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Complete List of Authors:	Spennati, Francesco; University of Florence, Department of Civil and Environmental Engineering Ricotti, Andrea; CEntro Ricerca Reflui Conciari (CER2CO) Mori, Gualtiero; CEntro Ricerca Reflui Conciari (CER2CO), Siracusa, Giovanna ; Universita degli Studi di Pisa Dipartimento di Biologia, Biology Becarelli, Simone; Universita degli Studi di Pisa Dipartimento di Biologia, Biology Di Gregorio, Simona; Universita degli Studi di Pisa Dipartimento di Biologia, Biology Tigini, Valeria; Universita degli Studi di Torino Scuola di Scienze della Natura, Department of Life Sciences and Systems Biology Varese, Giovanna ; Universita degli Studi di Torino Scuola di Scienze della Natura, Department of Life Sciences and Systems Biology Munz, G. ; University of Florence, Civil and environmental engineering; University of Manitoba, Civil Engineering
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THE ROLE OF COSUBSTRATE AND MIXING ON FUNGAL BIOFILM EFFICIENCY IN THE REMOVAL OF TANNINS

Francesco Spennati[a], Andrea Ricotti[b], Gualtiero Mori[b], Giovanna Siracusa[c], Simone Becarelli[c], Simona Di Gregorio[c], Valeria Tigini[d], Giovanna Cristina Varese[d], Giulio Munz[a].

[a] Department of Environmental and Civil Engineering, University of Florence, Via Santa Marta 3, 50139, Firenze, Italy.

[b] Cer2co lab, consorzio Cuoiodepur, Via Arginale Ovest 81, 56020, San Romano - San Miniato, Pisa, Italy.

[c] MUT, Department of Life Sciences and Systems Biology, University of Turin, Viale Mattioli 25, 1012, Torino, Italy.

[d] Department of Biology, University of Pisa, Via Luca Ghini 13, 56126, Pisa, Italy.

Abstract

Tannins are polyphenolic compounds produced by plants and they are used in industrial vegetable tanning of leather. Tannins represent one of the low biodegradability substances in tannery wastewaters with high recalcitrant soluble chemical oxygen demand, furthermore high concentration of tannins can inhibit biological treatment. In the present study four novel rotating submerged packed bed reactors were inoculated with a selected fungal strain to reach a biological degradation of tannins in non-sterile conditions. The selected fungal strain, *Aspergillus tubingensis* MUT 990, was immobilized in polyurethane foam cubes carriers and inserted inside a submerged rotating cage reactors. The reactors were feed with a solution composed by four tannins: Quebracho (*Schinopsis spp.*), Wattle (*Mimosa spp.*), Chestnut (*Castanea spp.*) and Tara (*Cæsalpinia spp.*). Four reactors with a volume of 4 L each were used, the co-substrate was pure malt extract, the hydraulic retention time was 24 hours and the pH setpoint was 5.5. The reactors configuration was chosen to allow the study of the effect of rotation and the co-substrate addition on tannins removal. The experiment lasted two months and it was achieved 80% of chemical oxygen demand and up to 90% dissolved organic carbon removal, furthermore it was detected an important tannase activity.

Keywords: natural tannins removal; *Aspergillus tubingensis*; tannase; malt extract; rotation.

1 Introduction

Tannins are the most abundant and widely distributed polyphenolic compounds in nature. Natural tannins can be subdivided into hydrolysable (gallotannins and ellagitannins), condensed (or proanthocyanidins) and complex tannins [1]. These compounds have a range of effects on various organisms from toxic effects on animals to growth inhibition of microorganisms [2]. Tannins differ from most other natural phenols in their prerogative of precipitating proteins [3] and they are used in tanning process to bind to the collagen proteins of the animal skin and make the leather more durable and not putrescible. The most used tannins in leather industry are Quebracho (*Schinopsis spp.*), Wattle (*Mimosa spp.*), Chestnut (*Castanea spp.*) and Tara (*Cæsalpinia spp.*). Tannins are among the most recalcitrant compounds in tannery wastewater and can inhibit biological treatments [4]. Despite the antimicrobial properties of tannins, many fungi, bacteria and yeast are quite resistant to tannins and can use tannins as carbon and energy sources; the biodegradation of natural tannins in the environment is mainly associated with fungi rather than bacteria. In particular, *Aspergillus spp.* and *Penicillium spp.* have been observed in tannery wastewaters and have been exploited in the biotransformation of tannins [5]. Fungi are able to express a large number of degradative enzymes among which peroxidases, laccases and tannases. Tannin acyl hydrolase (E.C.3.1.1.20) is commonly referred as tannase and catalyzes the hydrolysis of ester and bonds releasing glucose, gallic acid and galloyl esters. The enzyme finds application in many industrial sectors (i.e. pharmaceutical, food, chemical and beverages industry) and one of the most expensive enzymes since an economically feasible process need to be developed for tannase production [6]. *Aspergillus* tannins-degrading strains (section Nigri) were found in tannery wastewater rich in Quebracho tannin [7] and *Aspergillus* section Nigri are able to grow on tannic acid [8] and express degradative enzymes such as tannase [5]. Tannase activity from fungi in phenols-rich wastewater and the removal of soluble Chemical Oxygen Demand (sCOD), Dissolved Organic Carbon (DOC) and phenols content has been already investigated [9,10]; However, the effect of tannase on condensed tannins has not yet been adequately defined. In this context, the potential of fungi for the

bioremediation of wastewater streams from the tanning industry cannot be exploited until suitable technologies for their selection in a typical wastewater treatment train can be developed. In fact, the main operational concerns are related to bacterial contamination and to the robustness of the bioprocess in the long-term, since fungi are easily outcompeted by bacteria. Currently, a bioreactor able to maintain the stable growth and performance of fungi under sterile and non-sterile conditions is still a challenging task. Nowadays, fungi are rarely applied in environmental biotechnology for wastewater treatment: autochthonous microorganisms of real wastewaters could be a source of microbial stress to fungi, negatively affecting the fungal degradation capacity in non-sterile conditions [11,12]. Bacterial contamination in fungal-based reactors causes, particularly during the start-up period, damage to the fungal mycelium [13] and the reduction or inhibition of fungal enzymes and in general their activity [14]. Bacteria outcompete fungi in a bioreactor for several reasons. Fungi are eukaryotes and their grow rate is therefore intrinsically much slower than bacteria. Fungi prefer an acidic environment as opposed to most bacteria. They are able to brake complex bonds, but sometimes they require the addition of substrates for co-metabolism (co-substrates) [15]. The majority of fungi grow in temperatures ranging from 5 to 35°C, but optimal growth is usually around 25°C. Filamentous fungi are able to grow submerged, but they naturally grow at the water-air interface. For these reasons, it is clear that the typical conditions of conventional WWTPs are unfavourable to fungi. In the literature, multiple strategies have been suggested [11,12] to enhance the resistance of fungi in non-sterile conditions:

- immobilisation of fungal cultures;
- reduction of medium pH;
- limitation of nitrogen in the medium;
- elevated hydraulic retention time (HRT) (1-3 days);
- selective disinfection or microscreens;
- addition of co-substrates;

- fungal inoculation;
- bioreactor configurations;
- starting with mature and well-developed cultures of fungi.

Immobilised mycelia reactors present several advantages. They are more resistant to mechanical stresses and pH changes, have a higher degradation capacity, and minimal clogging is usually observed [16]. Moreover, immobilisation of the fungal biomass is preferable when the effluent includes a toxic pollutant with high concentrations and packed bed long-term stability and efficiency is significant higher than stirred tank reactors [17]. Mixing in a bioreactor plays an important role in oxygen and mass transfer in biofilms and reduces non-uniformity and gradients of relevant parameters such as temperature, pH, gases, and solids. However, mixing also influences the biofilm shape and thickness [18]. Rotating biological contactors with fungal biofilms have been investigated for their removal performance and resistance to microbial stress [19]. Rotation could also control biomass growth in immobilised systems [20], even though fungi, due to their structure, are sensitive to shear stress, and excessive agitation could damage biofilms or pellets. Furthermore, the fungal degradation of most of the recalcitrant compounds frequently require the supply of an external carbon source (co-substrate), since recalcitrant compounds may not be a suitable growth substrate [11,21]. Most research on the biodegradation of natural tannins and polyphenolic compounds by fungi has been performed in petri dishes or flasks: using tea by-products, tannin extracts or on tannic acid. There have been no previous experiments using continuously fed bioreactors (under sterile or non-sterile conditions) for the removal of natural tannins with fungi. Table 1 lists the operational conditions employed in a typical non-sterile fungal reactor used for the treatment of phenol rich wastewaters as olive mill wastewater (OMW) and molasses from wastewater distillery (MW). It is important to design the process conditions by also taking in account the characteristics of the target compounds.

Table 1. Non-sterile fungal bioreactor conditions used for phenol rich wastewaters.

Fungal strains	**Target compound**	**Reactor**	**Carriers**	**Temp. °C**	**pH**	**Co-substrate**	**Removal**	**Ref.**
N. crassa	phenols	MBfR	Membrane	-	-	Sucrose	92-100%	[22]
Fungal consortium	MW	Novel reactor (14 L)	Straw	24 ± 2°C	8.2	Straw	65%	[23]
A. tubingensis	MW	Novel reactor (6 L)	No carriers	20-30°C	4.45	-	90%	[24]
Fungal consortium	phenols	BTF (6.3 L)	chips Pinewood	19-25°C	-	Pinewood	81%	[25]
P. ostreatus	OMW	airlift (5 L)	no carriers	28°C	5	Potato dextrose	95%	[26]

Based on the considerations described above, a novel packed bed reactor type, with the ability to rotate, was designed and built as described in Materials and Methods. The experiment was designed to evaluate the role of specific process parameters: initial inoculum, co-substrate addition, and rotation. The evaluation was aimed at assessing the stability of the process and performance in the removal of the target recalcitrant compounds (tannins) in non-sterile conditions in a bioreactor.

2 Materials and Methods

2.1 Fungus selection and inoculum preparation

The strain tested in this study, *Aspergillus tubingensis* MUT 990, was isolated from commercial tannin powder and it is preserved at the Mycotheca Universitatis Taurinensis collection (MUT, University of Turin, Department of Life Sciences and Systems Biology) on Malt Extract Agar (MEA) at 4 °C. *A. tubingensis* was inoculated in a generic MEA (20 g L^{-1} malt extract, 20 g L^{-1} glucose, 2 g L^{-1} peptone, 20 g L^{-1} agar) plates (150 mm diameter) and incubated at 25°C in a dark for seven days. This fungal stain was selected among other suitable strains with tests in petri dishes and flasks (data not shown). After the incubation, the biomass was used as inoculum for immobilization on carriers [16]. The Polyurethane Foam carriers (PUF) were constituted by cubes of about 2 cm side (specific surface 600 m^2 m^{-3}, density 25 kg m^{-3}, Pores/Volume ratio: 0.97). The cubes were inserted in 1000 mL Erlenmeyer flasks containing 500 mL of GLY medium (5 g L^{-1} Glucose and 1.9 g L^{-1} of Yeast extract) and were autoclaved at 121 °C for 30 min. In each flask 10 mL of fungus homogenate were added. The flasks were cultured in agitated condition at 110 rpm with a shaking table at 25 °C in a dark for seven days. The obtained immobilized fungal biomass was weighted: the initial average dry weight of solids in a PUF cube was 0.2 g and consequently the fungal biomass concentration in the reactors was about 3 g L^{-1} (in each reactor were added 60 PUF cubes). The dry weight was calculated by difference, after 1 hour in an oven at 105 °C, measuring PUF weight in triplicates in presence and absence of fungal biomass.

2.2 Experimental set-up

The reactor design was based on the scientific literature and the outcomes of previous work. PUF cubes were adopted as carriers since they have already been demonstrated as the most suitable based on previous experience [27]. The packed bed reactor type was chosen based on the outcomes of previous work with different substrates [21,28]. The reactor was built with a rotating cage where

the immobilised cubes could be inserted. The cage rotation was a process parameter able to improve the mixing inside the reactor and the biomass growth control due to the shear stress [20]. The submerged configuration was also chosen for safety reasons due to sporulation. The final rotating, submerged, packed bed reactor scheme is represented in Figure 1 and described below.

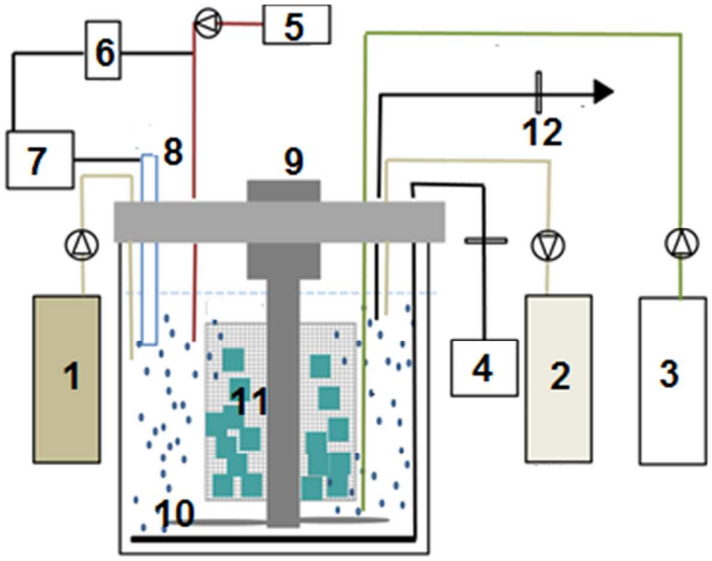

Figure 1. Rotating, submerged, packed bed reactor scheme: 1) medium tank; 2) discharge tank; 3) co-substrate tank; 4) air blower; 5) acid tank; 6) valve; 7) pH control panel; 8) pH probe; 9) engine to allow cage rotation; 10) air diffuser; 11) the metal cage and the PUF cubes; 12) air filter 0.2 µm.

The experimental set-up consisted of four aerated and completely mixed reactors with a volume of 5 L each (total volume, leaving 4 L of effective volume). The vessel had a cylindrical shape with a diameter of 18 cm and a height of 27.5 cm. The four rotating, submerged, packed bed reactors were designed, built and installed in the Cer2co laboratory inside the Cuoiodepur WWTP. The reactors had a cylindrical cage filled with 60 PUF cubes immobilised with the procedure described above. The cage was completely submerged at a liquid level of 4 L, and, consequently, the immobilised fungi were submerged. The cage had a cylindrical shape with a diameter of 5 cm and a height of 18 cm, assembled with a wide open stainless steel grid and perforated stainless steel. Each cage was connected with an engine through a stainless steel rod in the cylindrical axis and was able to rotate.

The engines were brushless (BL018.240 P42/3B, Intecno, Italy), electrically powered by Intecno card BLD07, and were modified to reach a maximum rotation velocity of 6 rpm. Each vessel was covered with a plastic device with nine circular openings along the border for probes and pipes and a central opening for the metal rod that connected the packed bed with the engine. The inlet and outlet flow was controlled by four peristaltic pumps (TEC-R, Aqua, Italy) and controlled by a PLC (LOGO! 230RC, Siemens, Italy). The co-substrate flow rate and the acid solution were controlled by peristaltic pumps (300 series, Watson and Marlow, USA) equipped with a multichannel head. In each reactor, pH was measured with Hamilton pH probes and automatically controlled with a pH controller (M20R-pH) by dosing sulphuric acid at 1 mol L^{-1}. An oxygen concentration close to saturation was ensured by air diffusers located at the bottom of the reactors. The reactors were located in a temperature-controlled room. The DO concentration was measured regularly with a portable galvanic DO sensor (Oxi 340i with CellOx 325, WTW, Germany). Outlet air was filtered at 0.2 µm (for safety reasons). The immobilised fungal biomass obtained was weighed: the initial average dry weight of solids in a PUF cube was 0.2 g and, consequently, the fungal biomass concentration in the reactors was about 3 g L^{-1} (60 PUF cubes were added in each reactor). The dry weight was calculated by subtraction after 1 h in an oven at 105°C, measuring the PUF weight in triplicate in the presence and absence of fungal biomass.

2.3 Process operation

The first experiment was designed to evaluate the role of specific process parameters: initial inoculum, co-substrate addition, and rotation. The evaluation was aimed at assessing the stability of the bioprocess and performance in the removal of the target recalcitrant compounds (tannins) in non-sterile conditions. Non-sterile conditions are simulated in lab-scale studies by using non-sterilised tap water, non-filtered air for aeration, industrial grade chemicals without sterilisation and by allowing direct air-liquid contact inside the reactor. The reactors were fed with synthetic wastewater, and a solution of malt extract (analytic grade) at 1 g L^{-1} was dosed as a co-substrate.

The experiment was also aimed at providing an evaluation of the degradation performance in the presence of a co-substrate for fungal growth [29]. Malt extract as a co-substrate could allow the fungi to inhibit the growth of competitive bacteria [30]. The synthetic wastewater was a mixture of four tannins for a total concentration of 1 g L^{-1} (0.25 g L^{-1} each) in tap water. Chimont International Spa, Montopoli (Italy) provided the industrial tannins (Quebracho, Wattle, Chestnut and Tara powders). These tannins are the most commonly used in the tannery factories in the Tuscany tannery district. All other reagents used in the present study were of analytic grade (Sigma-Aldrich). The operating conditions were designed to enhance the development and degradation capacity of the selected fungal strain: the acidic pH (5-6) of the medium [31], the HRT equal or higher than 24 h [32], and the immobilisation on carriers [33]. The process operation was designed to achieve a pH set point at 5.5 ± 0.2 and HRT of 24 h to enhance the exoenzyme tannase production [34]. The inlet flow of co-substrate medium and synthetic wastewater was equal. Reactor R1 was inoculated with the immobilised PUF cubes, and it was the only reactor without co-substrate dosing. In this way, Reactor R1 served as the control reactor to evaluate the effect of the co-substrate compared to the other reactors. Reactor R2 was filled with empty PUF cubes and an inoculum of 2 mL of activated sludge from the Cuoiodepur WWTP (Pisa, Italy). Even though the activated sludge of tannery WWTP is acclimated to natural tannins, their degradation is poor at the conventionally used WWTP operational conditions. The objective of the test was to evaluate the tannin removal capacity of a WWTP autochthonous biomass in the steady-state conditions of the new reactor configuration (pH set point, co-substrate feeding, etc.) and to compare the performance of reactors inoculated with the selected fungal strain. Reactor R3 was inoculated with the immobilised PUF cubes and was fed with co-substrate. R4 was identical to R3, except that the cage of reactor R4 rotated at 3 rpm. The R4 was used to evaluate the effect of rotation, in terms of tannase production and diffusion in the reactor and natural tannin degradation. The test was conducted for 50 days.

2.4 Analytical methods

The medium and co-substrate solutions were prepared three times a week, the pH and temperature measured with the pH reader (M20R-pH) were recorded. The inlet solutions were without suspended particles. The DOC and sCOD were measured from (filtrated at 0.45 µm) composite samples twice a week and were determined using a DOC and nitrogen analyzer (TOC-L Shimazdu Analyser) and Hach-Lange's probes. The DOC was measured after 20 days of activity. The dissolved oxygen was measured with a Hach-Lange LDO probe. Laccase, manganese peroxidase and the tannase activity were measured. The measurements of enzymes were based on enzymatic oxidation with DMP (2,6-dimethoxyphenol) for laccases and manganese peroxidases [35], while the tannase was measured by incubating the samples and tannic acid and measuring the produced glucose [36]. Blank control and controls on tannin suspensions/solutions were performed.

2.5 Statistical analysis

The sCOD of malt solution at 1 g L^{-1} was 690 mg L^{-1} and the tannins solution (the medium) at 1 g L^{-1} was 720 mg L^{-1}. The average DOC of malt solution at 1 g L^{-1} was 470 mg L^{-1} and the tannins solution (the medium) at 1 g L^{-1} was 540 mg L^{-1}. The standard deviation of these solutions and the outlet samples were less than 10%.

3 Results and discussion

3.1 Bioreactor performance

Bioreactors R1, R2, R3 and R4 operated with continuous feeding for two months under non-sterile conditions. They were stable and able to reach a high removal efficiency (RE) for DOC and sCOD. R1, working in the absence of malt extract as the co-substrate for fungal biomass growth, initially showed good performance with sCOD removal of up to 70-80% (Figure 2).

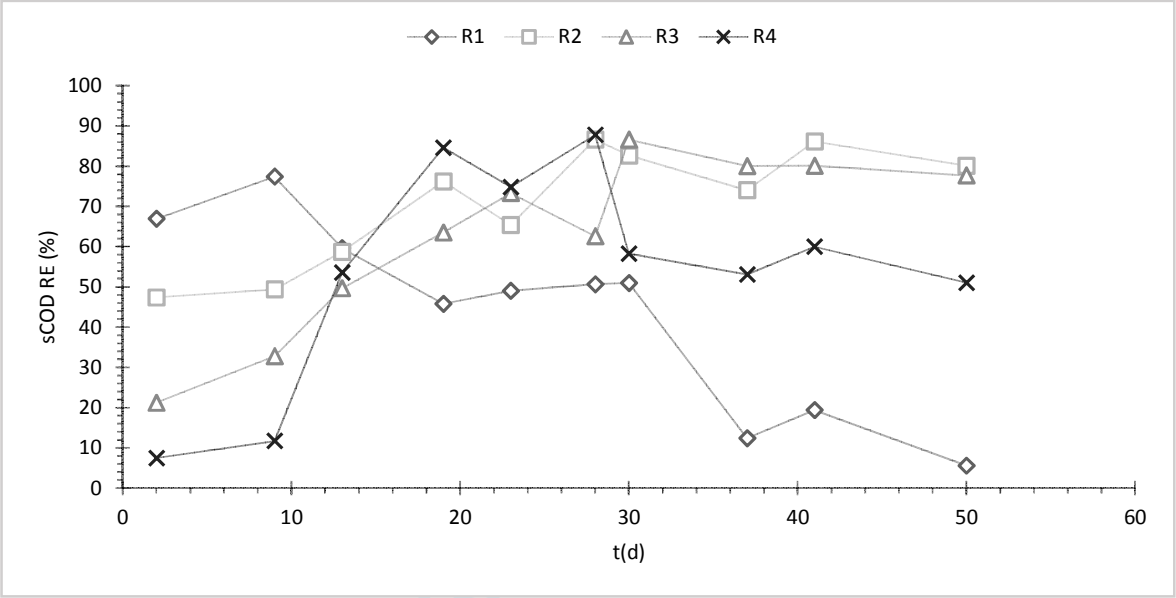

Figure 2. The sCOD RE during preliminary tests with continuous bioreactor operation.

However, after the first ten days of operation, tannin removal was reduced to lower values and the sCOD RE dropped to about 20% in the final period. As shown in the comparison between R1 and R3 (with co-substrate addition), the malt extract dosage was able to support the biomass and the RE. In fact, during the first 10 days of incubation, reactor R3 showed decreased performance with respect to R1. However, within 30 days, R3 achieved a high level of removal performance that was stable (Figure 2 and 3), with 80-90% RE of DOC and sCOD recorded. The observed trend can be explained by an initial fungal growth on malt extract (the co-substrate), with a shift to tannins as the carbon source when the fungal biomass reaches a critical point allowing it to overcome the inhibition of tannins and maintain competitiveness within the microbial community found in the non-sterile environment.

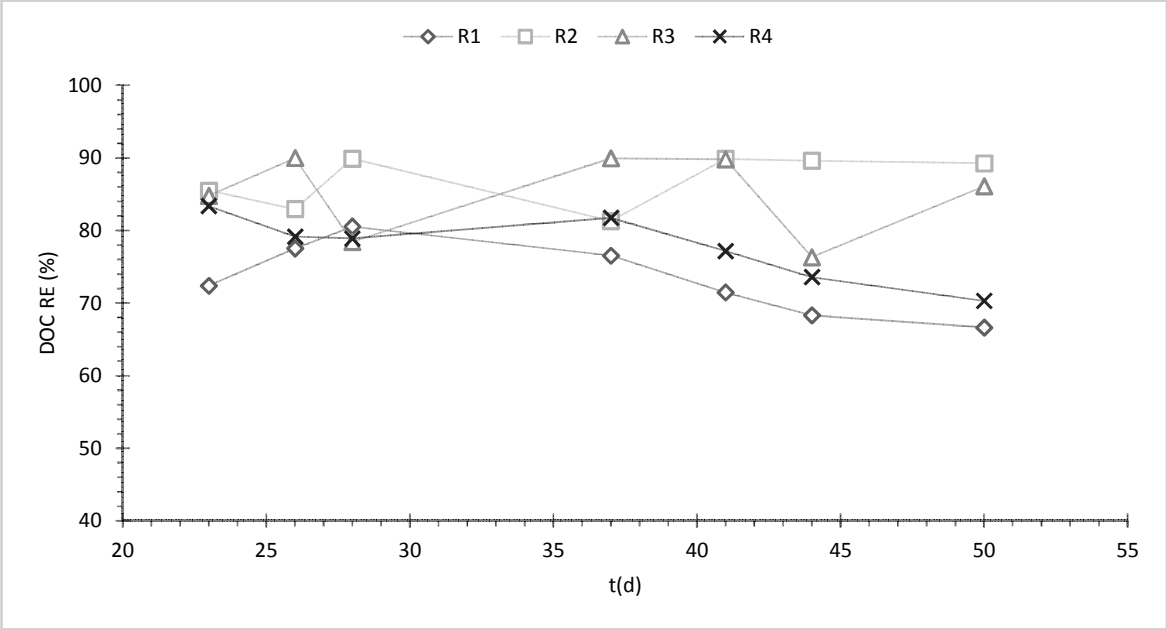

Figure 3. The DOC RE during preliminary tests with continuous bioreactor operation.

At the same time, it was evident that the fungal biomass in R1 showed a significant sCOD RE, suggesting that the fungal strain was a candidate for tannin removal in non-sterile conditions once the growth conditions were optimised. Reactor R4 operated under the same conditions of R3, except for the addition of cage rotation at 3 rpm with respect to R3, which lacked rotation. Reactor R4 showed lower performance at the beginning and at the end of the experiment. However, after 30 days of operation, it showed encouraging sCOD RE percentages (up to 90%) with a similar trend in RE compared to R3. Generally, mechanical stirring devices impact the system by both improving the contact between phases (i.e., air bubbles and microorganisms) and causing damage to cells at high turbulent flow due to shear stress [37]. However, in this case the rotation rate was very low and the fungal mycelium was protected by both the PUF and the metal cage. Thus, the negative stress due to shear forces was negligible and an important decrease in RE was not recorded. Reactor R2 was inoculated with activated sludge and represents the theoretical performance of WWTP autochthonous biomass developed in the operational conditions used in the fungal reactors. As shown in Figure 2, the sCOD RE was stable at approximately 80% after 30 days and, as shown in

Figure 3, the DOC RE was at approximately 90%. The performances obtained were similar to those recorded in reactor R3 inoculated with the selected fungal strain. An inhibitory effect of the tannins on the biomass was evident during the first 10 days of incubation when the RE in R2 was significantly lower than that observed in R1. Results obtained suggested that the fungal strain was more resilient to the eventual inhibition of tannins than WWTP activated sludge, confirming the possibility of exploiting the fungal candidate's resilience.

3.2 Tannase activity

Tannase activity was measured in all reactors as shown in Table 2. On the contrary, neither laccases nor manganese peroxidases were detected in the reactors. Similar results have been described in the literature. Hanafi [9] and Öngen [38] found 70% sCOD removal from OMW and 370-650 AU L^{-1} of tannase activity, with negligible laccase and peroxidase activity. Enzyme measurements revealed a maximum tannase activity of up to 360 AU L^{-1} achieved in R4 at day 9. This high tannase activity recorded in R4 could be partially related to the rotation that improved the tannase diffusion in the liquid. However, shaking conditions have also been demonstrated to improve tannase production by *Aspergillus spp*. [39]. The mechanisms regulating microbial tannase production are currently not well understood. Some authors suggest that gallic acid (a degradation product of tannin degradation) could act as both an inducer and inhibitor of tannase production, depending on its concentration [40]. This could partially explain the behaviour observed in R4, which was inversely proportional to the sCOD RE, with an initial high tannase activity in the first week followed by a decrease. In reactor R2, which was not inoculated with fungi, measurable tannase activity was recorded (ranging from a maximum of 62 to a minimum of 9 AU L^{-1}). However, the presence of tannase activity cannot be considered the only variable in evaluating fungi activity, since some bacteria can also produce tannase [41]. Tannase activity is, nevertheless, an index of the natural presence of organisms with the ability to biotransform tannins via tannase in

activated sludge from tannery wastewater treatment plants. Actually, each wastewater treatment plant selects a typical community of activated sludge able to degrade the pollutants that are present in the plant [42]. The autochthonous microorganisms in R2, on the whole, achieved similar sCOD RE performance, but the enzymatic activity of the selected fungus in R3 is substantially higher. A comparison of the tannase activity in R1 and R3 provides information about the effect of malt extract as a co-substrate in the treatment. The results indicated that the additional source of carbon promoted tannase production, contrary to that observed by other authors [39]. Alternatively, the co-substrate may have supported the growth of the fungal biomass and, potentially, tannase production. Finally, the low enzymatic activity recorded in the first 10 days in R1 and R2 confirms the hypothesis that biosorption was the main process involved in the pollution removal during that initial period.

Table 2. Maximum tannase activity measured in each reactor during preliminary tests with continuous bioreactor operation.

Tannase (AU L^{-1})	**R1**	**R2**	**R3**	**R4**
Day 9	56	49	163	360
Day 23	63	62	109	205
Day 41	119	46	162	145
Day 50	88	9	94	186

3.3 Biomass degradation capacity

Even though *Aspergillus tubingensis* has been successfully used in a biotrickling filter reactor to treat gas with monoethylene glycol [43], to the best of our knowledge no fungal-based bioreactors have been used for the biological degradation on natural tannins (either non-sterile or sterile). A stable system based on a fungal consortium was reached in R3 thanks to the selection of the operating conditions. It is possible to hypothesise that the conditions chosen for R2 allowed for the development of a tannin-degrading microbial consortium, including both fungi and bacteria. Nevertheless, further studies are required to better characterise the organisms developed. It was observed that the fungal biomass changed to a darker colour, probably due to the adsorption of tannins onto the biomass as previously suggested by other authors [38]. Adsorption is a relatively fast phenomenon, and it played a role in removal within the first few days of operation. The recorded decrease of sCOD and DOC at the later sampling times could be plausibly ascribed to biotransformation processes. The ratio between sCOD and DOC allowed insight into whether the biotransformation of tannins occurred. The typical ratios COD/TOC in municipal wastewater is in the range 3.5-2 [44]. The average ratio between sCOD and DOC was 1.48 for the inlet malt extract solution and 1.35 for the inlet tannin solution. However, the ratio between sCOD and DOC in the outlet changed with a higher value (as represented in Table 3), and this behaviour could be related to a tannin depolymerisation.

Table 3. Average ratio between sCOD and DOC in the outlet during the whole testing period of preliminary tests with continuous bioreactor operation.

	R1	**R2**	**R3**	**R4**
sCOD/DOC	2.8 ± 0.6	2.3 ± 0.6	2.5 ± 0.2	2.7 ± 0.6

To summarise, it was possible to run a fungal reactor in non-sterile conditions for two months thanks to the appropriate choice of design and operating conditions. The selected fungal strain seemed able to cope with tannins, but it was hypothesised that the degradation ability of the fungal strain may be different for different tannins. Rotation could be applied to control the biomass retention, but the frequency and duration of the rotation should be evaluated accurately to avoid excessive stress on the biofilm and obtain a fine tuning of the biomass retention time. Malt extract seemed to favour the fungal inoculum and also the development of a microbial consortium bioactive against one or more of the four tannins introduced (given the selected operational conditions and reactor design).

4 Conclusions

The reactor R3 inoculated with *Aspergillus tubingensis* MUT 990 allowed to obtain a stable system in non-sterile condition and promising results in removal of tannins. The co-substrate (pure malt extract) was able to support the fungal growth and improve the performance in long term. The rotations of the cage (R4) seemed improve the diffusion of tannase exoenzymes in the liquid, but do not improve the removal efficiency in the long time (due to the excessive shear stress). It was hypothesised that different tannins may have a different effect on the systems and may influence the consortium. It is necessary to perform further test with each tannin medium and different co-substrates (types and concentrations). In R2 the chosen conditions allowed to develop a microbial consortium able to degrade tannins, but further studies are needed. However, the results obtained in the experiment encourage to apply the tested condition in further tests with real tannery wastewater.

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