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## THE ROLE OF COSUBSTRATE AND MIXING ON FUNGAL BIOFILM EFFICIENCY IN THE REMOVAL OF TANNINS

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5 **THE REMOVAL OF TANNINS**  
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## 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 (*Caesalpinia 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

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

- 22 • immobilisation of fungal cultures;
- 23 • reduction of medium pH;
- 24 • limitation of nitrogen in the medium;
- 25 • elevated hydraulic retention time (HRT) (1-3 days);
- 26 • selective disinfection or microscreens;
- 27 • 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 significantly 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.
<i>N. crassa</i>	phenols	MBfR	Membrane	-	-	Sucrose	92-100%	[22]
Fungal consortium	MW	Novel reactor (14 L)	Straw	24 ± 2°C	8.2	Straw	65%	[23]
<i>A. tubingensis</i>	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]
<i>P. ostreatus</i>	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

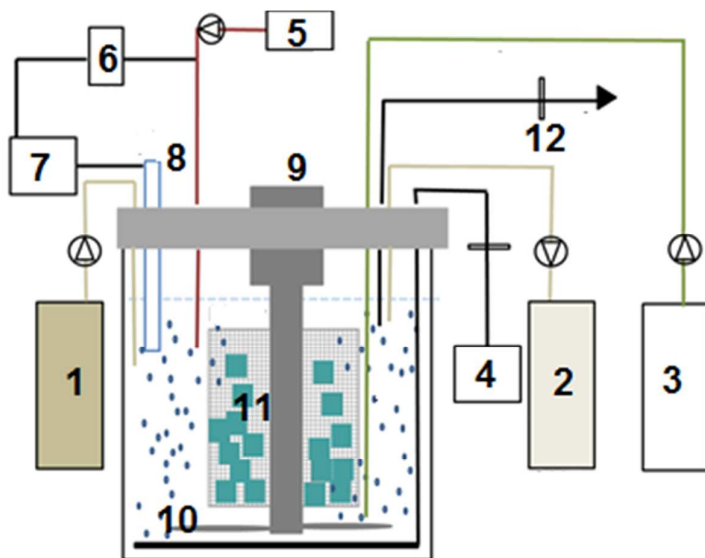
### 2.1 Fungus selection and inoculum preparation

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<sup>-1</sup> malt extract, 20 g L<sup>-1</sup> glucose, 2 g L<sup>-1</sup> peptone, 20 g L<sup>-1</sup> agar) plates (150 mm diameter) and incubated at 25°C in a dark for seven days. This fungal strain 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<sup>2</sup> m<sup>-3</sup>, density 25 kg m<sup>-3</sup>, Pores/Volume ratio: 0.97). The cubes were inserted in 1000 mL Erlenmeyer flasks containing 500 mL of GLY medium (5 g L<sup>-1</sup> Glucose and 1.9 g L<sup>-1</sup> 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<sup>-1</sup> (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  $\mu\text{m}$ .

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.

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

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## 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<sup>-1</sup> was dosed as a co-substrate.

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

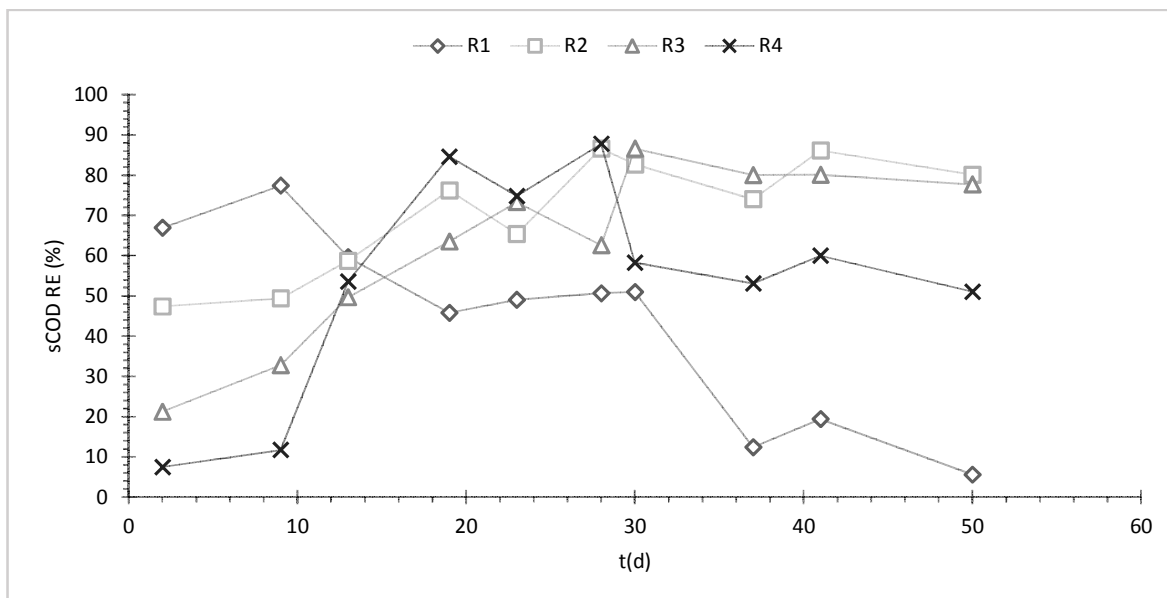
## 2.5 Statistical analysis

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

# 3 Results and discussion

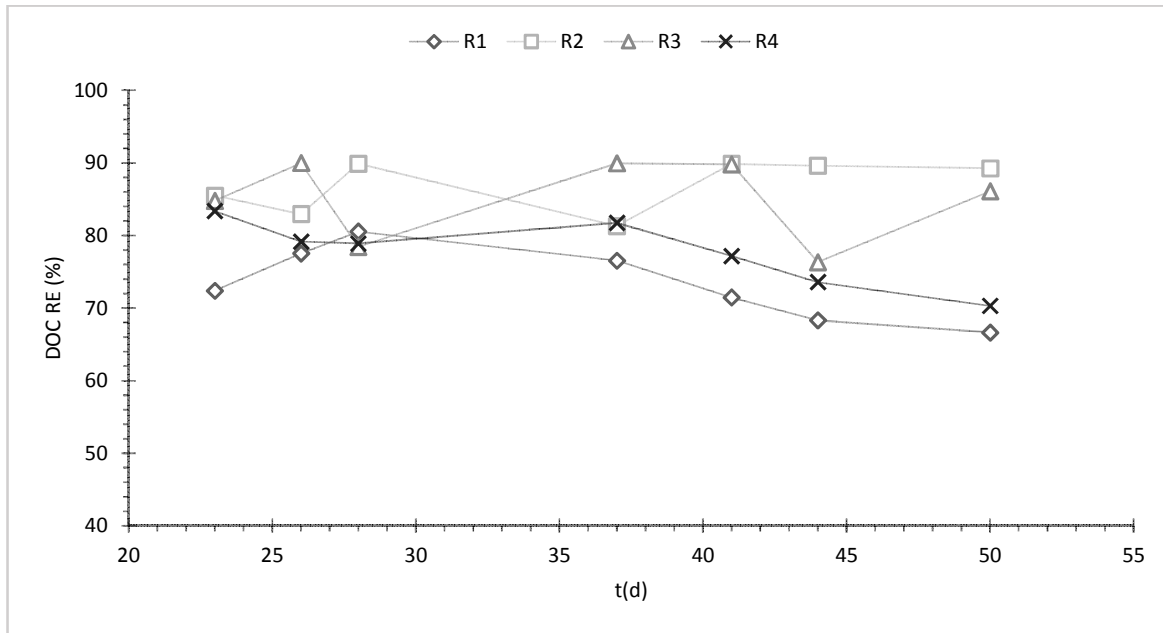
## 3.1 Bioreactor performance

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

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3 Figure 3, the DOC RE was at approximately 90%. The performances obtained were similar to those  
4 recorded in reactor R3 inoculated with the selected fungal strain. An inhibitory effect of the tannins  
5 on the biomass was evident during the first 10 days of incubation when the RE in R2 was  
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7 significantly lower than that observed in R1. Results obtained suggested that the fungal strain was  
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9 more resilient to the eventual inhibition of tannins than WWTP activated sludge, confirming the  
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11 possibility of exploiting the fungal candidate's resilience.  
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### 15 16 17 18 **3.2 Tannase activity** 19

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21 Tannase activity was measured in all reactors as shown in Table 2. On the contrary, neither  
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23 laccases nor manganese peroxidases were detected in the reactors. Similar results have been  
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25 described in the literature. Hanafi [9] and Öngen [38] found 70% sCOD removal from OMW and  
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27 370-650 AU L<sup>-1</sup> of tannase activity, with negligible laccase and peroxidase activity. Enzyme  
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29 measurements revealed a maximum tannase activity of up to 360 AU L<sup>-1</sup> achieved in R4 at day 9.  
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31 This high tannase activity recorded in R4 could be partially related to the rotation that improved the  
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33 tannase diffusion in the liquid. However, shaking conditions have also been demonstrated to  
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35 improve tannase production by *Aspergillus spp.* [39]. The mechanisms regulating microbial tannase  
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37 production are currently not well understood. Some authors suggest that gallic acid (a degradation  
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39 product of tannin degradation) could act as both an inducer and inhibitor of tannase production,  
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41 depending on its concentration [40]. This could partially explain the behaviour observed in R4,  
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43 which was inversely proportional to the sCOD RE, with an initial high tannase activity in the first  
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45 week followed by a decrease. In reactor R2, which was not inoculated with fungi, measurable  
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47 tannase activity was recorded (ranging from a maximum of 62 to a minimum of 9 AU L<sup>-1</sup>).  
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49 However, the presence of tannase activity cannot be considered the only variable in evaluating  
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51 fungi activity, since some bacteria can also produce tannase [41]. Tannase activity is, nevertheless,  
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53 an index of the natural presence of organisms with the ability to biotransform tannins via tannase in  
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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.

<b>Tannase (AU L<sup>-1</sup>)</b>	<b>R1</b>	<b>R2</b>	<b>R3</b>	<b>R4</b>
<b>Day 9</b>	56	49	163	360
<b>Day 23</b>	63	62	109	205
<b>Day 41</b>	119	46	162	145
<b>Day 50</b>	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

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3 To summarise, it was possible to run a fungal reactor in non-sterile conditions for two months  
4 thanks to the appropriate choice of design and operating conditions. The selected fungal strain  
5 seemed able to cope with tannins, but it was hypothesised that the degradation ability of the fungal  
6 strain may be different for different tannins. Rotation could be applied to control the biomass  
7 retention, but the frequency and duration of the rotation should be evaluated accurately to avoid  
8 excessive stress on the biofilm and obtain a fine tuning of the biomass retention time. Malt extract  
9 seemed to favour the fungal inoculum and also the development of a microbial consortium bioactive  
10 against one or more of the four tannins introduced (given the selected operational conditions and  
11 reactor design).  
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#### 23 **4 Conclusions**

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

- [1] Khanbabaee K, Ree T van. Tannins: Classification and Definition. *Nat. Prod. Rep.* . 2001;18:641–649.
- [2] Barbehenn R V., Peter Constabel C. Tannins in plant-herbivore interactions. *Phytochemistry*. 2011;72:1551–1565.
- [3] Lorenz MM, Alkhafadji L, Stringano E, et al. Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. *J. Sci. Food Agric*. 2014;94:963–968.
- [4] Tilli S, Mori G, Mannucci A, et al. Natural tannins for leather treatments : biodegradation by *Penicillium chrysogenum* MUT 4444 on a fixed bed bioreactor. *Proc. "Oxidative Enzym. as Sustain. Ind. Biocatal. Santiago Compost. 14-15 Sept. 2010, Spain. 2010;1–6.*
- [5] Batra A, Saxena RK. Potential tannase producers from the genera *Aspergillus* and *Penicillium*. *Process Biochem*. 2005;40:1553–1557.
- [6] Belur P, Mugeraya G. Microbial Production of Tannase : State of the Art. *Res. J. Microbiol*. 2011;6:25–40.
- [7] León-Galván F, Torres-Pacheco I, Jiménez-Espinoza F, et al. Molecular and biochemical characterization of extracellular tannin acyl hydrolase activity from a mexican isolate of *aspergillus niger*. *J. Sci. Ind. Res. (India)*. 2010;69:942–947.
- [8] Van Diepeningen AD, Debets AJM, Varga J, et al. Efficient degradation of tannic acid by black *Aspergillus* species. *Mycol. Res*. 2004;108:919–925.

- 1  
2  
3 [9] Hanafi F, Mountadar M, Etahiri S, et al. Biodegradation of Toxic Compounds in Olive Mill  
4 Wastewater by a Newly Isolated Potent Strain: *Aspergillus niger* van Tieghem. *J. Water Resour.*  
5 *Prot.* 2013;5:768–774.  
6  
7  
8  
9  
10 [10] Salgado JM, Abrunhosa L, Venâncio A, et al. Combined bioremediation and enzyme  
11 production by *Aspergillus* sp. in olive mill and winery wastewaters. *Int. Biodeterior. Biodegrad.*  
12 2016;110:16–23.  
13  
14  
15  
16  
17 [11] Espinosa-Ortiz EJ, Rene ER, Pakshirajan K, et al. Fungal pelleted reactors in wastewater  
18 treatment: Applications and perspectives. *Chem. Eng. J.* 2016;283:553–571.  
19  
20  
21  
22 [12] Svobodová K, Novotný Č. Bioreactors based on immobilized fungi: bioremediation under  
23 non-sterile conditions. *Appl. Microbiol. Biotechnol.* 2018;102:39–46  
24  
25  
26  
27 [13] Rene ER, Veiga MC, Kennes C. Biodegradation of gas-phase styrene using the fungus  
28 *Sporothrix variecibatus*: Impact of pollutant load and transient operation. *Chemosphere* .  
29 2010;79:221–227.  
30  
31  
32  
33  
34 [14] Libra JA, Borchert M, Banit S. Competition strategies for the decolorization of a textile-  
35 reactive dye with the white-rot fungi *Trametes versicolor* under non-sterile conditions. *Biotechnol.*  
36 *Bioeng.* 2003;82:736–744.  
37  
38  
39  
40  
41 [15] Bardi A, Yuan Q, Siracusa G, et al. Effect of cellulose as co-substrate on old landfill  
42 leachate treatment using white-rot fungi. *Bioresour. Technol.* . 2017;241:1067–1076.  
43  
44  
45  
46 [16] Anastasi A, Spina F, Romagnolo A, et al. Integrated fungal biomass and activated sludge  
47 treatment for textile wastewaters bioremediation. *Bioresour. Technol.* . 2012;123:106–111.  
48  
49  
50  
51 [17] Rodarte-Morales AII, Feijoo G, Moreira MTT, et al. Operation of stirred tank reactors  
52 (STRs) and fixed-bed reactors (FBRs) with free and immobilized *Phanerochaete chrysosporium* for  
53 the continuous removal of pharmaceutical compounds. *Biochem. Eng. J.* 2012;66:38–45.  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 [18] Picioreanu C, Van Loosdrecht MCM, Heijnen JJ. Two-dimensional model of biofilm  
4 detachment caused by internal stress from liquid flow. *Biotechnol. Bioeng.* 2001;72:205–218.  
5  
6  
7 [19] Svobodová K, Petráčková D, Kozická B, et al. The use of the fungus *Dichomitus squalens*  
8 for degradation in rotating biological contactor conditions. *Bioresour. Technol.* 2012;114:241–246.  
9  
10  
11 [20] Spennati F, Mannucci A, Munz GMG. Moving Bed BioTrickling Filter for hydrogen  
12 sulphide removal from gas streams. 2017;61:215–221.  
13  
14  
15 [21] Palli L, Gullotto A, Tilli S, et al. Biodegradation of 2-naphthalensulfonic acid polymers by  
16 white-rot fungi: Scale-up into non-sterile packed bed bioreactors. *Chemosphere* . 2016;164:120–  
17 127.  
18  
19  
20 [22] Luke AK, Burton SG. A novel application for *Neurospora crassa*: Progress from batch  
21 culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme Microb. Technol.*  
22 2001;29:348–356.  
23  
24  
25 [23] Pant D, Adholeya A. Development of a novel fungal consortium for the treatment of  
26 molasses distillery wastewater. *Environmentalist*. 2010;30:178–182.  
27  
28  
29 [24] Watanabe T, Tanaka M, Masaki K, et al. Decolorization and semi-batch continuous  
30 treatment of molasses distillery wastewater by *Aspergillus tubingensis* DCT6. *Water Sci. Technol.*  
31 2009;59:2179–2185.  
32  
33  
34 [25] Ehlers GA, Rose PD. Immobilized white-rot fungal biodegradation of phenol and  
35 chlorinated phenol in trickling packed-bed reactors by employing sequencing batch operation.  
36 *Bioresour. Technol.* 2005;96:1264–1275.  
37  
38  
39 [26] Olivieri G, Marzocchella A, Salatino P, et al. Olive mill wastewater remediation by means  
40 of *Pleurotus ostreatus*. *Biochem. Eng. J.* 2006;31:180–187.  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 [27] Spina F, Romagnolo A, Anastasi A, et al. Selection of strains and carriers to combine fungi  
4 and activated sludge in wastewater bioremediation. *Environ. Eng. Manag. J.* 2012;11:1789–1796.  
5  
6  
7 [28] Anastasi A, Spina F, Prigione V, et al. Scale-up of a bioprocess for textile wastewater  
8 treatment using *Bjerkandera adusta*. *Bioresour. Technol.* 2010;101:3067–3075.  
9  
10  
11 [29] Palli L, Gullotto A, Tilli S, et al. Effect of carbon source on the degradation of 2-  
12 naphthalenesulfonic acid polymers mixture by *Pleurotus ostreatus* in petrochemical wastewater.  
13 *Process Biochem.* 2014;49:2272–2278.  
14  
15  
16 [30] Svobodová K, Petráčková D, Kozická B, et al. Mutual interactions of *Pleurotus ostreatus*  
17 with bacteria of activated sludge in solid-bed bioreactors. *World J. Microbiol. Biotechnol.* 2016;32.  
18  
19  
20 [31] Mohan SK, Viruthagiri T, Arunkumar C. Statistical optimization of process parameters for  
21 the production of tannase by *Aspergillus flavus* under submerged fermentation. *3 Biotech.*  
22 2013;4:159–166.  
23  
24  
25 [32] More TT, Yan S, Tyagi RD, et al. Potential use of filamentous fungi for wastewater sludge  
26 treatment. *Bioresour. Technol.* 2010;101:7691–7700.  
27  
28  
29 [33] Hai FI, Yamamoto K, Nakajima F, et al. Degradation of azo dye acid orange 7 in a  
30 membrane bioreactor by pellets and attached growth of *Coriolus versicolour*. *Bioresour. Technol.*  
31 2013;141:29–34.  
32  
33  
34 [34] Sharma S, Agarwal L, Saxena RK. Statistical optimization for tannase production from  
35 *Aspergillus niger* under submerged fermentation. *Indian J. Microbiol.* 2007;47:132–138.  
36  
37  
38 [35] Liew CY, Husaini A, Hussain H, et al. Lignin biodegradation and ligninolytic enzyme  
39 studies during biopulping of *Acacia mangium* wood chips by tropical white rot fungi. *World J.*  
40 *Microbiol. Biotechnol.* 2011;27:1457–1468.  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 [36] Darah I, Sumathi G, Jain K, et al. Involvement of Physical Parameters in Medium  
4 Improvement for Tannase Production by *Aspergillus niger* FETL FT3 in Submerged Fermentation.  
5 Biotechnol. Res. Int. 2011;2011:897931.  
6  
7  
8  
9  
10 [37] Serrano-Carreón L, Galindo E, Rocha-Valadéz JA, et al. Hydrodynamics, Fungal  
11 Physiology, and Morphology. In: Krull R, Bley T, editors. Filaments in Bioprocesses . Cham:  
12 Springer International Publishing; 2015. p. 55–90.  
13  
14  
15  
16 [38] Öngen G, Güngör G, Kanberoglu B. Decolourisation and dephenolisation potential of  
17 selected *Aspergillus* section Nigri strains - *Aspergillus tubingensis* in olive mill wastewater. World  
18 J. Microbiol. Biotechnol. 2007;23:519–524.  
19  
20  
21  
22 [39] Aboubakr HA, El-Sahn MA, El-Banna AA. Some factors affecting tannase production by  
23 *Aspergillus niger* Van Tieghem. Brazilian J. Microbiol. 2013;  
24  
25  
26  
27 [40] Aguilar CN, Augur C, Favela-Torres E, et al. Induction and repression patterns of fungal  
28 tannase in solid-state and submerged cultures. Process Biochem. 2001;36:565–570.  
29  
30  
31  
32 [41] Vaquero I, Marcobal Á, Muñoz R. Tannase activity by lactic acid bacteria isolated from  
33 grape must and wine. Int. J. Food Microbiol. 2004;96:199–204.  
34  
35  
36  
37 [42] Shchegolkova NM, Krasnov GS, Belova AA, et al. Microbial community structure of  
38 activated sludge in treatment plants with different wastewater compositions. Front. Microbiol.  
39 2016;7:1–15.  
40  
41  
42  
43 [43] Esmaeili A, Loghmani K. Removal of Monoethylene Glycol from Gas Field Wastewater  
44 Using *Aspergillus tubingensis* and a New Bioreactor. Waste and Biomass Valorization.  
45 2016;7:151–156.  
46  
47  
48  
49 [44] Henze M, van Loosdrecht MCM, Ekama GA, et al. Biological Wastewater Treatment . IWA  
50 Publishing; 2008.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60