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Comparing carriers as a support media of white-rot fungi in natural tannins removal

A. Bardi [a,*], Y. Ciummei [b], F. Spennati [b], I.C. Moga [d], S. Di Gregorio [c], G. Petroni [c], G. Munz [a]

[a] Department of Civil and Environmental Engineering, University of Florence, Via Santa Marta 3, Firenze 50139, Italy
[b] Cer^{2}co, Consorzio Cuoiodepur, Via Arginale Ovest 81, San Romano - San Miniato, Pisa 56020, Italy
[c] DFR Systems SRL, 46 Drumul Taberei, Bucharest, Romania
[d] Department of Biology, University of Pisa, Via Luca Ghini 13, Pisa 56126, Italy

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ABSTRACT

In the last decades, White-rot Fungi bioremediation potential has been widely investigated, providing remarkable results toward several recalcitrant molecules. However, full-scale applications based on fungi are not employed yet and little is known about their optimal operating conditions, such as (i) their ability to grow without sterile conditions, (ii) co-substrate requirements and (iii) the accurate carrier design for fungal growth. In this study, several batch tests were performed as preliminary steps to evaluate the possible design of a pilot-scale reactor based on fungal biomass to be operated under not-sterile conditions in the removal of Quebracho natural tannin. The tests were performed to verify fungal affinity, including Basidiomycetes and Ascomycetes for innovative cellulose-containing carriers compared to commonly employed PolyUrethane Foam Cubes. In particular, four fungi, including three Basidiomycetes White-rot Fungi, *Bjerkandera adusta, Phanerochaete chrysosporium* and *Tyromyces chioneus* and the Ascomycota strain *Aspergillus tubingensis*, were employed. As a first step, fungi were tested to evaluate their ability to attach and grow onto 12 types of innovative carriers made by High-Density PolyEthylene and containing cellulose in different percentages. Other tests were performed without sterile conditions. In particular, fungal abilities (i) to attach and grow onto two different types of support, including cellulose-containing carrier and polyurethane foam cubes and (ii) to biotransform recalcitrant molecules (Quebracho natural tannin) (iii) to grow and operate synergistically in a consortium of two fungi, were evaluated. The main parameters evaluated were soluble Chemical Oxygen Demand (sCOD) reduction and dry weight increase. Basidiomycetes showed high affinity for cellulose-containing carrierswith the highest cellulose percentage (7%) achieving full colonization and 60% coverage, in sterile conditions and not- sterile conditions, respectively. These results were associated with a Quebracho sCOD removal of 25 ± 4%, without sterility. When combined, the two selected strains, *Bjerkandera adusta* and *Aspergillus tubingensis* were able to grow on carriers and to remove up to 15 ± 4% of tannins recalcitrant sCOD. This study provides evidence of (i) Basidiomycetes high affinity for cellulose-containing carriers that could favour fungi attachment in sterile and not-sterile conditions and (ii) the feasibility of a combined use of Ascomycetes and Basidiomycetes in bioremediation.

1. Introduction

White Rot Fungi (WRF) have been widely studied as bioremediation agents in many fields, from biotechnological to industrial ones (He et al., 2017; Fang et al., 2018), including tannins removal and tannery wastewater treatment (Prigione et al., 2018a, b; Spennati et al., 2019); however, full-scale applications based on their use still need to be developed (Spennati et al., 2021). Most of studies involving fungi as bioremediation agents have been performed on batch-scale with a preliminary cultivation step in sterile conditions (Ellouze and Sayadi, 2016).

In bioremediation applications, fungi can be exploited either in suspended or in attached form, using a variety of inert or biodegradable carriers. The use of fungi in attached form has been reported as

Abbreviations: COD, Chemical Oxygen Demand; GLY, GLucose Yeast (rich liquid media); HDPE, High Density PolyEthylene; MEA, Malt Extract Agar; PUFC, PolyUrethane Foam Cubes; sCOD, soluble Chemical Oxygen Demand; TOC, Total Organic Carbon; WRF, White Rot Fungi.

* Corresponding author.
E-mail addresses: alessandrabardi85@gmail.com, alessandra.bardi@for.unipi.it (A. Bardi).

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advantageous from many points of view, including higher enzymatic production, the reduction of clogging problems caused by free biomass and of the time and the resources needed to separate the mycelium (Spina et al., 2012); moreover, the selection of an appropriate carrier plays a crucial role since it deeply affect fungal active biomass development (Spina et al., 2012). The carriers used at industrial scale in moving bed bioreactors for wastewater treatment have been mainly developed for bacteria and could not represent the most suitable solution for fungi, which usually present different growth habitus (hyphae vs single cells) (Spina et al., 2012). Among the diverse carriers tested, Polyurethane Foam Cubes (PUFC) were reported as promising when considering the potential enzymatic production and the homogeneity and persistence of colonization (Spennati et al., 2021; Spina et al., 2012; Bardi et al., 2017).

Fungi can biotransform several compounds commonly recalcitrant to bacteria, including polyphenolic substances, such as tannins (Spennati et al., 2021; Lorenz et al., 2014), a major component of tannery wastewaters (Prigione et al., 2018a). Although such compounds are naturally produced by plants, they are poorly biodegradable by conventional treatment technologies, mainly based on bacteria (Munz et al., 2009); as a consequence filamentous fungi capable of degrading tannins have been regarded as a promising solution for tannery wastewater treatment (Cassano et al., 2003; Romero-Dondiz et al., 2015).

Most of tannins biodegradation tests were performed using Ascomycetes. These studies showed the ability of several Ascomycota species to grow on culture media containing different types of industrial tannins as sole carbon source (Prigione et al., 2018a, 2018b). The genus *Aspergillus*, is well-known for its abilities in tannins biotransformation (Chaudhary et al., 2019; Prigione et al., 2018a, 2018b; Pottevin, 1900) and as tannase producer (Dhiman et al., 2018).

On the contrary, Basidiomycetes were scarcely employed as bioremediation agents towards tannins; however, owing to their ability to exploit cellulose as substrate (Bardi et al., 2017), which can be directly added inside carriers (Moga et al., 2019a), Basidiomycetes could be promising bioremediation candidates, reducing the costs of additional carbon source employment in fungi-based systems.

In the present study, three WRF strains and an Ascomycete candidate were employed toward the natural tannin Quebracho. The Ascomycota strain *Aspergillus tubingensis* MUT 990, was isolated from commercial tannin powder and has been object of study for its potential towards tannins (Sigona et al., 2020; Spennati et al., 2019, 2021; Prigione et al., 2018b). Different types of supports for fungal attachment and grow were considered, including (i) innovative carriers made by High-Density PolyEthylene (HDPE) and cellulose and (ii) the commonly employed PolyUrethane Foam Cubes (PUFC). To our knowledge, this is the first study in which (i) fungal cultures were directly inoculated in the recalcitrant solution without a first growth step in sterile conditions and (ii) Ascomycetes and a Basidiomycete were synergistically used to remove tannins and iii) carriers containing HDPE and cellulose were used support media for fungal growth.

2. Material and methods

2.1. Fungal strains, chemicals and carriers

All chemicals employed in the present study were of analytical grade. Within this study, four fungal strains were employed, including the WRF *Bjerkandera adusta* MUT 2295, *Phanerochaete chrysosporium,Tyromyces chioneus* and the Ascomycota *Aspergillus tubingensis* MUT 990,. *Bjerkandera adusta* MUT 2295 and *Aspergillus tubingensis* MUT 990, were obtained from *Mycotheca Universitatis Taurinensis* (MUT). These two strains have been employed in several bioremediation studies to treat leachate and tannins (Spennati et al., 2021; Bardi et al., 2017; Sigona et al., 2020). *Phanerochaete chrysosporium* Burdsall DSMZ 6909 (ATCC 24725), was purchased from DSMZ, (Germany) and *Tyromyces chioneus* was isolated from a sample of landfill leachate (Winnipeg, Canada). All strains were preserved on Malt Agar plates (MEA, glucose 20 g/L, malt extract 20 g/L, yeast extract 20 g/L and peptone 2 g/L) at +4 °C and periodically inoculated in new Petri dishes to preserve the colony. In each petri dish, were poured 10 mL of MEA (Micheluz et al., 2015). In the experiments, two types of carriers were employed, including Poly-Urethane Foam Cubes (PUFC) and wheel-shape carriers, which were designed and provided by DFR Systems s.r.l (Moga et al., 2019a).

2.2. Batch test: attachment to carriers in sterile conditions

In this experiment, 12 types of carriers made with High Density PolyEthylene (HDPE), talcum and cellulose, were employed, including BIG (internal diameter: 2.2 cm) and SMALL ones (internal diameter: 1.2 cm). Talcum (5%) was added in combination with HDPE, resulting in a more hydrophilic material (Moga et al., 2019b). For each group (big and small), 4 compositions were employed, characterized by different percentages, in weight, of cellulose and HDPE. Cellulose and talc were directly poured in the mixture. Among the small carriers, two types were tested: regular or with additional spokes (Fig. 1, carriers drawing are available in supplementary material, Fig. ES1). Details of the carriers employed are reported in Table 1a,b.

Fungal attachment to carriers was operated according to a modified protocol from Bardi et al. (2017). After the pre-cultivation on MEA, four fungi (*Aspergillus tubingensis, Bjerkandera adusta* MUT 2295, *Phanerochaete chrysosporium* and *Tyromyces chioneus*) were homogenized under sterile conditions with a saline solution (9.0 g/L NaCl). For each $cm^2$of mycelium, 1 mL autoclaved saline solution (9 g/L NaCl) was added (Bardi et al. 2017).The resulting homogenate was poured into 500 mL flasks containing 250 mL of glucose and yeast extract liquid medium (GLY = 5.0 g/L glucose; 1.9 g/L yeast extract). Homogenate was added to all flasks, using 10 mL/flask. HDPE-cellulose carriers, previously sterilized through autoclave (at 121 °C for 20 min), were added to the flasks as follows: in the trials with big carriers, 6 pieces were added to each flask, while in the trials with small carriers, 24 pieces were located into each flask. Each condition was repeated twice for a total of 24 flasks, which were incubated in agitation (150 rpm) on a shaker for one week at room temperature and without adjusting illumination condition, to allow fungal adhesion to carriers. During the incubation, the progress of the attachment was monitored daily with visual observation and with the measurement of the fungal biomass at the end of the experiment (Bardi et al., 2017). Carriers were weighted after drying in the oven at 105 °C overnight, obtaining 12 measurements for big carriers and 48 for small ones. Empty carriers dry weight was calculated as

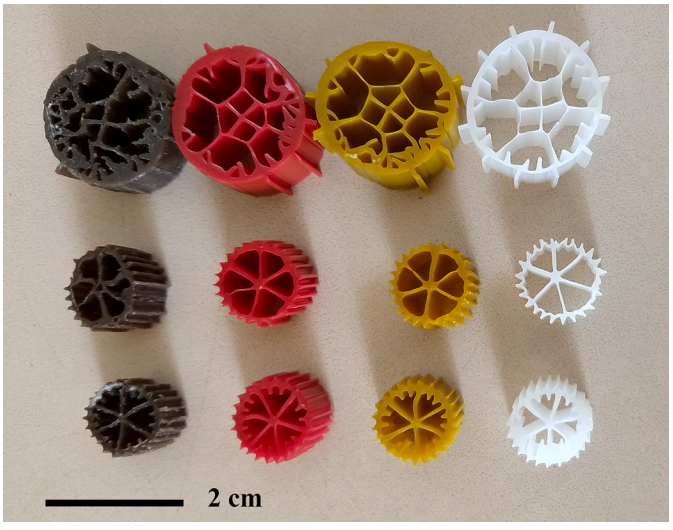

Fig. 1. from left to right, First line: BW, BY, BR and BB; Second line: SW, SY, SR and SB; Third line: SSW, SSY, SSR and SSB.

Table 1
(a) composition, size and shape of each carriers tested in the experiment, b) specific characteristics of each carrier category, including Big (BW, BY, BR and BB), Small (SW, SY, SR and SB) and Small with additional spokes (SSW, SSY, SSR and SSB).

a) Colour	Composition	Size	Spokes	Abbreviation
White	100% HDPE	BIG		BW
		SMALL	Regular	SW
			With Spokes	SSW
Yellow	3% cellulose, 5% talcum 92% HDPE	BIG		BY
		SMALL	Regular	SY
			With Spokes	SSY
Red	5% cellulose, 5% talcum 90% HDPE	BIG		BR
		SMALL	Regular	SR
			With Spokes	SSR
Brown	7% cellulose, 5% talcum 88% HDPE	BIG		BB
		SMALL	Regular	SB
			With Spokes	SSB

b) Carrier type	Specific Internal area (m^2/m^3)	Specific External area (m^2/m^3)	Internal surface/ total surface (%)	Specific surface (m^2/m^3)
Big	544.95	197.05	73	742
Small	356.89	425.11	46	782
Small with spokes	461.62	304.38	60	766

the average among three replicates. Dry weight increase was calculated as the difference between the dry weight of fungal biomass/flask at the end of the experiment and the average weight of three empty carriers. Empty carriers dry weight, cellulose/carrier (g) and cellulose/flask is reported in Table 2 at the end of Section 2.4.

2.3. . Batch test: attachment to carriers in not-sterile condition

The ability of fungal biomass to grow in attached form in not-sterile conditions was assessed using two types of supports: PolyUrethane Foam Cubes (PUFC), and, based on the results of the previous experiment, the Big Brown (BB) carriers with 7% cellulose. Fungal attachment to carriers was performed following a modified protocol from Bardi et al. (2017). The test was performed, using *Bjerkandera adusta* MUT 2295, whose affinity for cellulose was suggested by the previous experiment and was already been investigated (Bardi et al. 2017). The ability of the selected strain to attach to the carriers was assessed with and without co-substrate addition. Indeed, after the pre-cultivation on MEA, *Bjerkandera adusta* was homogenized under sterile conditions in 9.0 g/L NaCl, and inoculated into 500 mL flasks containing: (a) Quebracho medium 1g/L (250 mL/flask) (b) Quebracho medium 0.5 g/L and GLY medium (250 mL/flask), employed as a co-substrate.Five BB carriers or five PUFC were added to each flask and each condition was repeated in triplicates for a total of 12 flasks, which were incubated with agitation (150 rpm) for 45 days at room temperature. During the incubation, the progress of the attachment was monitored daily and, at the end of the test. In addition, 3 mL aliquots were taken and filtered through 0.45 μm PTFE membranes, at the beginning and at the end of the test, to carry out the soluble COD (sCOD) analyses, in order to understand if the inoculated fungus was able to degrade the organic substance present. sCOD was measured according to Standard Methods for the Examination of Water and Wastewater (SMEW, 21st Edition, APHA, 2005).

Table 2
Empty carriers dry weight (g); measurements are given as average among three replicates +/- S.D. The following calculation are reported: (1) cellulose weight/ carrier (0, 3, 5, 7%), (2) total cellulose/ flask (cellulose weight/carrier*number of carriers) and (3) cellulose (g)/ L of medium employed in each experiment.

Carrier (g)			Cellulose (g)		
Name	Average (g)	S.D.	g/Carrier	g/flask	g/L GLY
BW	1.774	0.054	0.00	0.00	0.00
SW	0.554	0.010	0.00	0.00	0.00
SSW	0.699	0.011	0.00	0.00	0.00
BY	2.566	0.118	0.08	0.46	1.85
SY	0.705	0.012	0.02	0.51	2.03
SSY	0.768	0.003	0.02	0.55	2.21
BR	2.682	0.027	0.13	0.80	3.22
SR	0.713	0.011	0.04	0.86	3.42
SSR	0.767	0.004	0.04	0.92	3.68
BB	2.649	0.081	0.19	1.11	4.45
SB	0.686	0.026	0.05	1.15	4.61
SSB	0.716	0.093	0.05	1.20	4.81

To assess fungal growth on PUFC/carriers the flasks were monitored daily by visual observation, and by measurement of the dry weight of fungal biomass at the end of the experiment. PUFC/carriers were weighted as described in Section 2.2. Empty carriers dry weight, cellulose/carrier (g) and cellulose/flask is reported below in Table 2. The ANOVA Two Ways, followed by Bonferroni test were used to detect significant differences ($p < 0.05$, $p < 0.01$, and $p < 0.001$) among the single conditions, in terms of sCOD concentration (mg/L). When performing the ANOVA the two factors were (i) the type of the treatment and (ii) the time, to consider the sCOD removal along the experiment. The statistical analyses were performed with StatPlus (statistical software package for Excel), produced by AnalystSoft (https://www.analystsoft.com/).

2.4. Efficiency of one fungal (Ascomycetes) in degrading Quebracho tannin, evaluated alone and combined with another strain (Basidiomycetes)

The abilities of *Bjerkandera adusta* MUT 2295 and *Aspergillus tubingensis* MUT 990 to (1) attach and grow onto BB carriers and PUFC and to (2) remove a recalcitrant solution containing tannins were evaluated in not sterile condition, considering the two strains separately or combined. After the pre-cultivation on MEA, both fungi were homogenized (separately) under sterile conditions in 9.0 g/L NaCl and inoculated into 500 mL flasks containing Quebracho medium 1 g/L (250 mL/flask).BB (3) and PUFC (3) were added to each flask following the timing summarized in Fig. 2. Each condition was repeated in triplicates for a total of 12 flasks, which were incubated with agitation (150 rpm) for 45 days at room temperature, to allow fungal attachment to carriers. During the incubation, the progress of the attachment was monitored daily and, at the end of the test. In addition, 3 mL aliquots were taken, and filtered through 0.45 μm PTFE membranes, at the beginning (T0), T15, T24, and at the end (T49) of the test, in order to carry out the sCOD analyses.

To assess fungal growth on PUFC and carriers the flasks were monitored daily with visual observation and to quantitatively estimate the growth the measurement of the dry weight of fungal biomass was performed at the end of the experiment. Dry weight, sCOD measurements and statistical analyses were performed as described in Sections 2.2 and 2.3, respectively. Empty carriers dry weight, cellulose/carrier (g) and cellulose/flask is reported below in Table 2.

3. Results

3.1. Batch test: attachment to carriers in sterile conditions

Results of the attachment to the carriers in sterile conditions are shown in Fig. 3. Only carriers on which fungal growth could be visually observed are represented in the Figure. Several pictures of fungal attachment to carriers are reported in supplementary material (Fig. ES2). Out of 12 carriers tested, fungal growth could be observed on 4 types. However, the high Standard Deviations (S.D) provide evidence of the relevant differences among replicates. Such differences can be partially attributed to an effective variability in the growth within technical and biological replicates. In case of technical replicates, it is

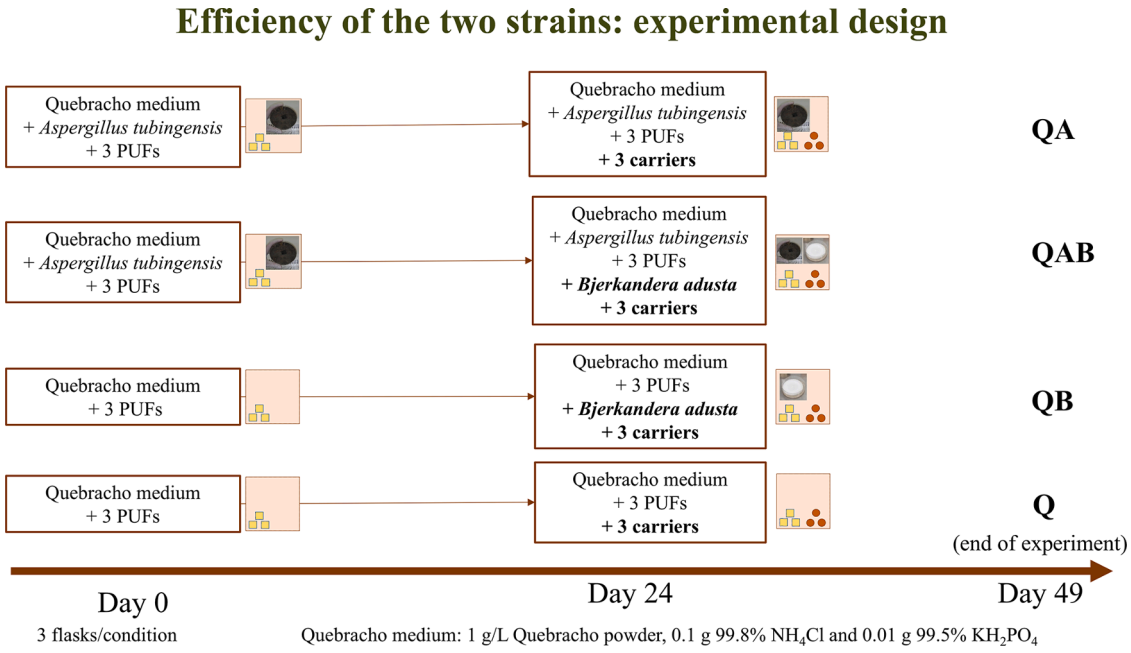

Fig. 2. Experimental design of the batch test described in Section 2.4: (Day0: inoculum of *A. tubingensis*; Day24: inoculum of *B. adusta*). Day0: QA) Quebracho + *A. tubingensis* + PUFC; QAB) Quebracho + *A. tubingensis* + PUFC; QB) Quebracho + PUFC; Q) Quebracho + PUFC; Day24: QA) Quebracho + *A. tubingensis* + PUFC; QAB) Quebracho + *A. tubingensis* + *B. adusta* + PUFC; QB) Quebracho + *B. adusta* + PUFC; Q) Quebracho + PUFC.The average dry weight of empty carriers is provided below in Table 2, in which are reported (1) empty carriers dry weight, (2) g of cellulose/carrier and data concerning the first experiment (Section 2.2), including (1) g of cellulose/flask that is cellulose (g)/ carrier * number of carriers/flask and (2) g of cellulose/liquid (L) in each condition tested. With regard experiments in not-sterile condition (Section 2.3) and the combination of two strains (described in Section 2.4) in which only BB carriers were employed, cellulose (g)/flask were 0.93 (BB = 5) and 0.56 (BB = 6), respectively and g/L liquid were 3.7 and 2.23, respectively.

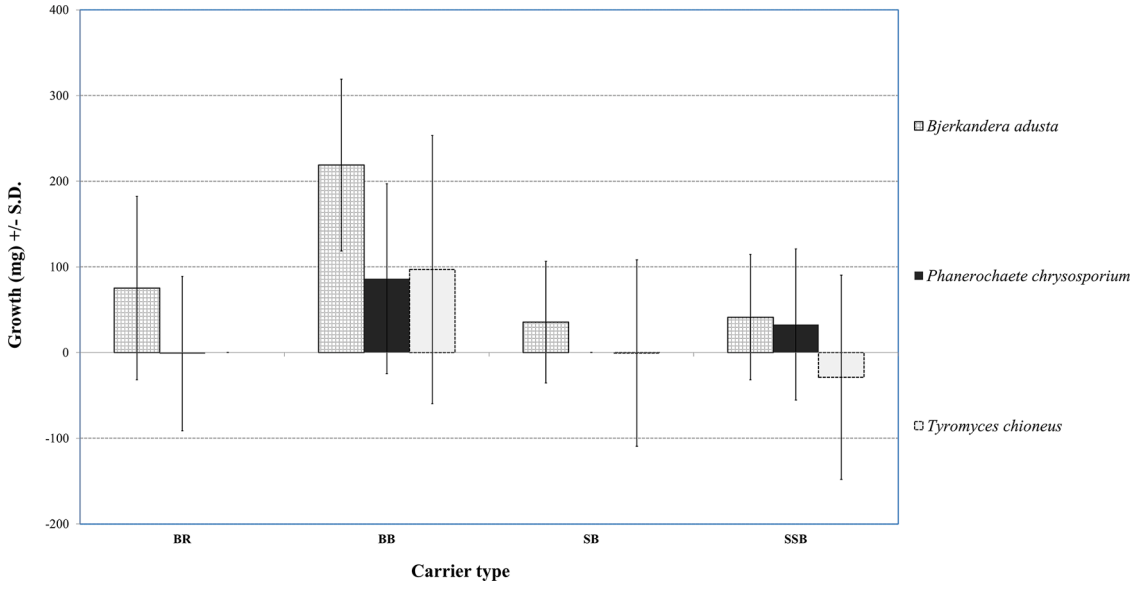

Fig. 3. Fungal growth on different types of carriers after one week of experiment. Big carriers $n = 12$, small carriers $n = 48$. Measures are given as average among replicates ± S.D. For the abbreviations see Table 1.

reasonable to consider a certain level of variability in the age of the original colony, which could have led to differences in the attachment ability of fungi. Each flask represents a unique system, and many variables could have affected fungal attachment (Spina et al., 2012). The variability in the weight among empty carriers should also be taken into account. Indeed, the drying process of a carrier does not allow its employment in batch tests and an average weight of empty carriers was used to calculate the growth. Although all carriers were obtained through the same process, a certain variability was present; this is particularly true in case of high cellulose percentages. Indeed, from visual observation of empty carriers a slight deformation in 7% cellulose carriers could be noticed, probably due to the presence of cellulose itself.

It is worth noting that fungal growth occurred on the carriers with higher cellulose percentages: 3 out of 4 carriers were brown (7% cellulose) and one type was red (5% cellulose). No fungal growth could be observed on the other types of carriers. It is clear that *B. adusta* showed the highest affinity for cellulose, being able to attach partially or completely to all carrier types reported in the picture, with particular

predilection for BB carriers, on which the growth was 219 mg ± 100. However, only in case of BB carriers the attachment interested all carriers employed and, in many cases, covered the whole carrier surface. In case of BR, *B. adusta* could grow on 10 out of 12 carriers employed and the coverage did not cover the whole carriers surface. Even though the growth was lower compared to that achieved by *B. adusta*, all Basidiomycetes could grow on BB carriers. Indeed, neither *P. chrysosporium* nor *T. chioneus* could grow on the great majority of BB carriers surface. Comparing to *B. adusta*, the growth was less homogeneous, as understandable from the high S.D.s. In case of *P. chrysosporium,* the growth interested 10 out of 12 carriers employed, while in case of *T. chioneus* fungal growth could be quantified on 9 out of 12 carriers employed.

On small carriers, the growth was clearly lower compared to big ones and hardly noticeable from visual observation. Higher fungal growth could be observed on SSB, on which all strains showed a modest growth, suggesting that the presence of additional spokes could favour fungal mycelia development/attachment. In all conditions, *B. adusta* showed the highest average growth values. However, as understandable from the high S.D.s, the growth did not interest all the carriers employed; out of 48 carriers employed for each fungus, a growth could be quantified on 38 carriers for *B. adusta*, 41 *for P. chrysosporium* and 27 for *T. chioneus*. No attachment was observed in the flasks inoculated with *A. tubingensis*.

3.2. Batch test: attachment to carriers in not-sterile condition (with or without co-substrate)

Results of *B. adusta* growth in not-sterile conditions on different types of carriers, (PUFC and BB), are shown in Fig. 4a. Fungal attachment on BB carriers at the end of the experiment can be found in supplementary material. Although the biomass coverage on carriers was not complete, a modest growth could be observed (visual observation and quantitative measurements) in all conditions, reaching higher values on BB carriers, with or without co-substrate. In case of PUFC, without co-substrate, a growth could be observed in 2 out of 3 flasks onto 7 out of 15 PUFC, resulting in very high S.D.s. In fact, in these trials, the average growth was 2 ± 8 mg. High variability could be detected also within each flask PUFC, suggesting possible inhomogeneity in the inoculum and/or a certain variability within PUFC size/weight. In presence of GLY the growth was clearly higher, which is consistent with the ability of *B. adusta* to exploit GLY for its growth. Also, in this case, the growth was not homogeneous and clearly higher in one out of three flasks, reaching an average growth among PUFC of 16 ± 22 mg. It is worth noting that, also in presence of co-substrate, the number of colonized PUFC did not change since fungal growth could be quantified onto 7 out of 15 cubes, as occurred without co-substrate addition. Owing to PUFC shape and considering the modest growth achieved, especially without co-

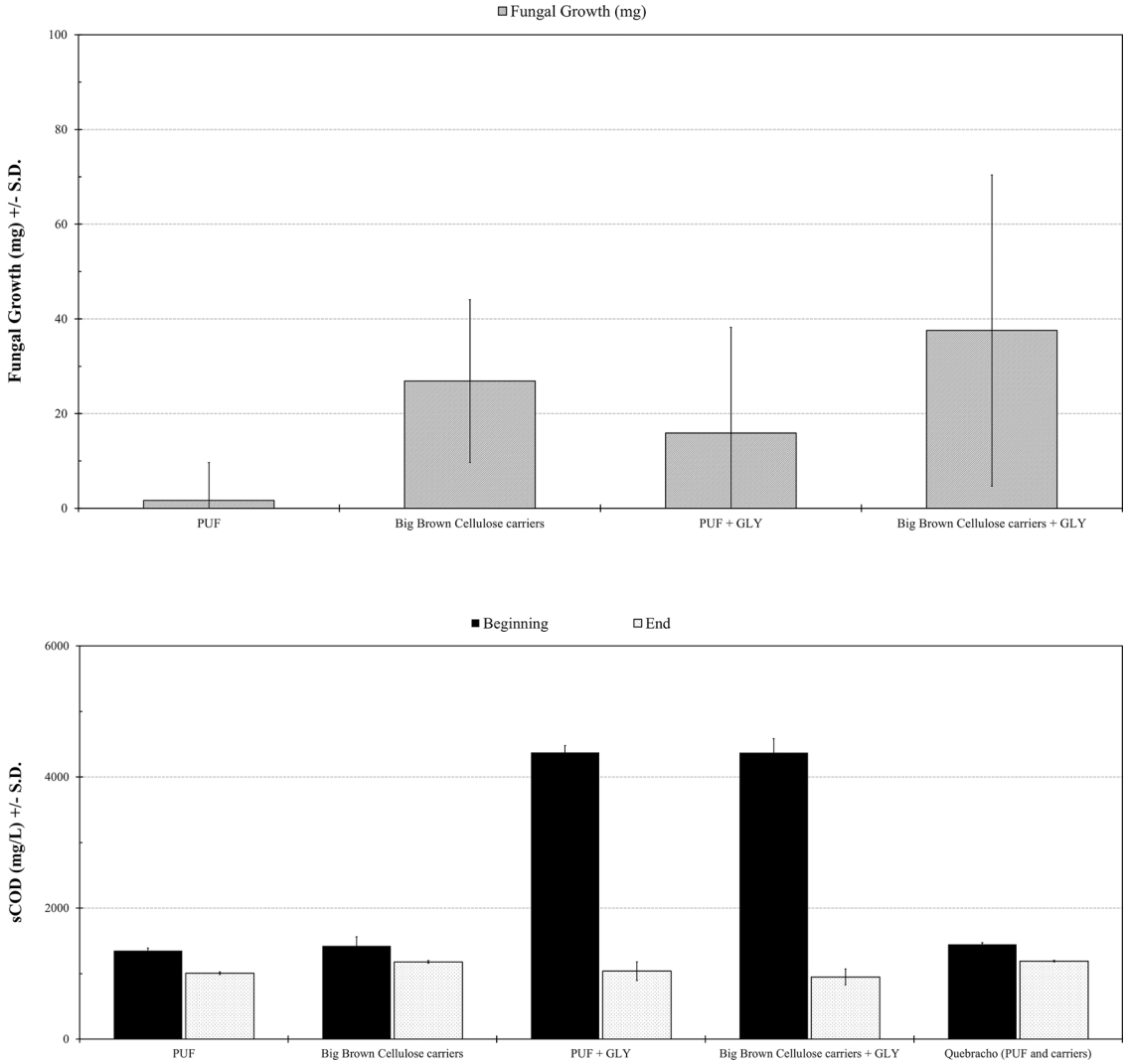

Fig. 4. (a) *B. adusta* growth on BB and PUFC with/without cellulose at the end of the experiment (45 days). Measures are given as average among replicates ± S.D (5 carriers/PUFC/ flask and 3 flasks/conditions, $n = 15$/ condition). (b) initial and final value of soluble Chemical Oxygen Demand (sCOD) mg/L; values are given as the average among replicates ± Standard Deviations (SD).

substrate, visual observation did not allow to quantify fungal growth coverage onto PUFC. The higher growth on BB is consistent with fungal ability to exploit the cellulose in the support as co-substrate for fungal growth. Without GLY, fungal growth could be quantified in all flasks, for a total of 10 out 15 carriers and an average growth of 27 ± 17 mg. In presence of GLY, which supplied an additional co-substrate for the growth of *B. adusta,* fungal growth could be quantified in all flasks even if in one of them it was almost negligible, for a total of 11 out of 15 carriers and an average growth of 38 ± 33. From visual observation on BB carriers, it was possible to quantify an approximate coverage growth of 60 ± 10% without co-substrate and 67 ± 32% in presence of co-substrate.

Concerning the results of sCOD trend, sCOD removal without co-substrate addition was higher when using PUFC (25±4%) compared to cellulosic carriers (17 ± 7%) and to unseeded controls (18 ± 1%). On the other hand, when co-substrate was added, sCOD removals were comparable using both types of carriers, reaching final values of 1038 ± 5 mg/L for PUFC and 950 ± 5 mg/L with for BB carriers. Removal percentages increase of 51% was obtained in both cases, achieving 76 ± 4% and 78 ± 3% when using PUFC and BB carriers, respectively. Results of statistical analyses are reported in supplementary material. From ANOVA tests, it is possible to observe significative differences for both factors, treatment and time and for their interaction (supplementary Material, Table 1). Significant differences have been recorded concerning sCOD trend in the comparison between conditions with-without co-substrate. Indeed, comparison among groups highlighted the presence of significant differences in the trials containing GLY toward those trials in which GLY was not added (supplementary Material, Table 2 i.e. BB versus BB + GLY). These results are consistent with the high sCOD associated to GLY. Through Bonferroni test is possible to observe significative differences between the beginning and the end of the experiment (Table 2 supplementary material); such difference is significant in all the conditions tested. However, p value is higher in the control without fungal inoculum compared to most conditions using fungi, suggesting that fungi could lead to higher removal rates.

3.3. *Efficiency of one fungal (Ascomycetes) in degrading Quebracho tannin, evaluated alone and combined with another strain (Basidiomycetes)*

Fungal growth measured at end of the experiment is reported in Fig. 5. Only conditions on which biomass growth could be observed and quantified are reported in the Figure. The highest biomass growth was detected where the two strains were combined (QAB) using PUFC, achieving an average growth/PUF of 67 ± 20 mg. Even if the growth in such condition (QAB) did not show additive properties, the two fungi could grow together. Dry weight measurement did not provide any information about each strain coverage on the PUFC; accurate molecular analyses would be necessary to further understand the pattern. Considering the strains employed separately, *B. adusta* showed higher biomass growth, being able to grow using both types of carriers. It is particularly interesting considering that *B. adusta* was inoculated only after 24 days after the beginning of the experiment.

No biomass growth was detected on BB in presence of *A. tubingensis* employed alone, confirming the results achieved in all the previous experiments and the lack of affinity for this strain toward cellulose. Since *A. tubingensis* was not able to grow on BB carriers, it is clear that the growth observed on BB carriers when the two fungi were combined (QAB) would be represented mainly by *B. adusta,* with the exception of some autochthonous microorganisms, which can be present in the solution itself (Anastasi et al., 2010), being able to contribute to the biofilm developed onto carriers. It is worth noting that the growth in QAB (16 ± 6 mg) is clearly lower than QB (36 ± 11), indicating that *B. adusta* can grow together with *A. tubingensis* but its growth ability seems reduced. It is reasonable to hypothesize that, by inoculating the two fungi on different timing, *A. tubingensis* could attach and grow onto PUFC in QAB before the addition of *B. adusta*. Besides that, *B. adusta* could achieve higher growth onto PUFC (62 ± 7 mg) when used alone compared to *A. tubingensis* employed alone (QA, 42 ± 15 mg) in less days.

The results of sCOD removal in the experiment on the use of alone and combined with Bjerkandera adusta to treat Quebracho tannin are shown in Fig. 6.

B. adusta and *A. tubingensis*, employed separately, led to similar sCOD removal% (22 ± 2 and 21 ± 3, respectively, corresponding to a depletion of 329 ± 32 and 299 ± 53 mg/L of sCOD). However, *B. adusta* achieved such results in less days (added at day 24).

In batch conditions, the combination of the two strains was associated with a slight reduction in removal efficacy compared to the conditions in which they were not associated, achieving a sCOD removal of 15 ± 4% in 21 days, which corresponded to 227 ± 49 mg/L of sCOD depletion. Higher sCOD removal percentages have been achieved in presence of inoculated fungi compared to unseeded controls, in which 17 ± 1% of sCOD removal was achieved (corresponding 256 ± 16 mg/L) in 45 days, suggesting that fungal inoculation would represent an advantage in the treatment of such tannin. The results of two ways ANOVA showed significant differences for the time and for the interaction of treatment versus time (supplementary material, Table 4). From the comparison among groups (supplementary material, Table 5), it is possible to observe significant differences from the values detected at the beginning and the end of the treatment. On the contrary, no significant differences have been observed among the beginning of the

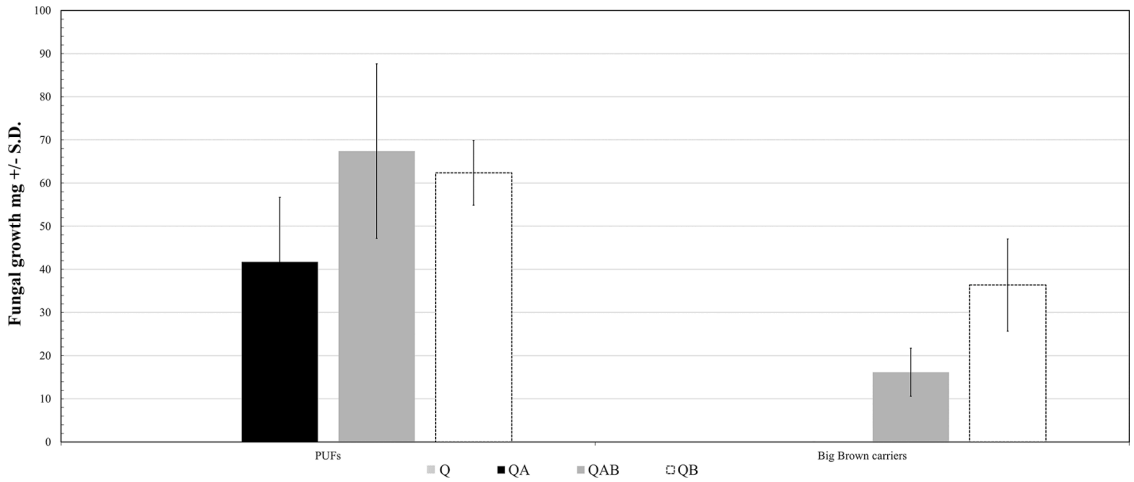

Fig. 5. Fungal growth at the end of the experiment; values are given as the average among replicates ± Standard Deviations (S.D.).

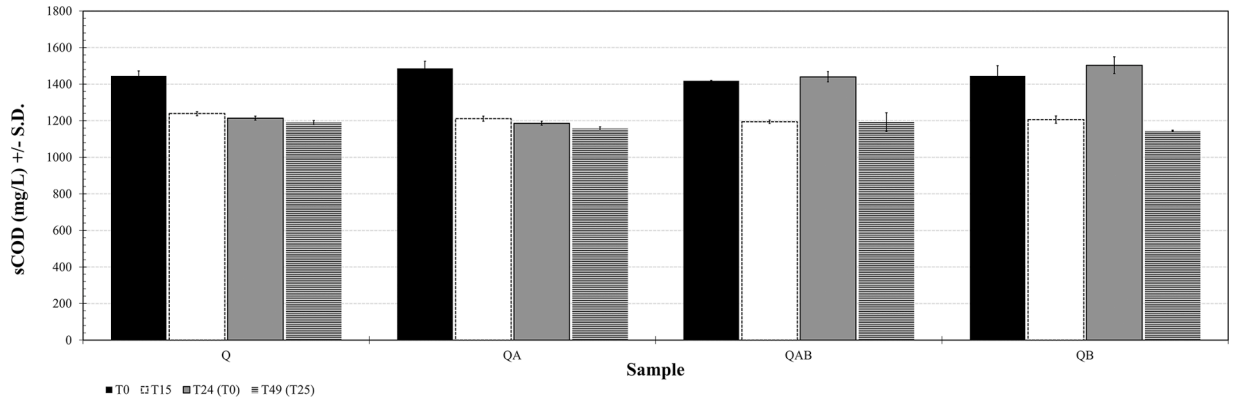

Fig. 6. value of soluble Chemical Oxygen Demand (sCOD) mg/L monitored during the experiment (T0: immediately after the beginning of the experiment, T15: after 15 days, T24: after 24 days (when *B. adusta* was inoculated) and T49: after 49 days from the beginning (25 days after inoculation); values are given as the average among replicates ± Standard Deviations (SD).

experiment and day 24 when *B. adusta* was added with residuals of MEA from cultivation. Comparisons among groups of factor 1 (Treatment) within each factor 2 (Time) level (Table 7, supplementary material), showed several significant differences related to the addiction of fungal inoculum (at day 0 for *A. tubingensis* and day 24 for *B. adusta*), which is associated with increases in sCOD related to the presence of residuals from fungal growth medium. On the contrary, significant differences have not been observed at the end of the experiment, suggesting that *B. adusta* could achieve similar removal percentages in less days.

4. Discussion

4.1. Fungal growth in diverse experimental conditions

Concerning the affinity towards the innovative carriers containing cellulose, a clear difference between the Ascomycota candidate and Basidiomycetes strains could be observed. Although only one Ascomycota strain was included in the experiment, such strain could not grow on innovative carriers in any conditions tested. On the contrary, Basidiomycetes could grow on, at least, two types of carriers tested. The attachment coverage and the growth were clearly higher on bigger carriers and among them, growth could be detected only on red and brown ones, containing the higher cellulose percentages. Basidiomycetes could grow and fully colonize BB carriers, characterized by the highest cellulose percentage (7%), suggesting the role of cellulose in facilitating fungal growth and attachment. The positive effect of cellulose in fungal attachment could be explained due to Basidiomycetes cellulolytic properties (Mir-Tutusaus and Sarrà, 2020). In the case of *B. adusta,* whose cellulolytic capabilities have been already documented (Bardi et al., 2017; Quiroz-Castañeda et al., 2009), full attachment occurred in 7% cellulose carriers and only partial adhesion occurred in 5% ones. Hence, it is reasonable to consider such concentration (7%, corresponding to 0.19 g/carrier, as specified in Table 2) as a threshold or minimal concentration for the growth and full carriers coverage by *B. adusta* MUT 2295.

Considering small carriers, only negligible attachment could be observed onto small brown carriers, slightly greater on those with additional spokes. The pattern is in accordance with the results reported by Spina et al. (2012). In fact, these authors did not observe biomass attachment when inoculating *B. adusta* in agitated conditions on circular industrial carriers with similar shape and size (Spina et al. 2012). Further evidence of Basidiomycetes ability to attach to small carriers in static conditions have been provided by Moga et al. (2019a). These authors could observe a high coverage on SSB carriers when using the Basidiomycete WRF *Cerioporus squamosus* without agitation. A possible explanation is due to the different exposition to shear stress between big and small carriers. As small carriers (without additional spokes) present greater relative external specific surface (about 425.11 m^2/m^3) compared to both, big carriers (about 197.05 m^2/m^3) and compared to small carriers with additional spokes, (304.38 m^2/m^3). Hence, it is reasonable to hypothesize that in agitated conditions the biomass attached onto small carriers detached during the experiment because of shear stress. When comparing the two types of small carriers, the percentage of internal vs. total surface is clearly greater in small carriers with additional spokes compared to small carriers without additional spokes (60% and 46%, respectively), supporting the small growth detected on SSB carriers and providing evidence that the presence of spokes enhances fungal ability to attach onto carriers. Tests in static condition may be helpful to understand whether *B. adusta* could attach and grow also onto small carriers, as reported by Spina et al. (2012), with similar carriers shape and size.

Innovative carriers are made by HDPE that is commonly employed for biomedia in Moving Bed Biofilm Reactor (MBBR) for wastewater treatment (Moga et al., 2019b). Moreover, the current design and composition, supplemented with cellulose would avoid or reduce the costs required for co-substrate addition. On the other hand, PUFC have been proved suitable media for fungal attachment. Owing to their tridimensional structure with a high specific surface (close 600 m^2/m^3) fungal hyphae can develop easily into PUFC pores space (Spina et al., 2012). Although their efficacy for fungal growth, with a preliminary sterile cultivation step, was already proven, they are seldom employed (Sandip et al., 2019) as biomedia in MBBR due to several limitations of polyurethane foam, such as ash accumulation inside the carrier pores, which reduces mass transfer, negatively affecting biofilm activity (Yuan et al., 2015).

When comparing the highest growth, achieved on BB carriers, with traditional PUFC, the timing of 7 days was suitable to fully colonize both type of carriers for all Basidiomycetes. According to the values obtained, the growth on BB carriers is clearly greater compared to that achieved with the same medium by *B. adusta* on PUFC (31 ± 18 mg, data not shown). Although the values are clearly different, it is worth taking into account that PUFC and carriers present two different shapes and structures, hardly comparable. Spina et al. (2012) reported a higher growth in sterile condition when using the same strain onto PUFC in the presence of a nitrogen rich liquid medium, achieving 350 mg of growth. In the tested conditions, the presence of cellulose inside BB carriers could have further promoted fungal growth. From the results achieved in the present study, it is clear that the carriers tested and particularly, BB carriers, are suitable for the employment in bioremediation tests using Basidiomycetes as agents. *B. adusta* proved to be an excellent candidate, being able to grow better compared to the other strains in all the conditions tested.

The ability of *B. adusta* to attach and grow onto BB carriers was further confirmed in not-sterile conditions. Although the growth values

were certainly lower compared to those achieved in sterile conditions, the strain could grow with and without GLY, confirming the role of the cellulose present in the carriers in supporting fungal growth and metabolism. It is worth noting that such result was achieved in presence of a tannin-containing recalcitrant solution, which did not inhibit *B. adusta* growth. Besides other studies conducted with a precultivation step in sterile condition using the same strain (Spina et al., 2012), reported that the use of PUFC allowed the most homogeneous and persistent biomass colonization, BB carriers have proven to be suitable for the attachment of *B. adusta* MUT 2295 without sterile steps.

The combination of two strains, *B. adusta* and *A. tubingensis,* was proved to be feasible also without sterile condition nor co-substrate addition. With the present set of analyses, it is not possible to quantify eventual contribution of autochthonous microorganisms on carriers/ PUFC. Although microorganism growth was negligible on the carriers contained in unseeded trials, it is not possible to exclude that the presence of inoculated fungi could have promoted the attachment and growth of other microorganisms already present in the synthetic solution (Anastasi et al., 2010). The presence of other microorganisms could explain the higher growth values achieved in QB (Fig. 5) on PUFC compared to the second experiment (Fig. 4a, without co-substrate addition), in similar conditions. Alternatively, another explanation could be due to the different experimental duration of the two experiment. In the first case, the experiment lasted 49 days, while *B. adusta* was added in QB after 24 days from the beginning of the experiment, for a total of 21 experimental days. Since dry weight was measured only at the end of the experiment, it is not possible to exclude that in the first experiment, without co-substrate addition, *B. adusta* could have grown onto PUFC during the experiment using as co-substrate eventual residuals from solid pre-grown MEA solid medium. Once finished co-substrate consumption, some biomass could have detached (Spennati et al., 2019), leading to the final growth values achieved on PUFC. Similarly, in QB *B. adusta* could have preferred PUFC in comparison with BB carriers, mainly due to the shape and size of foam cubes, which have proven to be very suitable for *B. adusta* mycelia growth (Spina et al., 2012). In case of *A. tubingensis*, except for the different experimental duration (45 days), similar considerations on the potential colonization by other microorganisms may be done. The inhomogeneity detected in QA trials may be explained as a consequence of a partial biomass detachment from cubes. However, in QA high average growth values were achieved in not-sterile conditions (42 ± 15 mg). Such values are higher than those achieved by *B. adusta* with GLY in the previous experiment, providing further evidence of PUFC efficacy for the growth of *A. tubingensis* and confirming the affinity of this strain for the tannins solution in which was inoculated (Sigona et al., 2020; Bardi et al., 2019; Spennati et al., 2019, 2021). The detected growth demonstrated the feasibility of bioremediation tests without preliminary precultivation steps in sterility. As a future perspective, *B. adusta* and *A. tubingensis* could be exploited synergistically in the treatment of natural tannins on wider scale, using PUFC and BB carriers, as support for fungal growth. Since cellulose could be fully consumed during long-term experiment, the periodic addition of empty BB carriers and/or spikes of co-substrate may be taken into account, to sustain inoculated fungal cultures.

4.2. sCOD removal in diverse experimental conditions

With regard to the results obtained towards tannins sCOD removal, significative differences have not been observed between the two types of carriers tested, PUFC or BB carriers. On the other hand, significative differences have been observed when using GLY as co-substrate, suggesting that a co-substrate addition, could be considered to enhance fungal growth.

While a certain sCOD removal when using *A. tubingensis* was expected as previously reported by others (Spennati et al., 2019, 2021), unexpected removals of 25 ± 4 and 21 ± 3% were achieved by *B. adusta*, when using PUFC in not-sterile conditions in 49 days (Fig. 4b) and when using both PUFC and BB carriers in 21 days, confirming the versatility of this strain (Anastasi et al. 2012), even though we did not measure particulate COD (pCOD), which could also provide information on fungal growth/detachment (Islam et al., 2019). Considering the recalcitrance and the concentration of Quebracho (1 g/L), the removals obtained are remarkable. In fact, when evaluating the results obtained, it is worth taking into account the lack of fungal pre-grown steps nor co-substrate addition. Other studies were carried out using lower Quebracho concentrations, such as 0.1 g/L, in Spennati et al. (2019) or with pre-cultivation in rich media (Sigona et al., 2020). Although the combination of the two strains, lead to a slight reduction in sCOD removal, it cannot be excluded that longer experiment, in continuous, would allow strains acclimation to the environment. Despite the slight reduction in sCOD removal achieved when using the two strains combined, compared to their use alone, a certain removal of sCOD was achieved also in such condition associated with fungal growth, which onto PUFC reached higher values compared to those achieved by using only one strain (Fig. 5). The pattern of results achieved allows to say that the two fungi are compatible.

4.3. Limit of the study and future recommendations

With regard to fungal growth analyses, the present set of analyses did not allow to quantify eventual contribution of autochthonous microorganisms on carriers/ PUFC. Further molecular analyses would be needed to get a better understanding of microorganisms colonization. It is worth taking into account that in the tested conditions, only one Ascomycota strain was employed and the lack of affinity toward innovative carriers could not be extended to all Ascomycetes strains. With regard to the results obtained towards tannins sCOD removal, further chemical analyses could help quantifying the sCOD reduction due to co-substrate consumption from recalcitrant compounds degradation. As a future perspective, the compatibility and eventual synergistic effects may be tested on bench-scale or pilot scale in association with a wider panel of analyses such as chemical and molecular types.

5. Conclusion

Three Basidiomycetes fungi were able to grow on big polyethylene carriers containing cellulose (0.19 g/carrier), providing evidence of the efficacy of innovative cellulose-containing carriers for bioremediation experiments with Basidiomycetes fungi in both, sterile and not-sterile conditions. On the contrary, these carriers were not suitable for the Ascomycota strain tested whose growth was higher when using PUFC. Among Basidiomycetes, the strain *B. adusta* MUT 2295 was able to obtain a Quebracho sCOD removal of 25 ± 4%, without sterility. This strain was able to grow and operate toward Quebracho recalcitrant fraction employed alone and combined with the Ascomycota strain. These results represent the first step for longer and scaled-up experiments using Ascomycetes and Basidiomycetes with PUFC and cellulose-containing carriers for tannins removal, including a wider panel of community structure and recalcitrant fraction composition analyses.

CRediT authorship contribution statement

A. Bardi: Project administration, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Y. Ciummei:** Formal analysis, Data curation, Writing – original draft. **F. Spennati:** Investigation, Writing – review & editing. **I.C. Moga:** Funding acquisition, Methodology, Project administration. **S. Di Gregorio:** Conceptualization, Funding acquisition, Project administration. **G. Petroni:** Conceptualization, Supervision. **G. Munz:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100311.

References

Anastasi, A., Spina, F., Prigione, V., Tigini, V., Giansanti, P., Varese, G.C., 2010. Scale-up of a bioprocess for textile wastewater treatment using *Bjerkandera adusta*. Bioresour. Technol. 101, 3067–3075. https://doi.org/10.1016/j.biortech.2009.12.067.

Anastasi, A., Spina, F., Romagnolo, A., Tigini, V., Prigione, V., Varese, G.C., 2012. Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation. Bioresour. Technol 123, 106–111. https://doi.org/10.1016/j.biortech.2012.07.026.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21sted. American Public Health Association, Washington DC, USA. https://doi.org/10.4236/health.2010.26086.

Bardi, A., Yuan, Q., Siracusa, G., Chicca, I., Islam, M., Spennati, F., Tigini, V., Di Gregorio, S., Levin, D.B., Petroni, G., Munz, G., 2017. Effect of cellulose as co-substrate on old landfill leachate treatment using white-rot fungi. Bioresour. Technol. 241, 1067–1076. https://doi.org/10.1016/j.biortech.2017.06.046.

Bardi, A., Yuan, Q., Siracusa, G., Becarelli, S., Di Gregorio, S., Tigini, V., Levin, D.B., Petroni, G., Munz, G., 2019. Stability of fungal biomass continuously fed with tannic acid in a non-sterile moving-packed bed reactor. J. Environ. Manag. 247, 67–77. https://doi.org/10.1016/j.jenvman.2019.06.036.

Cassano, A., Adzet, J., Molinari, R, Buonomenna, M.G., Roig, J., 2003. Membrane treatment by nanofiltration of exhausted vegetable tannin liquors from the leather industry. Water. Res. 37, 2426–2434. https://doi.org/10.1016/S0043-1354 (03) 00016-2.

Chaudhary, P., Beniwal, V., Kaur, R., Kumar, R., Kumar, A., Chhokar, V., 2019. Efficacy of *Aspergillus fumigatus* MCC 1175 for bioremediation of tannery wastewater. CLEAN–Soil Air Water 47 (6), 1900131. https://doi.org/10.1002/clen.201900131.

Dhiman, S., Mukherjee, G., Singh, A.K., 2018. Recent trends and advancements in microbial tannase-catalyzed biotransformation of tannins: a review. Int. Microbiol. 21 (4), 175–195. https://doi.org/10.1007/s10123-018-0027-9.

Ellouze, M., Sayadi, S., 2016. White-rot fungi and their enzymes as a biotechnological tool for xenobiotic bioremediation. Management of Hazardous Wastes. InTech, London, pp. 103–120. https://doi.org/10.5772/64145.

Fang, W., Zhang, P., Zhang, X., Zhu, X., van Lier, J.B., Spanjers, H., 2018. White rot fungi pretreatment to advance volatile fatty acid production from solid-state fermentation of solid digestate: efficiency and mechanisms. Energy 162, 534–541. https://doi.org/10.1016/j.energy.2018.08.082.

He, K., Chen, G., Zeng, G., Huang, Z., Guo, Z., Huang, T., Pen, M., Shi, J., Hu, L., 2017. Applications of white rot fungi in bioremediation with nanoparticles and biosynthesis of metallic nanoparticles. Appl. Microbiol. Biotechnol. 101 (12), 4853–4862. https://doi.org/10.1007/s00253-017-8328-z.

Islam, M., Wai, A., Hausner, G., Yuan, Q., 2019. Effect of lignocellulosic enzymes on the treatment of mature landfill leachate. J. Environ. Manag. 233, 400–409. https://doi.org/10.1016/j.jenvman.2018.12.045.

Lorenz, M.M., Alkhafadji, L., Stringano, E., Nilsson, S., Mueller-Harvey, I., Udén, P., 2014. Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. J. Sci. Food Agric. 94 (5), 963–968. https://doi.org/10.1002/jsfa.6344.

Michelu, A., Manente, S., Tigini, V., Prigione, V., Pinzari, F., Ravagnan, G., Varese, G.C., 2015. The extreme environment of a library: xerophilic fungi inhabiting indoor niches. Int. Biodeterior. Biodegrad. 99, 1–7. https://doi.org/10.1016/j.ibiod.2014.12.012.

Moga, I.C., Bardi, A., Di Gregorio, S., Spennati, F., Munz, G., Batistini, S., Iordache, O G, Mitran, C.E., Petrescu, G., 2019a. Improved biofilm carriers for fungal exploitation in wastewater treatment. In. In: Proceedings of the IOP Conference Series: Materials Science and Engineering, 572. IOP Publishing, 012104. https://doi.org/10.1088/1757-899X/572/1/012104.

Mir-Tutusaus, J.A., Sarrà, M., 2020. Fungal reactors: a solution for the removal of pharmaceuticals in urban and hospital wastewater. The Handbook of Environmental Chemistry. Springer, Berlin, Heidelberg. https://doi.org/10.1007/698_2020_660.

Moga, I.C., Petrescu, G., Anghelache, G., Pantazi, A.G., Doroftei, B.I., 2019b. Biotechnologies used in wastewater treatment. In. In: Proceedings of the IOP Conference Series: Materials Science and Engineering, 572. IOP Publishing, 012069. https://doi.org/10.1088/1757-899X/572/1/012069.

Munz, G., De Angelis, D., Gori, R., Mori, G., Casarci, M., Lubello, C., 2009. The role of tannins in conventional and membrane treatment of tannery wastewater. J. Hazard. Mater. 164 (2-3), 733–739. https://doi.org/10.1016/j.jhazmat.2008.08.070.

Pottevin, H, 1900. La tannase. Diastase dedoublant l'acide gallotannique. Compt. Rend 131, 1215–1217.

Prigione, V., Spina, F., Tigini, V., Giovando, S., Varese, G.C., 2018a. Biotransformation of industrial tannins by filamentous fungi. Appl. Microbiol. Biotechnol. 102 (24), 10361–10375. https://doi.org/10.1007/s00253-018-9408-4.

Prigione, V., Trocini, B., Spina, F., Poli, A., Romanisio, D., Giovando, S., Varese, G.C., 2018b. Fungi from industrial tannins: potential application in biotransformation and bioremediation of tannery wastewaters. Appl. Microbiol. Biotechnol. 102 (9), 4203–4216. https://doi.org/10.1007/s00253-018-8876-x.

Quiroz-Castañeda, R.E., Balcázar-López, E., Dantán-González, E., Martinez, A., Folch-Mallol, J., Martínez Anaya, C., 2009. Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium. Electron. J. Biotechnol. 12 (4), 5–6. https://doi.org/10.4067/S0717-34582009000400005.

Romero-Dondiz, E.M., Almazán, J.E., Rajal, V.B., Castro-Vidaurre, E.F., 2015. Removal of vegetable tannins to recover water in the leather industry by ultrafiltration polymeric membranes. Chem. Eng. Res. Des. 93, 727–735. https://doi.org/10.1016/j.cherd.2014.06.022.

Sandip, M., Kalyanraman, V., 2019. Enhanced simultaneous nitri-denitrification in aerobic moving bed biofilm reactor containing polyurethane foam-based carrier media. Water. Sci. Technol. 79 (3), 510–517. https://doi.org/10.2166/wst.2019.077.

Sigona, C., Bardi, A., Modeo, L., Mori, G., Potekhin, A., Verni, F., Munz, G., Petroni, G., 2020. Role of bacterivorous organisms on fungal-based systems for natural tannin degradation. Heliyon 6 (4), e03604. https://doi.org/10.1016/j.heliyon.2020.e03604.

Spennati, F., Mora, M., Tigini, V., La China, S., Di Gregorio, S., Gabriel, D., Munz, G., 2019. Removal of Quebracho and Tara tannins in fungal bioreactors: performance and biofilm stability analysis. J. Environ. Manag. 231, 137–145. https://doi.org/10.1016/j.jenvman.2018.10.001.

Spennati, F., La China, S., Siracusa, G., Di Gregorio, S., Bardi, A., Tigini, V., Mori, G., Gabriel, D., Munz, G., 2021. Tannery wastewater recalcitrant compounds foster the selection of fungi in non-sterile conditions: a pilot scale long-term test. Int. J. Environ. Res. Public Health 18 (12), 6348. https://doi.org/10.3390/ijerph18126348.

Spina, F., Romagnolo, A., Anastasi, A., Tigini, V., Prigione, V., Varese, G.C., 2012. Selection of strains and carriers to combine fungi and activated sludge in wastewater bioremediation. Environ. Eng. Manag. J. 11 (10), 1789–1796. https://doi.org/10.30638/eemj.2012.223.

Yuan, Q., Wang, H., Hang, Q., Deng, Y., Liu, K., Li, C., Zheng, S., 2015. Comparison of the MBBR denitrification carriers for advanced nitrogen removal of wastewater treatment plant effluent. Environ. Sci. Pollut. Res. 22 (18), 13970–13979. https://doi.org/10.1007/s11356-015-4546-z.