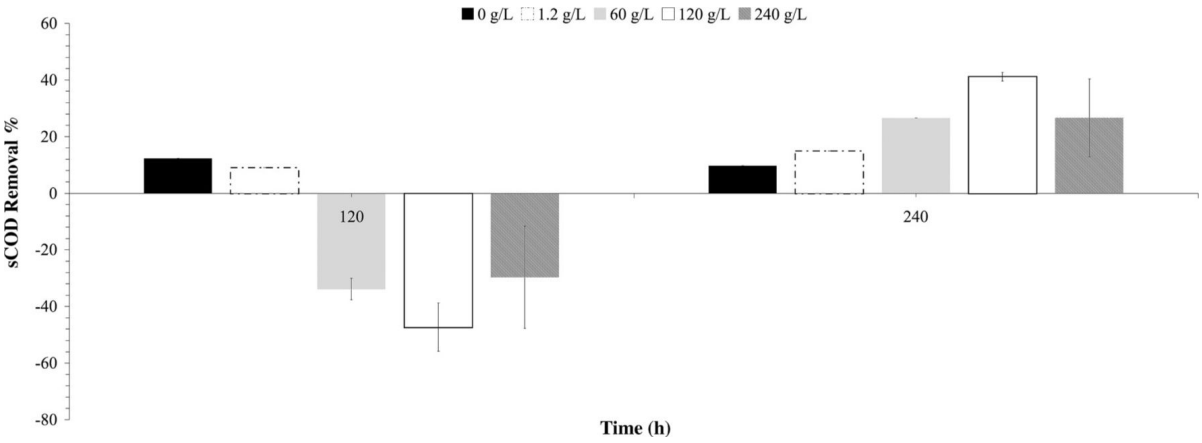

Fig. 3. A) sCOD removal percentages in the experiment with tannic acid and aerobic granular sludge (0 g/L, 1.2 g/L, 60 g/L, 120 g/L, and 240 g/L). Values are given as average among triplicates ± standard deviation (SD).

Results of Lignin Peroxidase (LiP) activity assays are shown in Fig. 4. LiP activity ranged between 1.3 ± 0.1 and 13.8 ± 8.1. LiP detection is not surprising considering that this enzyme, together with MnPs, and Versatile Peroxidases (VP) are considered part of lignin-attacking system of *B. adusta* (Ruiz-Duenas et al., 2013). However, the detected LiP concentration did not suggest a major role of LiP in fungal degradative process occurred in the reactor R1. Since LiP are involved in the oxidation of non-phenolic aromatic compounds, it is reasonable to suppose that additional enzymes were involved in the process operated by *B. adusta.* An enzyme that could have been involved in the degradation of tannic acid could have been Tannase, which catalyze the depolymerisation of gallotannins producing gallic acid and glucose (Tilli et al., 2010).

3.2.1. Bavendamm assays

The high COD and sCOD removal suggested that other enzymes, such as Tannase, could have been involved in the degradative process observed. Hence, the ability of *B. adusta* MUT 2295 to grow on tannic acid and produce a brown ring in presence of such compound was assayed outside the reactor. Although the coverage of the fungus on the plate was not complete as occurred on MEA, the results of the Bavendamm assay after 1 week of incubation of *B. adusta* showed that the strain was able to grow on the plate with TA (0.5%) (see Fig. S3). Hence, the concentration of TA used resulted in only a slight reduction in the growth rate and no clear growth inhibition related to TA presence was noted. In addition, a dark zone or “corona” was clearly visible on the back of the plate, indicating the degradative capability of *B. adusta* MUT 2295 toward TA. All the results suggested the ability of *B. adusta* to produce tannase, which could be responsible of the degradative process observed during the treatment in R1.

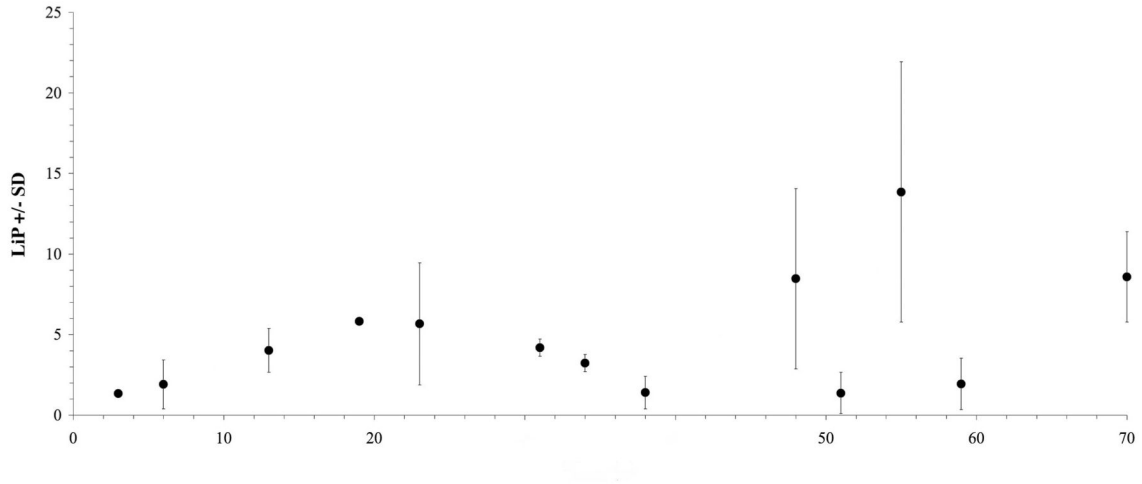

Fig. 4. Results of Lignin Peroxidase activity assays during the treatment with *B. adusta.* Values are given as average among triplicates ± standard deviation (SD).

3.2.2. Tannase production

An additional test was performed to confirm Tannase production outside the reactor. Results of the Tannase production capability of *B. adusta* MUT 2295 are reported in supplementary material (see Fig. S4). The shape of the spectrum of tannic acid is represented by the sample at the beginning of the experiment, which is indicated as T0. In particular, tannic acid spectrum is characterized by a peak at 280 nm and a shoulder at 310 (Tilli et al., 2010). The reductions of the absorbance at 280 and 310 nm indicate the hydrolysis of tannic acid and the diminution at 260 nm the disappearance of gallic acid (Tilli et al., 2010). Absorbance reduction at 280 nm and 310 have been detected in the trials inoculated with *B. adusta*. On the contrary, spectrum reductions did not occur in the unseeded control, in which the spectrum shape after one week of incubation does not show clear differences compared to the T0 spectrum. Indeed, only 3% of DP was achieved in the unseeded control after one week of incubation (data reported in supplementary material, Table S1). Reductions of the spectrum were detected in the trials inoculated with *B. adusta*, providing further evidence of the role of the fungus in the flattening of spectral shape and thus in the degradation of TA. In such trials, DP was 50, 53, and 46 at 260, 280. and 310 nm, respectively. These results suggested the presence of tannase enzyme, which is able of depolymerizing gallotannins (Aboubakr et al., 2013) and is responsible for the degradation of tannic acid into gallic acid and glucose. From these results, it is possible to conclude that the strain MUT 2295 was able to produce tannase in the tested conditions and tannase activity was not detected in the unseeded controls. According to our knowledge, the production of tannase was not reported before in *B. adusta*.

3.2.3. Tannases assay inside R1

Tannase activity was assayed in R1 at different times, during the first 50 days of the treatment with *B. adusta*. The results of the Tannase activity assays are reported in Fig. 5. Tannase activity was detected at all times assayed, and ranged between 1 and 65 U/L, with and average of 23 ± 15 U/L. The fluctuations observed can be explained as a consequence of several factors. One possible explanation could be related to the concentration of tannic acid. Since tannic acid can induce tannase production by fungi (Aboubakr et al., 2013), it is reasonable to expect that the Tannase concentration could have changed according to the concentration of tannic acid available in the reactor.

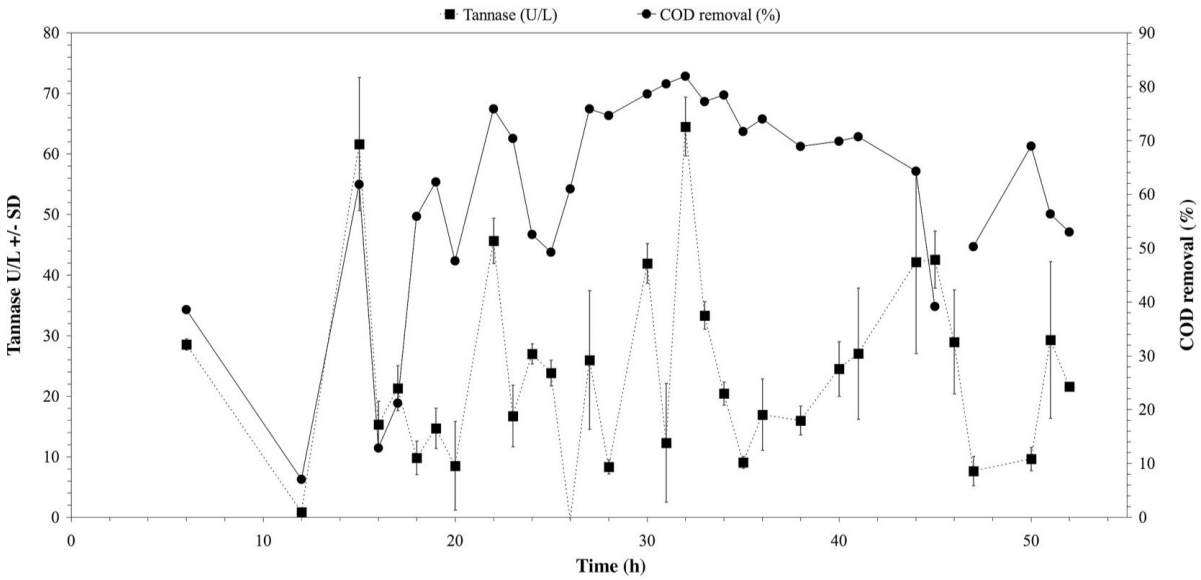

Fig. 5. Results of Tannase activity assays in the effluent and COD removal (%) during the treatment with *B. adusta* MUT 2295. All values are given as average among triplicates ± standard deviation (SD).

Tannic acid was continuously degraded in the reactor; however, new feed, containing tannic acid, was pumped in the reactor every 6 h, influencing tannase expression. As shown in Fig. 5, the pattern of Tannase activity showed several common features with system efficiency in removing COD. Indeed, the two parameters presented similar graphical shapes characterized by several corresponding peaks, as observed on days 15, 22, 32, and 44. In particular, the highest Tannase activity detected on day 32 corresponded to the highest COD removal detected during the whole reactor operating time (82%, corresponding to a residual COD of 420 mg/L), supporting the strong association within tannase activity and tannic acid degradation in the reactor.

One drawback of the method employed to measure tannase activity in this study is that Miller assay is not considered fully reliable and specific (Brahmbhatt and Modi, 2015). However, since in this study, the ability of the strain to produce tannase has been tested also outside the reactor with two independent methods, Bavendamm assay and spectra DP, it is possible to conclude that the strain used in this study was able to produce Tannase in the tested condition. In addition, it is reasonable to expect that such enzyme was involved in the degradative process observed during the treatment. As a control, the activity was measured in some cases also in the influent (data not shown). The maximum activity detected in the influent was 10 ± 2 U/L at day 24, providing further evidence of ability of the strain to produce tannase. The little enzymatic activity detected in the influent could be due to the presence of some bacteria in the non-sterile influent itself, which are also able to produce tannase.

3.3. Dry weight measurement

The cubes present in the cage at the end of the treatment are represented in Fig. 6A. Some images of the cubes employed to measure fungi dry weight at the end of the treatment may be seen in Fig. 6B. The dry weight of the biomass after one week of immobilization, before the inoculation in the reactor, was 18 ± 8 mg/PUF. At the end of the treatment, the biomass was 136 ± 40 mg/PUF. The dry weight of fungi biomass grow remarkably during the treatment, reaching 6.5-times the initial dry weight (Fig. 6C). However, the dry weight measurement by itself cannot be fully explained as an increase of the fungi biomass. Other bacteria and fungi, coming from reagents (i.e: tannic acid powder), could be present as part of the biofilm onto the cubes (Spennati et al., 2019; Badia-Fabregat et al., 2017). In addition, solids could also be

trapped within the biofilm. Other analyses, including molecular approaches could clarify the composition of the biofilm at the end of the treatment in R1. Nevertheless, a clear increase in the cubes biomass occurred during the treatment and the visual analyses of those cubes suggested that the fungal strain inoculated at the beginning of the test, *B. adusta* MUT 2295, was still present at the end of continuous test. The decline of fungi is generally associated with biomass loss from the cubes (Spennati et al., 2019), which was not observed in the present experiment.

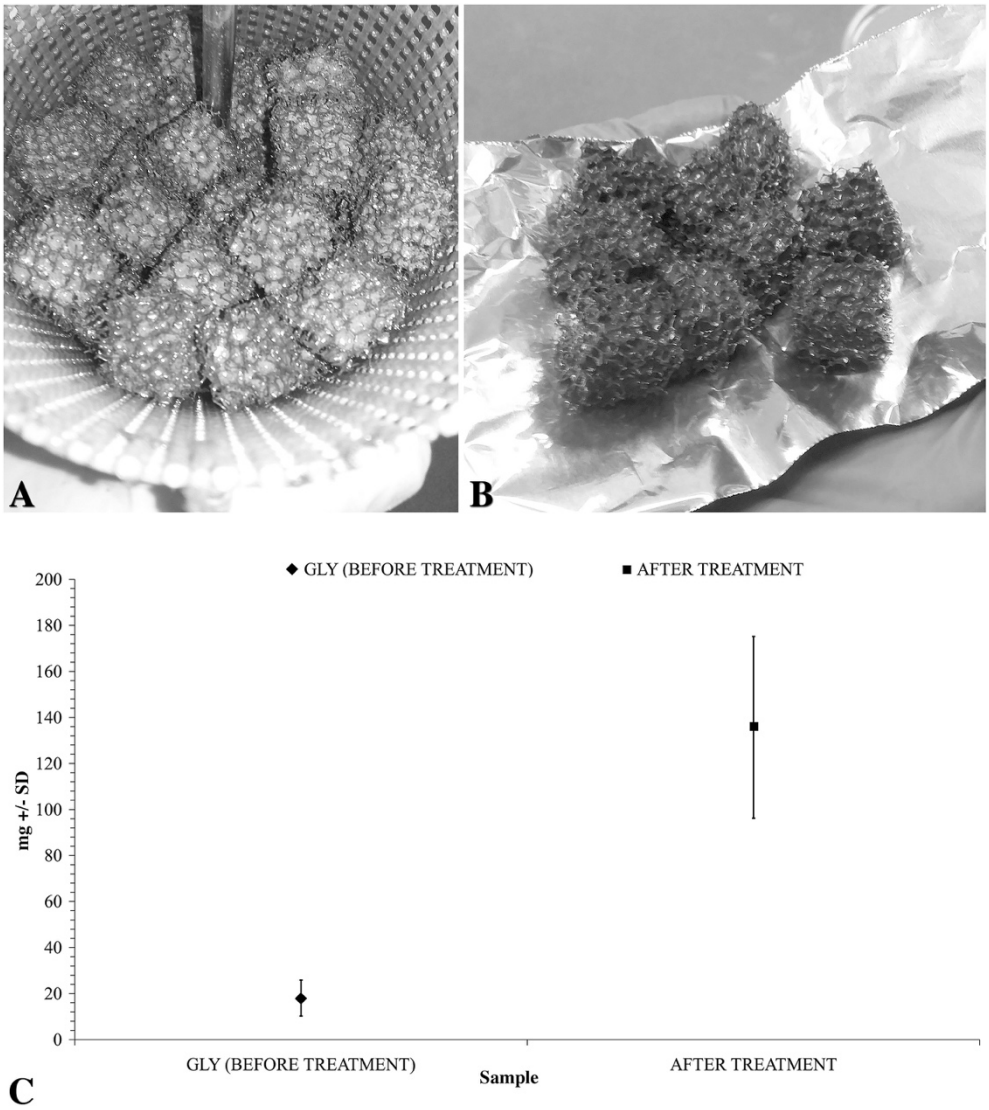

Fig. 6. PUFs at the end of the treatment. A) PUFs inside the cage at the end of the treatment in R1 (210 days). B) PUFs on aluminum foil before measuring dry weight at the end of the treatment in R1. C) PUFs dry weight at the end of the immobilization phase in GLY and at the end of the treatment in R1. All the values are given as the average among in triplicates ± SD.

3.4. Ergosterol concentration

Ergosterol chromatograms have been reported in supplementary material (Fig. S5). Ergosterol peaks were detected at the beginning and at the end of the tests with a retention time of 11.154 and 9.38 min, respectively. Ergosterol after the immobilization phase was 16.22 ppm/g of fungal humid biomass, corresponding to an ergosterol concentration of 0.9 ppm/mg of fungal dry biomass. At the end of the treatment 2.48 ppm of ergosterol/g of fungal humid biomass were found, which corresponded approximately to 0.02 ppm of ergosterol/mg of fungal dry weight.

Ergosterol has been considered an accurate indicator of fungal biomass, whose concentration can depend on several factors, including physiological state of the fungus and growth conditions (Beni et al., 2014). The results achieved in this study suggest the presence of fungal active metabolism at the end of the treatment. However, the presence of ergosterol does not confirm which fungi were present in the reactor since many basidiomycetes and ascomycetes are able to produce ergosterol (Beni et al., 2014). Ergosterol detection together with the visual analyses of the cubes, the lack of important biomass loss in terms of decrease of cubes dry weight and/or important increases of effluent pCOD, suggest that the fungal strain inoculated at the beginning of the experiment in R1, *B. adusta*, was active at the end of the treatment.

3.5. Ecotoxicological analyses: Vicia faba minor assay

The results of ecotoxicological analyses performed using *Vicia faba minor,* including root elongation assay, mitotic index and micronuclei frequency are shown in Table 3. Such parameters have been measured at the beginning of continuous experiment (T0) and after 36 (T1) and 66 (T2) days of treatment.

Table 3. Results of the assay using *Vicia faba minor*. All the values are given as average among triplicates ± standard deviation (SD).

Sample	Root Elongation		Micronuclei		Mitotic Index	
	cm	SD	(%)	SD	(%)	SD
Control®	2.99	1.43	0.99	0.04	11.54	0.42
T0[b]	1.74	1.16	4.37	0.60	5.75	0.21
T1[c]	1.78	0.88	3.38	0.69	6.99	0.63
T2[d]	1.69	1.10	0.67	0.58	7.41	1.57

[a] Control = deionized water. [b] TO = synthetic leachate at the beginning of the test. [c] Tl = after 36 days of treatment. [d] T2 = after 66 days of treatment.

The results of phytotoxicity assay and mitotic index did not show a clear improvement during the treatment. Indeed, no clear differences in root elongation could be observed among T0, T1, and T2. The average root length at the beginning of the experiment was 1.74 cm, which is 42% lower compared to the control. This result suggests the presence of a certain level of root growth inhibition due to tannic acid, although such reduction did not lead to a complete inhibition of the growth. The same conclusions could be drawn also in the case of mitotic index. Indeed, from the beginning of the test (T0) the index grow from 5.75 ± 0.01 to 7.41 ± 0.99, which is 36% lower compared to the control. On the contrary, micronuclei frequency is remarkably reduced along the treatment. The progressive reduction in micronuclei frequency was already visible after 36 days, dropping from 4.37 ± 0.54 to 3.38 ± 0.09. After 66 days the micronuclei frequency was reduced up to 0.67 ± 0.01, which was even lower compared to the control (0.99 ± 0.02), suggesting that the treatment with *B. adusta* positively affected the quality of the effluent in terms of genotoxicity.

Although, the three endpoints measured, namely root elongation, mitotic index and micronuclei frequency, did not provide the same pattern, we can conclude that the treatment did not lead to the production of highly toxic compounds. Indeed, the treatment proposed in this study resulted in 40% of phytotoxicity and 36% of cytotoxicity after 66 days, while no genotoxicity could be observed at that timing.

These results are in contrast with those previously reported in literature. Indeed, other authors have reported increase in toxicity during continuous experiments with fungi. An example is the work of Cruz-Morato et al. (2014), where aerobic degradation of clofibric acid by *Trametes versicolor* was scaled-up in an air-pulsed fluidized bioreactor operated in a continuous mode.

These authors indicated 80% of the fed concentration (160 mg/L) degradation at the steady state; however, the *Vibrio fischeri* acute toxicity test showed that the final culture broth was more toxic than the beginning. In this context, the results achieved in our experiment encourage further applications of the proposed treatment, which could be associated with regular monitoring of genotoxicity with *Vicia faba minor*.

The evidence presented here strongly supports the view that the use of *B. adusta* MUT 2295 led to significant reductions of COD, sCOD, and effluent ecotoxicity in the synthetic TA solution, and that these reductions were associated with the production of extracellular enzymes. Since tannins are commonly present in tannery wastewater and leachate (Spennati et al., 2019; Spina et al., 2018), the proposed system could be employed as pre-treatment for such wastewater, without sterilization or co-substrate addition.

4. Conclusions

A fungal-based system was employed in non-sterile conditions to treat a tannic acid containing solution. The selected fungal strain, *B. adusta* MUT 2295, previously reported as effective on several recalcitrant effluents (i.e. landfill leachate), was inoculated in attached form onto PUFs in a packed-bed bench-scale reactor. The process was stable for about five months. Organic matter removal was associated with biomass growth, ergosterol detection, enzymatic activity and ecotoxicity reduction. This study proved the feasibility and the efficacy of the proposed system for the treatment of tannic acid-containing wastewater in non-sterile conditions and without co-substrate addition. Hence, such system could be employed to substitute or complement current technologies for TA-containing wastewaters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2019.06.036.

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