Removal of Quebracho and Tara tannins in fungal bioreactors: Performance and biofilm stability analysis

REMOVAL OF QUEBRACHO AND TARA TANNINS IN FUNGAL BIOREACTORS: PERFORMANCE AND BIOFILM STABILITY ANALYSIS

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Abstract

Tannins are polyphenolic compounds produced by plants that are used in the vegetable tanning of leather at industrial scale. Quebracho tannin and Tara tannin are intensively used by the tanning industry and are two of the most recalcitrant compounds that can be found in tannery wastewaters. In this study two reactors fed with Quebracho tannin and Tara tannin, respectively, were inoculated with polyurethane foam cubes colonized with a fungal strain biofilm of *Aspergillus tubingensis* MUT 990. A stable biofilm was maintained in the reactor fed with Quebracho tannin during 180 days of operation. Instead, biofilm got detached from the foam cubes during the start-up of the reactor fed with Tara tannin and a bacterial-based suspended culture was developed and preserved along the operational period (226 days). Soluble chemical oxygen demand removals up to 53% and 90% and maximum elimination capacities of 9.1 g sCOD m^{-3} h^{-1} and 37.9 g sCOD m^{-3} h^{-1} of Quebracho and Tara tannins, respectively, were achieved in the reactors without the addition of co-substrates. Next generation sequencing analysis for bacteria and fungi showed that a fungal consortium was developed in the reactor fed with Quebracho tannin while fungi were outcompeted by bacteria in the reactor fed with Tara tannin. Furthermore, Quebracho and Tara tannins were successfully co-treated in a single reactor where both fungi and bacteria were preserved.

Keywords: recalcitrant compounds; tannins removal; non-sterile conditions; fungal reactor; biomolecular analyses.

1 Introduction

Tannins are abundantly applied in leather tanning industry due to their prerogative of precipitating proteins (Lorenz et al., 2014). In fact, tannins blind to the collagen proteins of the animal skin making leather more durable and not putrescible. Tannins are water-soluble polyphenolic compounds produced by plants (Barbehenn and Peter Constabel, 2011) and could be subdivided into hydrolysable (gallotannins and ellagitannins), condensed (or proanthocyanidins) and complex tannins (Khanbabaee and Ree, 2001). Tara tannin (TT) is obtained from the fruit pods of *Cæsalpinia spp*., which principal components are hydrolysable tannins based on a galloylated quinic acid structure (Sciences et al., 2007). Quebracho tannin (QT) is obtained from the wood of *Schinopsis spp*. and is composed mainly of condensed tannins that are more difficult to be biodegraded than those found in TT given that hydrolysable tannins are easier to be biodegraded (He et al., 2008). Conventional activated sludge systems (Li et al., 2009) and anaerobic digestion (Mannucci et al., 2010) are not effective to treat tannins due to their low biodegradability and high soluble chemical oxygen demand (sCOD) (Lofrano et al., 2013). In fact, high concentration of tannins can inhibit the biological treatment (Munz et al., 2009). Then, tannins are usually removed by means of chemical processes in tannery wastewater treatment plants (WWTPs).

Although the leather tanning industry is known to be of prime economic importance in many countries, the concern about the environmental impacts related to the production of leather and the release of various recalcitrant pollutants in tannery wastewaters (Lofrano et al., 2013; Romer et al., 2011) has been increasing in the last decades. Current biological wastewater treatments are engineered biological ecosystems based on bacteria, which are ineffective in the removal of several recalcitrant compounds such as tannins (Mannucci et al., 2010). Fungi play a role in the biodegradation of phenols, chlorinated phenolic compounds, chlorinated alkanes and alkenes, polycyclic aromatic hydrocarbons, petroleum hydrocarbons and other emerging contaminants (Harms et al., 2011). Although fungi represent a promising biological resource in environmental

biotechnology, they have rarely been applied in wastewater treatment due to a lack of knowledge regarding the optimal process conditions and due to their lack of stability under non-sterile conditions (Espinosa-Ortiz et al., 2016). Most research in wastewater treatment with fungi has been focused on the degradation of pharmaceuticals, dyes and pesticides at lab-scale, with important limitations and, above all, mostly under sterile conditions (Svobodová and Novotný, 2017). In fact, the main operational concerns are related to bacterial contamination and to the robustness of the bioprocess in the long-run since fungi are easily outcompeted by bacteria. Nowadays, operation of a fungal-based bioreactor able to maintain stable fungal growth and performance, under sterile and non-sterile conditions, is still challenging. Furthermore, the fungal degradation of most recalcitrant compounds frequently requires the supply of an external carbon source (co-substrate), since recalcitrant compounds might not be suitable substrates for fungal growth (Palli et al., 2016). Nevertheless, among recalcitrant compounds present in tannery wastewaters, tannins represent a potential carbon source for fungi, despite their antimicrobial properties (Mingshu et al., 2006). Alternative biological treatment processes able to effectively remove this fraction could lead to environmental and economical advantages (Giaccherini et al., 2017). Moreover, *Aspergillus* spp. and *Penicillium* spp. have been isolated in tannery wastewaters and reported as microorganisms capable to biodegrade tannins (Murugan et al., 2007). In particular, *Aspergillus* (section Nigri) can grow on tannic acid (TA) as carbon source and was found in tannery wastewaters with high QT concentration (León-Galván et al., 2010). Based on this findings, it can be hypothesised that fungi would be able to grow in the recalcitrant fraction of wastewater containing a high concentration of tannins, such as tannery wastewater. Most of the research reported in the literature about the biodegradation of natural tannins and polyphenolic compounds by fungi has been performed in petri dishes or flasks using tea by-products (Ni et al., 2015), tannin extracts (Belmares et al., 2009) or on TA (Van Diepeningen et al., 2004). To the authors' knowledge, no previous experiments have been reported with continuously fed bioreactors (under sterile or non-sterile conditions) for the removal of QT, TT or other natural tannins with fungi.

The aims of the present work were i) to demonstrate the proper performance ii) to evaluate the stability and iii) to assess the bacterial and fungal diversity of a novel fungal bioreactor configuration operated under non-sterile conditions to reach an effective removal of Tara and Quebracho tannins. TT was selected as a representative of hydrolysable tannins and QT as a representative of condensed tannins, among the natural tannins applied as tanning agents. The evolution of the system was evaluated by means of combined physical-chemical analyses and molecular techniques for bacteria and fungi. Almost no works in literature have analysed both communities and their relationship during the treatment of tannins in bioreactors.

2 Materials and methods

2.1 Fungal strain, immobilization on supports and reagents

Based on previous tests (Tigini et al., 2015) and literature research, *Aspergillus tubingensis* MUT 990, a black *Aspergillus* belonging to the section Nigri, was chosen as the inoculum for the present study. This fungal strain was originally isolated from commercial TT powder and is preserved at Mycotheca Universitatis Taurinensis (MUT) on malt extract agar (MEA) (agar 20 g, glucose 2 g, malt extract 2 g, peptone 0.2 g, water up to litre) at 4°C. The selected fungal strain was inoculated on 20 generic MEA plates (150 mm diameter) and incubated at 25°C in the dark for seven days. After incubation, the fungal colonies were cut to obtain a homogenate. For each cm^2 of mycelium, 1 mL autoclaved distilled water was added (Anastasi et al., 2012). Polyurethane foam (PUF) cubes were chosen as carriers (Spina et al., 2012). PUF carriers were cubes of about 2 cm per side (density 25 kg m^{-3}, pores/volume ratio: 0.97 and specific surface 600 m^2 m^{-3}), which were inserted in six 1L-Erlenmeyer flasks (35 cubes each) containing 500 mL of GLY medium (5 g L^{-1} of glucose and 1.9 g L^{-1} of yeast extract) and autoclaved at 121°C for 30 min. Afterwards, 10 mL of fungal homogenate was added to each flask. Flasks were shaken in the dark for seven days at 110 rpm and 25°C. This procedure allowed the immobilisation of the selected fungal strain onto the PUF. The QT and TT commercially used for tanning were kindly provided by Chimont

International Spa, Montopoli (Italy). All other reagents used in the present study were of analytical grade (Sigma-Aldrich).

2.2 Analytical methods

The concentrations of dissolved organic carbon (DOC), COD and sCOD in the liquid samples collected from the system were determined using a TOC-TN analyser (TOC-L series, Shimazdu Analyser, Japan) and Lovibond COD cuvettes, respectively. The COD and sCOD measurements were performed immediately after sampling. DOC was measured once the samples were filtered and frozen at -20°C. DOC and sCOD were measured after the samples were filtered with 0.45 µm acetate filters.

Dry mass content in the colonized PUF cubes was periodically determined. The procedure followed consisted of drying a colonized PUF cube collected from the reactors at 105°C until constant weight (W_1). Then, the dried colonized PUF cube was submerged into a commercial solution of sodium hypochlorite and then in ultrapure water to obtain a clean PUF. The clean PUF was dried again at 105ºC until constant weight (W_2). The difference between W_1 and W_2 was the dry mass contained in 1 cm^3 of colonized PUF cube. Total suspended solids (TSS) in the liquid influent and effluent from both reactors were measured according to standard methods (APHA-AWWA-WPCF, 2005).

2.3 Experimental set-up

Two submerged, packed bed reactors were designed, built and installed at the Autonomous University of Barcelona (GENOCOV laboratories). Each reactor consisted of a 5 L vessel (4 L of effective volume and 1 L of headspace) equipped with pH control. NaOH (1M) and HCl (1M) solutions were dosed to maintain a pH of 5.8 ± 0.2 during the reactors operation. The acidic environment is generally more favorable for growth and metabolic activities of fungal strains and in particular for *Aspergillus*, as reported by Mohan et al. (2013).

Air was injected through a stone diffuser and manually controlled with a rotameter (100 NL h^{-1}). Outlet air was filtered at 0.2 µm for safety reasons. The air flow allowed for complete mixing inside the reactor. Inside the vessel, a submerged plastic cylindrical cage was inserted containing 100 immobilised PUF cubes. The cage had a radius of 5 cm and a square grid with 1.25 cm sides. Probes for continuous pH, dissolved oxygen (DO) and temperature measurements were located inside the vessels. DO, temperature and pH were measured with a galvanic DO sensor (Oxi 340i with CellOx 325, WTW, Germany) and pH electrodes (53 33, Crison, Spain). Temperature was maintained at 23 ± 2°C (room temperature) and the DO was maintained above 6.5 mg L^{-1} to avoid oxygen limitation. Liquid samples were also collected from the reactors and feeding tanks three times per week to analyse, sCOD and DOC. A computer and a SCADA system developed in-house were used for data acquisition and pH regulation. A schematic of the reactor is shown in Figure 1.

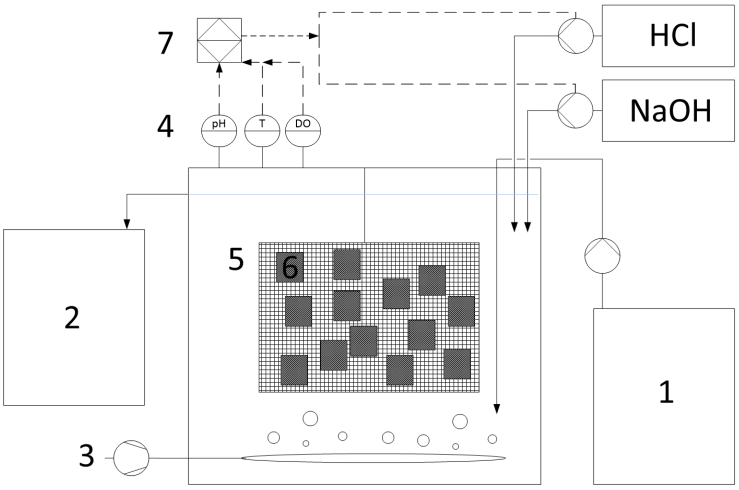


Figure 1. 1) feeding tank; 2) discharge tank; 3) aerator and air sparger; 4) pH, temperature and DO probes; 5) plastic cage containing PUF carriers; 6) PUF cubes; 7) computer.

2.4 Process operation

As previously mentioned, tests were carried out in two submerged, packed bed reactors run in parallel. Both reactors were fed with medium solution containing 1 g L^{-1} tannin (each reactor was fed with a different tannin), 0.1 g L^{-1} NH_4Cl and 0.01 g L^{-1} KH_2PO_4 dissolved in tap water. QT and TT were selected as the most representative recalcitrant compounds of condensed and

hydrolysable tannins contained in tannery effluents (Romer et al., 2011; Tilli et al., 2010). The reactor degrading QT (RQ) was initially fed with a sCOD concentration of 1433 ± 56 mg L^{-1} (1 g QT L^{-1}) while the reactor degrading TT (RT) was initially fed with a sCOD of 786 ± 67 mg L^{-1} (1 g TT L^{-1}). Both concentrations of COD corresponded to the same concentrations of soluble COD (sCOD) due to the absence of particulate COD (pCOD) in the medium. In RQ the biofilm was stable and active for five months of operation under non-sterile conditions leading to an average outlet TSS concentration of 0.03 g L^{-1} which means that the outlet COD was mainly sCOD. The pCOD was less than 4% of the total COD and likely composed of detached biomass. In addition, the ratio between sCOD and DOC at the reactor inlet was determined to be 3.02 ± 0.15. Unfortunately, DOC measurements in the effluent were not reliable due to an unexpected coagulation phenomenon that occurred after samples storage at -20°C. The hydraulic retention time (HRT) and tannin load (measured as volumetric organic loading rate or OLR) were varied during the operation in both reactors to study their performance in terms of elimination capacity (EC) and removal efficiency (RE). HRT values ranging from 52h to 9h were set by modifying inlet and outlet flows with peristaltic pumps (300 series, Watson and Marlow, USA). Tannin concentrations ranging from 0.1 to 8 g L^{-1} were also tested by changing the tannin concentration in the medium (see Table 1 and Table 2).

Despite the fact that both reactors were run in parallel, the operating conditions tested were different due to the completely different behaviour of the bioreactors. This fact led to design an independent experimental plan per reactor. Regarding to the RQ, different QT concentrations were tested to optimise the RE (Table 1) since experiments performed under different HRT in RQ were not effective (data not shown). Then, only data corresponding to the lowest and highest HRT tested in the RQ are presented. On the other hand, during the start-up of the RT the biofilm got completely detached from the PUF cubes and consequently a second start-up was required. Since similar results were obtained in the first start-up and the second one (see Section 3), the cage was removed and

different HRT and TT concentrations were tested. Then, the RT performance and the inhibition caused by the presence of TT on the suspended biomass could be assessed (Table 2). After 154 days of operation, the simultaneous treatment of QT and TT was tested in RQ (see Table 1) which was fed with a medium containing 1 g TT L^{-1} and 0.1 g QT L^{-1} (corresponding to a total inlet sCOD of 879 ± 63 mg L^{-1}).

Table 1. Operating conditions of RQ fed with QT.

Time (days)	0	56	84	100	106	113	141	154
HRT(h)	28	52	52	52	28	28	28	28
QT concentration (g QT L^{-1})	1	1	0.5	1	0.5	0.2	0.1	0.1*
OLR (g COD m^{-3} h^{-1})**	51.1	27.5	12.9	27.5	24.0	7.2	3.6	31.0
COD (mg O_2 L^{-1})	1,433	1,433	672	1,433	672	202	104	869

(*) the composition of the medium was 1 g TT L^{-1} and 0.1 g QT L^{-1} (**) OLR is the volumetric organic loading rate

Table 2. Operating conditions of RT fed with TT.

Time (days)	0	150	155	159	164	169	174	179	187	201	211
HRT(h)	28	52	28	16	12	9	52	28	28	28	28
TT concentration (g TT L^{-1})	1	1	1	1	1	1	1	1	2	8	1
OLR* (g COD m^{-3} h^{-1})	27.9	15.0	27.9	48.9	65.2	87	15.0	27.9	55.9	223.9	15.0
COD (mg O_2 L^{-1})	786	786	786	786	786	786	786	786	1,570	6,270	786

(*) OLR is the volumetric organic loading rate

2.5 Adsorption of tannins on the fungi mycelium

The adsorption capacity of QT and TT on the mycelium was determined in 1-week flask tests. The flasks were filled with 100 mL of tannins (QT and TT separately) plus two colonized and

autoclaved PUF cubes (with the same liquid/PUF ratio obtained in the bioreactors) and stored with agitation in an orbital shaker for one week. sCOD was measured per triplicate after 24 h, 72 h and 168 h (less than 15% of the total volume of the liquid was sampled). Flasks without PUF were used as control. The adsorption of QT and TT to the biomass was assessed considering that part of the sCOD was adsorbed to the flasks and clean PUF cubes (adsorption obtained in control flasks) and part of the sCOD was adsorbed to the inactivated biomass. Indeed, the autoclaved biomass generally show higher adsorption yields with respect to the living one, because of the heat inactivation cause the cell wall breakdown and, thus, the exposition of the inner binding sites (Casieri et al., 2008). It must be pointed out that the measure of COD also was substituted by the measure of sCOD to avoid the effect of the fungal biofilm detachment.

2.6 Study of tannins biodegradability with respirometric tests

To estimate the biodegradable sCOD fractions (sbCOD) of QT and TT, a medium solution containing 10 g tannins L^{-1} was prepared, filtered (filter pore of 0.45 µm) and spiked in a respirometer (MARTINA, Spes Srl, Italy) containing activated sludge. An LFF mode was set to perform the respirometric tests which means that the oxygen concentration was measured in the liquid phase (L) and that both liquid and gas phases were flowing (FF). The activated sludge used to run the respirometric tests was obtained from Cuoiodepur tannery WWTP (Pisa, Italy) to ensure its acclimation to tannins. A conventional procedure described elsewhere (Andreottola and Esperia, 2001) was applied for COD fractioning.

2.7 Metagenomic DNA analysis

Fungi and bacteria communities were analysed from the biofilm developed in the PUF cubes contained in RQ after 50 days of operation (RQ-28h). Suspended fungi and bacteria communities developed in RT were also analysed under steady-state conditions on days 159 (RT-28h) and 174 (RT-9h), corresponding to HRTs of 28 h and 9 h, respectively.

As mentioned in section 2.3, different biomass was developed in each reactor depending on the tannin used as substrate. Then, the biofilm immobilized in a PUF cube (RQ) as well as the suspended biomass (RT) were collected and washed with a phosphate buffered saline solution (0.01 M P-PO_4^{3-}). Afterwards, the samples were centrifuged at 8,000 rpm (Thermo Scientific Hareus Pico17, USA) and the supernatants were discharged. The total DNA of the pelleted biomass was extracted using a MoBio PowerBiofilm® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer instructions. DNA quality and quantity were measured using a NanoDrop® spectrophotometer (Thermo-Scientific, USA). A total of 50 ng of DNA was used for the production paired-end libraries and for the sequencing of the 16S and ITS2 fragments. The primers used are reported in Table 3. The resulting paired-end libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Bio-tek) and pooled in equimolar amounts according to the Qubit dsDNA high-sensitivity (HS) assay. The pools were sequenced using Illumina MiSeq PE300 run. The raw data obtained from sequencing was de-multiplexed, and the sequences of the primers were removed. The fastq files were assembled using a PEAR assembler (Zhang et al., 2014), followed by a quality check using BBmap tools and FastQC (Bushnell, 2014). Sequences with a length lower than 300 bp and with a Phred quality score lower than 20 were removed. Chimera sequences were removed using UCHIME (Edgar, 2016). The 16S sequences were analysed using QIIME pipeline (Caporaso et al., 2011) in order to obtain the Operational Taxonomic Units (OTUs) and to analyse the alpha diversity. The Ribosomal Database Project (RDP) 16S and the UNITE ITS database were used as reference databases in the selection step of the OTUs, performed by the SortMeRNA methods with a 97% threshold. The OTUs identified with low-confidence, related to less than 0.1% of the reads (0.1% is the estimate index miss-assignment on Illumina Miseq), were removed. The numbers of high-confidence sequences were normalised by using the Deseq2 method. The Shannon, Chao and Simpson alpha-diversity indexes were calculated by using QIIME and Mothur to characterise the microbial diversity.

Table 3. Primers used to amplify 16S and ITS2 regions to sequence bacterial and fungal rRNA**.**

16 S	515F: 5'- GTG CCA GCM GCC GCG GTA A -3' 909R: 5' - CCG TCA ATT YHT TTR AGT - 3'
ITS2	ITS4: 5' - TCCTCCGCTTATTGATATGC - 3' ITS3: 5' - GCATCGATGAAGAACGCAGC - 3'

2.8 Statistical analysis

The standard deviation of sCOD for the inlet solutions of QT was 0.04% and the standard deviation of sCOD for the inlet solutions of TT was 0.08%. The sCOD in the outlet was measured in triplicate only in few samples since the reactor was running as a long term experiment and not as a flask test. The standard deviation of the outlet samples for both reactors were less than 5%.

3 Results and discussion

3.1 Performance of Quebracho tannin degrading reactor

Despite some oscillations occurred in the first 56 days, the sCOD-RE was ranging from 4% to 34% which was in average (17% ± 7%) higher than that determined from respirometric tests carried out with an activated sludge sample collected from a tannery WWTP (sCOD-RE of 8%) as shown in Figure 2. This difference was related to the fungal biomass inoculated, which was more effective in QT removal than the autochthonous species of the activated sludge from the tannery WWTP. It may also depend on the chosen operating conditions of the reactor (acidic pH vs neutral pH), which could have influenced the performance of the inoculated fungal strain and the microbial communities developed during reactor operation. The operating conditions chosen generated enough selective pressure in the long-term to drive the evolution of the ecosystem towards an increase in the diversity of fungi, as described in a subsequent section.

As shown in Table 1, after a start-up phase of 56 days, the HRT was increased from 28 to 52 h maintaining the inlet COD concentration constant in order to verify if a longer contact time could improve the low RE obtained during this phase.

However, this strategy did not enhance the QT-RE, which pointed at limited removal probably due to biomass inhibition and not due to low QT biodegradation kinetics, as was initially hypothesized. The average RE obtained during this period was 12%. From day 100 on, the inlet concentration of QT was progressively reduced from 1 to 0.1 g QT L^{-1} (Table 1). As shown in Figure 2, as soon as the inlet concentration was reduced to 202 g COD m^{-3} (equivalent to 0.2 g QT L^{-1}), the RE started to increase, thus confirming the inhibition of process culture by the presence of QT. After 141 days of operation, a further inlet QT concentration decrease was set which allowed reaching a sCOD-RE increase up to 53% ± 13% in the steady-state and an optimal EC of 9 g sCOD m^{-3} h^{-1}. This result confirmed that the biomass was inhibited by QT, which is in agreement with previous works performed at lab-scale with fungi and bacteria using antifouling coatings based on tannins (Bellotti et al., 2012; He et al., 2008).

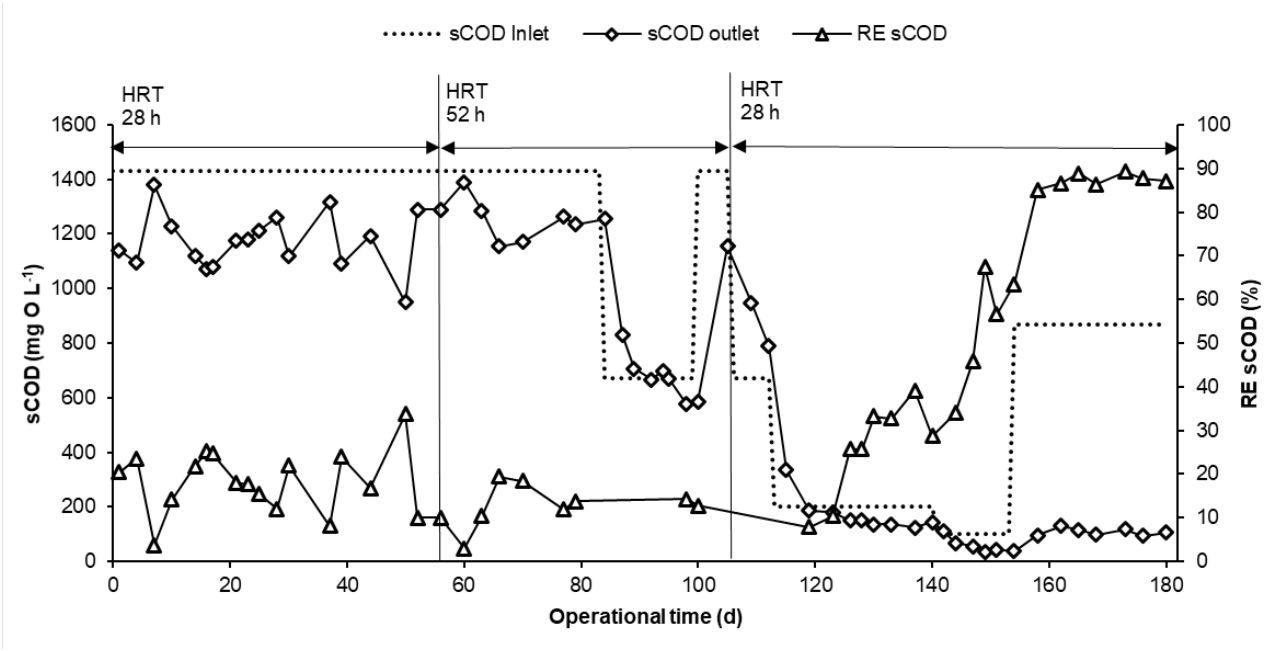


Figure 2. Inlet and outlet sCOD and sCOD removal percentage during continuous treatment in bioreactor RQ.

It is worth mentioning that some PUF cubes were collected from the reactor after 24 h of operation and cut into two halves. The cross section showed that diffusion and adsorption of tannins occurred from the external layers of the PUF cube to the inner layers. In order to assess the role of adsorption in COD removal, the adsorption of QT on mycelium was estimated with flask tests using colonized and autoclaved PUF cubes at the same volumetric ratio of liquid/PUF set in the reactor. After 72 h, the adsorption equilibrium was reached with a total sCOD adsorbed of 31%. Then, adsorption was considered as a relevant phenomenon only at the beginning of the reactor operation as well as right after each change of influent load (either flow rate or inlet concentration changes). However, adsorption was considered negligible with respect to the observed COD removal once the system reached a new steady-state (at least 5-6 days after each stepwise inlet change performed at 28 h HRT).

The biomass content of the reactor was estimated on the basis of the dry mass inside PUF cubes. The dry mass inside PUF increased for 100 days from an initial value of 0.03 g to 0.1 g of dry mass per cube. Afterwards, dry mass content in each PUF cube increased slowly until the end of the experiment when a dry mass content of 0.12 g per PUF cube was obtained. The sCOD balance during the first 100 days of operation (considering the amount of biomass grown in that period) lead to an observed yield coefficient of 0.19 g COD g^{-1} COD. This value was lower than the value reported in the literature for *Aspergillus niger* (this strain belongs to section Nigri) grown in glucose which is 0.37 g dry mass g $glucose^{-1}$ (which would correspond to approximately 0.51 g COD g^{-1} COD) (Viniegra-González et al., 2003). This result was somehow expected since the higher complexity of tannins compared to glucose probably caused less growth efficiency.

3.2 Performance of Tara tannin degrading reactor

Start-up of RT was performed as the start-up of RQ. However, detachment of the fungal biofilm and growth of the biomass in suspended form were observed during the first two weeks. A second start-up was performed with fresh *Aspergillus tubingensis* MUT 990 to verify the

repeatability of the observed phenomenon. The same results were obtained; after two weeks the PUF cubes were almost devoid of biofilm (the dry mass inside the PUF cubes was below the detection limit of the measurement procedure). The fungal biofilm detachment may have been caused by bacterial proliferation, which could create competition for the substrate and also damage the mycelium (Espinosa-Ortiz et al., 2016). Since the biomass was in suspended form, the support medium was removed, and the reactor became a standard continuous stirred tank reactor (CSTR). Despite the detachment, the performance of the reactor was promising. After 20 days of operation, a stable 90 ± 2% sCOD-RE was achieved (which corresponded to an EC of 40 g sCOD m^{-3} h^{-1}), much higher than the sCOD-RE of 19% found with respirometric tests using activated sludge from a tannery WWTP. Though TT (as other common tannins) has toxic or inhibiting effects on various organisms, some bacteria and yeast have the ability to degrade it. Since gallotannins, one of the main component of TT, are among the easiest tannins to be biodegraded, it is reasonable to think that there was a fast development of naturally selected suspended biomass in RT with an active role in TT biotransformation (Mingshu et al., 2006). The hypothesis that bacteria were the main agent responsible for TT degradation was further confirmed in the microbial diversity analysis (see Section 3.4).

As mentioned in Section 2 (Materials and Methods), the reactor performance was evaluated under different tannin loads. Firstly, the HRT was reduced stepwise from 52 h to 9 h while maintaining the inlet TT concentration (experiments beginning after 154 days of operation). Secondly, the HRT was set at 28 h (experiments beginning after 179 days of operation), and the inlet concentration was increased stepwise up to 8 g L^{-1} of TT (Table 2).

Performance was assessed once the steady-state was reached at each step (i.e. at least four times the HRT). It was observed that at an HRT of 9 h, the biomass was almost washed out and the sCOD-RE dropped to 43% (Figure 3). In addition, the ratio between sCOD and DOC in the inlet and outlet

were 2.80 ± 0.30 and 2.69 ± 0.44, respectively, which was another indicator of the large degree of mineralisation of TT. Similar results were obtained in terms of DOC (data not shown). As mentioned above (Section 2), the performance of the RT was also assessed by setting different inlet concentrations in the medium (1, 2, and 8 g TT L^{-1}). Inhibition was observed mainly over an inlet TT concentration of 2 g L^{-1}, in which the RE decreased from 90% to 62%. The system was almost completely inhibited at 8 g L^{-1}. No toxicity was observed, since the RE recovered after day 210 to the initial RE of 90% when the inlet concentration was again reduced to 1 g L^{-1}. In fact, the reported minimum inhibitory concentrations (MICs) of TT for most bacteria is up to 1 g TT L^{-1} (Aguilar-galvez et al., 2014). The optimal operating conditions of the system were obtained at 1 g TT L^{-1}.

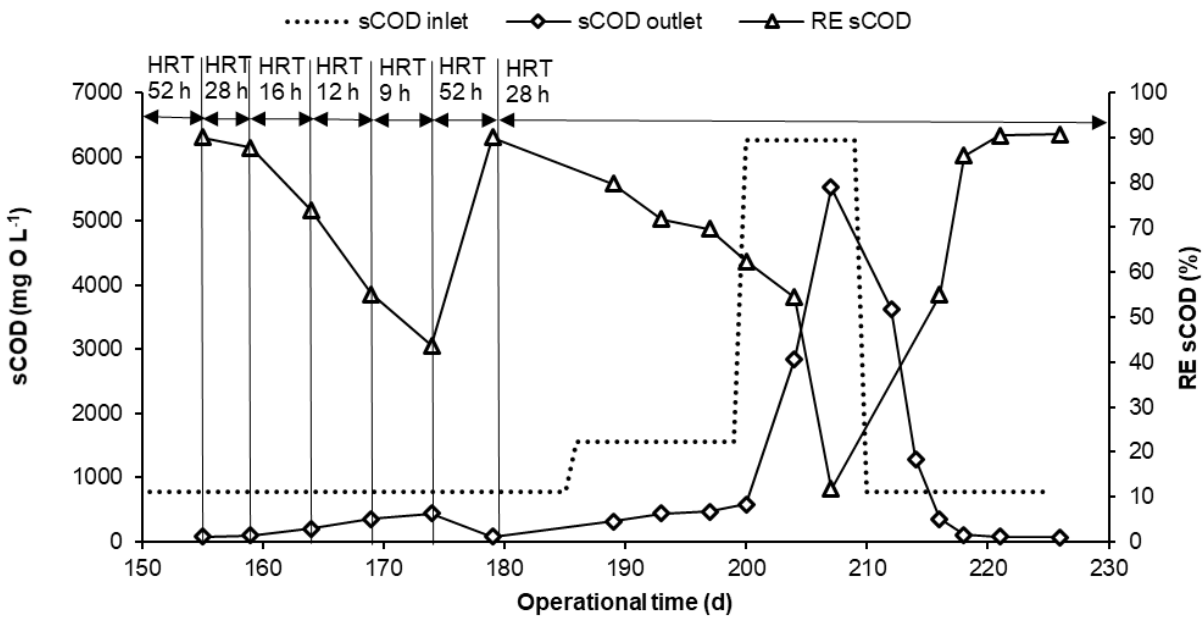


Figure 3. Inlet and outlet sCOD and sCOD-RE profiles obtained from RT performance from day 150 until the end of the operation. Vertical lines show the different HRT set during the operation.

3.3 Simultaneous treatment of TT and QT

After the previous tests, RQ was fed with a medium composed of TT (1 g L^{-1}) and QT (0.1 g L^{-1}) (corresponding to a total inlet sCOD of 879 ± 63 mg L^{-1}) beginning on day 154 (Figure 2). It was also inoculated with the bacteria developed in RT. Since the inhibition caused by QT was strong even at a relatively low COD concentration, it was hypothesised that QT contributed to

biofilm preservation from bacterial growth. The RE of COD after 4 days of simultaneous QT and TT feeding was stable at approximately 88% on average. No synergistic effects were detected when comparing the weighted individual RE during simultaneous treatment with the RE for QT and TT obtained when fed individually during RQ and RT operation. The simultaneous feeding test lasted 26 days, and the biofilm did not get detached from the PUF cubes, maintaining a stable removal performance similar to the previous test phase with QT only. Fungi were shown to be more tolerant to condensed tannins (QT) compared to bacteria and, as previously observed in literature (Mutabaruka et al., 2007), the presence of condensed tannins could increase the ratio between fungi and bacteria in a microbial ecosystem. In the literature, TA has been demonstrated to be an efficient bacterial quorum quenching molecule (QQM) (Patel et al., 2017), and others tannins also show quorum-sensing inhibitory activity (Yang et al., 2016). Moreover, QT is known for its antifouling features (Bellotti et al., 2012). Thus, it is possible that QT could also act as an efficient QQM as one of its mechanisms of action against bacteria. Despite the fact that inhibition of tannins increases with the concentration, the inhibition mechanism is still partially unclear. These results, together with the selected operating conditions, could be used as a possible strategy to increase fungal competitiveness over bacteria and favour the development of a fungal-bacterial consortium that is symbiotically effective in recalcitrant compounds removal. It is important to highlight that in a co-culture, microbial interaction could promote alternative intermediate compounds or metabolites and therefore different degradation pathways of the target pollutant and possible different end products (Bertrand et al., 2014).

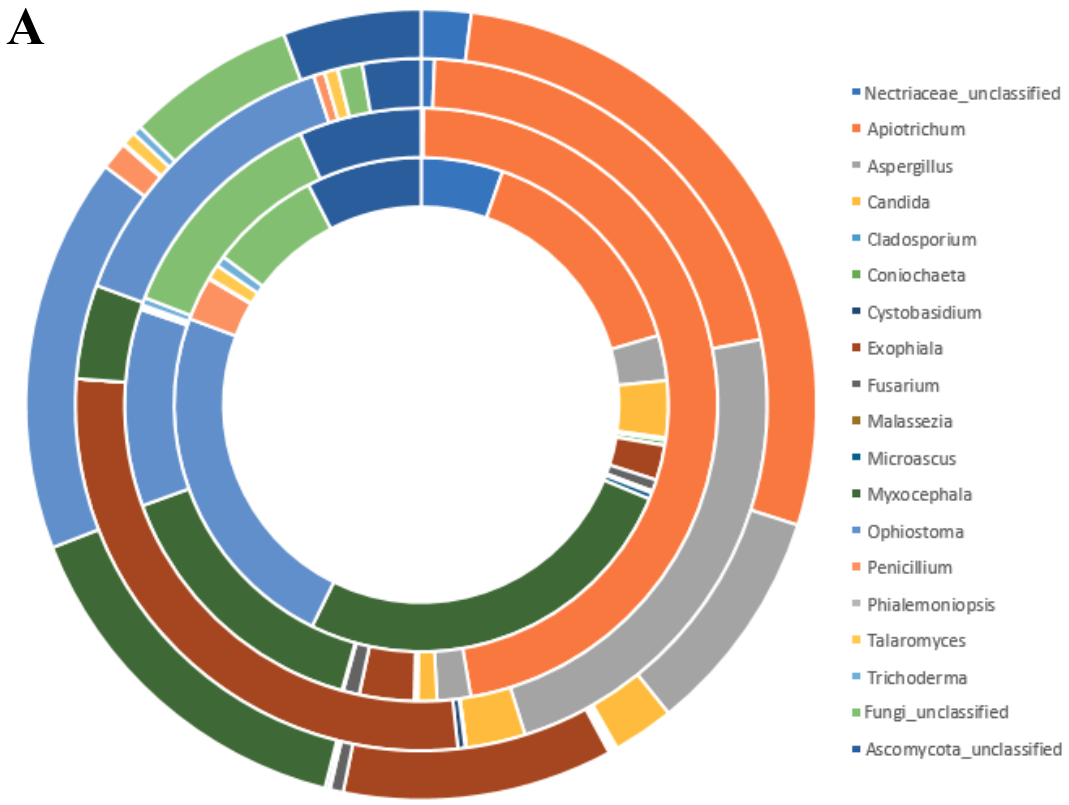
3.4 Microbial community analysis

The total number of high prokaryotic sequences obtained across the three samples was 229,296, with an average of 76,432 each. Regarding fungi, the amount of high-confidence sequences across the three samples was 85,259, with an average of 28,419 each. Rarefaction curves for both bacterial and fungal communities (see Supplementary Information) showed that sequencing

results covered bacterial and fungal diversity, since the rarefaction curves tended toward a saturation plateau. The alpha diversity, Chao, Shannon and Simpson indexes (see Supplementary Information) showed that the bacterial and fungal communities in the bioreactor were well characterised. Regarding the bacterial community, 97% of sequences were classified into 113 OTUs by using the 97% similarity threshold. At phylum level, OTUs were characterised by four phyla. The *Proteobacteria* phylum was the most abundant, with 88.1% representation among the total sequences. The other phyla were *Actinobacteria* (8.2%), *Bacterioides* (2.4%) and *Proteobacteria* (0.5%). At the genus level, most of the sequences were attributed to a taxonomical identity (Figure 4). The most abundant genus was *Novosphingobium* (13.7%). Other representative genera were *Kaistia* (12.9%), *Comamonas* (11.9%), *Sphingobium* (7%) and *Microbacterium* (5.2%). The above-mentioned genera were distributed across the samples (from reactor RT, RT-9h and RT-28h, and reactor RQ, RQ-28h) in different ways: *Novosphingobium* was distributed with a percentage of 33.2% (RT-9h), 5.2% (RT-28h) and 2.7% (RQ-28h); *Kaistia* was very abundant in the RQ reactor (37.4 % into RQ-28h), while it reached only 0.7% (RT-9h) and 0.6% (RT-28h) of the total in the RT reactor. On the other hand, the *Comamonas* genus was most abundant in the RT reactor (with 5.6% in RT-9h and 28.5% in RT-28h) and less abundant in the RQ reactor (1.5% in RQ-28h). The genus *Sphingobium* was recorded at 15% in the RQ reactor, but less abundant in RT reactor (2.7% in RT-9h and 3.4% in RT-28h). The *Microbacterium* genus was abundant in the RT bioreactor (15.2% in RT-28h) and less abundant in the RQ bioreactor (less than 1% in RQ-28h). The remainder of the genera was represented with lower percentages than those reported above or not classified at the genus level. In the case of the fungal community, 93.1% of the total number of sequences represented 46 different OTUs. At phylum level, OTUs were divided into two phyla: *Ascomycota*, with an abundance of 65%, and *Basidiomycota*, with an abundance of 28.1%. The remaining 6.9% of sequences were uncharacterised. As shown in Figure 4, the *Basidiomycota* phylum *Apiotrichum* was the most-represented genus (27.9% of total sequences). Within the phylum *Ascomycota*, the genera *Ophiostoma* (16.2%), *Myxocephala* (15.2%), *Exophiala* (11.1%)

and *Aspergillus* (9.3%) were most common. The above-mentioned genera were distributed within the two bioreactors as follows: *Apiotrichum* genus was predominant in RT, with an abundance of 24.4% in RT-9h, as compared to RQ where it accounted for just 15%. The *Ophiostoma* genus was most abundant in the RT bioreactor (23.4% in RT-28h) as compared to RQ where it accounted for just 14%. The *Myxocephala* genus accounted for 26% in the RT bioreactor (RT-9h), and for 4.4% in RQ. The *Exophiala* genus was more abundant in RQ (27.9%) compared to RT (2.3%). The *Aspergillus* genus accounted for 23% in RQ-28h and approximately 3% in RT (RT-9h and RT-28h). Since *Aspergillus tubingensis* was the fungal strain inoculated (immobilised onto the PUF), it might be reasonable to assume that the strain constituted the majority of the total *Aspergillus ssp*. in the RQ (23% of the total fungi in the RQ) and RT (about 3% of the total fungi in the RT in both HRT). Furthermore, biomolecular analysis showed that there was an important fungal consortium development at steady-state conditions in RQ. Starting from a single fungal strain inoculum, the *Aspergillus* relative abundance was reduced to a residual value in RT. It is worth mentioning that among the most abundant bacterial genera found in the reactors (*Kaistia* and *Sphingobium* in RQ; *Comamonas*, *Novosphingobium* and *Microbacterium* in RT), several species were identified that have not been previously reported in literature to be tannin degraders. Instead, such species are reported to be related to the biotransformation of phenols. Likewise, for the fungal genera *Exophiala*, *Aspergillus*, *Apiotrichum*, and *Ophiostoma* in RQ and *Apiotrichum*, *Myxocephala* and *Ophiostoma* in RT, several species were identified that were not previously reported as tannin degraders. In the experimental context, the presence of a diversified microbial community in addition to the inoculated *Aspergillus* was reasonably linked to the non-sterile nature of the commercial tannins used and the environmental contamination. The involvement of these communities in the tannin removal process under the experimental conditions adopted herein is reasonable. The relationship between organisms in a fungi and bacteria consortium is complex. A comparison between RQ and RT nevertheless provides some indications about the organisms more suitable to degrade TT or QT. Further studies are required to reach a better understanding about the

interactions between microorganisms and the influence of the operating conditions of the reactor. As an example, the different HRT in RT was, as expected, a reactor operating condition able to strongly influence the microbial populations. Thus, *Basidiomycota* phylum was present with an abundance of 47.3% in RT-28h while decreasing sharply to 15.3% in RT-9h. In fact, the fungi of *Basidiomycota* phylum are characterised, in general, by a slower growth rate than *Ascomycota.* Looking to the relative abundance, is it possible that the genera *Novosphingobium, Comamonas,* and *Microbacterium* could better tolerate/degrade TT with respect to QT and could be an invading species for most fungi. *Kaistia* and *Sphingobium*, on the other hand, could have symbiotic interactions with fungi and could better tolerate/degrade QT.



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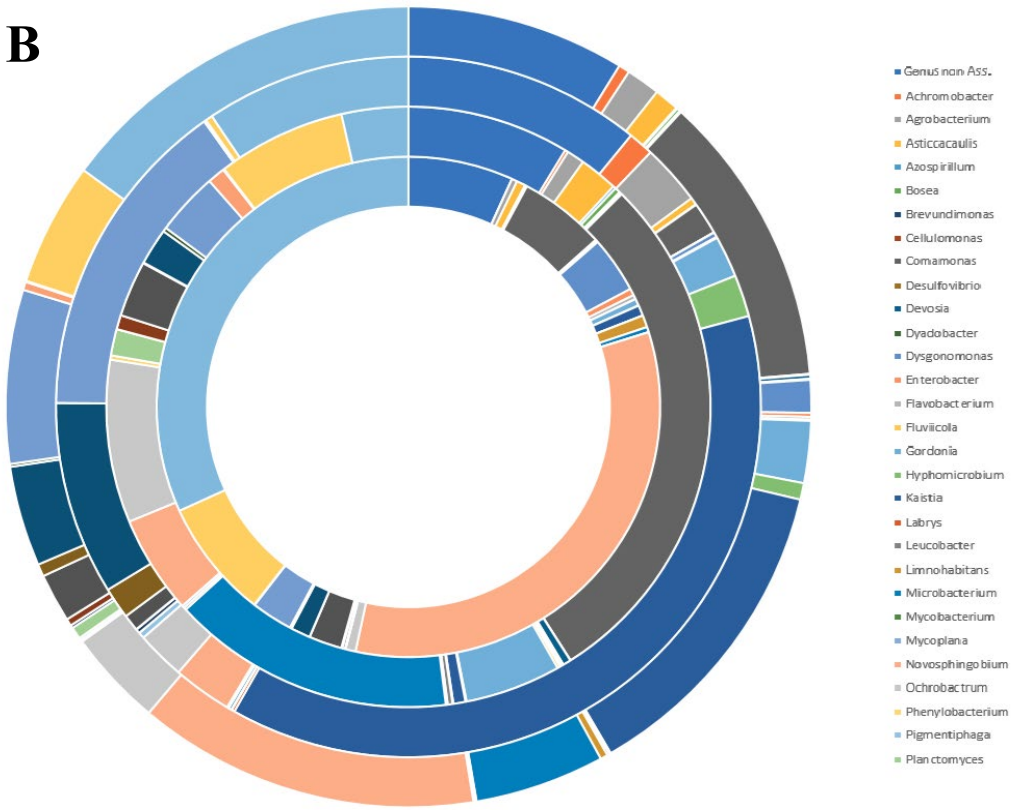

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3 **Figure 4.** Genus percentage in both bioreactors for fungal (A) and bacterial (B) communities. From inside to

4 outside: The total percentage; RQ-28h, RT-28h and RT-9h.

3.5 Comparison between RQ and RT

The volumetric organic loading rate (OLR) and the volumetric organic removal rate (ORR) in RQ and RT are compared in Figure 5. Under the conditions tested in this work, a maximum ORR of 37.9 mg sCOD L^{-1} h^{-1} was obtained for TT while a maximum ORR of 9.1 mg sCOD L^{-1} h^{-1} was obtained for QT, which can be used for further sizing of larger scale reactors. Because of the difficulty to measure biomass concentration in the QT, specific rates could only be compared at an OLR of 28 mg sCOD L^{-1} h^{-1}. The specific ORR of RT was 61.2 mg sCOD g^{-1} TSS h^{-1} while that estimated for RQ was 2.7 mg sCOD g^{-1} TSS h^{-1}.

The different performance of RQ and RT bioreactors in terms of maximum ORR reached as well as the diversity changes in both reactors, can be explained because of the different competition for substrates between fungi and bacteria. It has been previously observed that bacterial growth can lead to the disruption of the fungal mycelium (Rene et al., 2010). Moreover, TT was mainly composed of hydrolysable tannin structures based on gallic acid and quinic acid (Garro Galvez et al., 1997) and, in agreement with previous studies, the minimum inhibitory concentration (MIC) of TT for most bacteria was found to be in the range of 0.16 to 16 g TT L^{-1} (Aguilar-galvez et al., 2014). As a consequence, the selected fungal strain was outcompeted by bacterial strains able to degrade TT (Mingshu et al., 2006), with a MIC higher than the concentration used in the medium solution (1 g TT L^{-1}). The operating conditions in RT (such as slightly acidic pH) allowed the development of bacterial-based suspended biomass with faster growth kinetics that lead to larger volumetric and specific rates compared to the RQ, which was mostly a fungal based reactor. In the RQ, the RE was satisfactory only when QT was fed at a concentration below 0.2 g QT L^{-1}. A QT removal rate of 53% was obtained with 0.1 g L^{-1} concentration in the inlet solution. Then, the RQ fed with QT outlasted the colonisation of bacteria and allowed a stable fungal biofilm thanks to the operating conditions, the QT concentration and consequently the condensed tannins load. This

finding was also verified during the simultaneous feeding of TT and QT since biofilm was preserved in the PUF cubes as well as the suspended biomass in the liquid phase.

Overall, results obtained in the present study represent an important progress in the application of biological processes in tannins removal from wastewater. To the authors' knowledge, this work is the first to report the continuous treatment of QT and TT, providing removal rates and operating conditions that could aid the design of industrial scale reactors. The reactor design and operating conditions demonstrated successful, stable tannin removal under non-sterile conditions, even without the addition of any co-substrate. In both RT and RQ, the development of a fungal and bacterial consortium was observed. Moreover, results allow designing a process for simultaneous treatment of different types of tannins as well as to expand the range of application of fungal-based technologies.

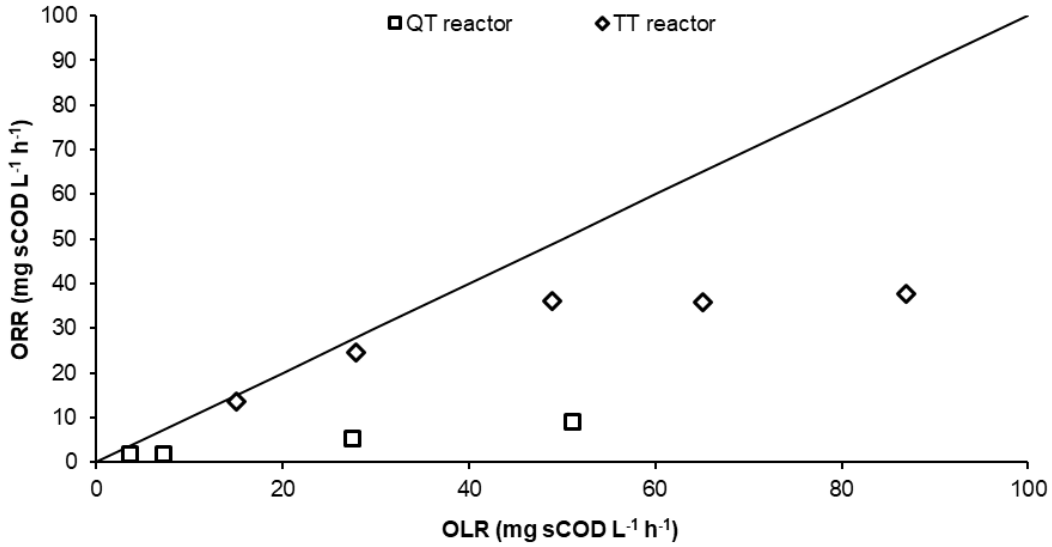

Figure 5. Overall performance rates of RQ and RT reactors.

4 Conclusions

The effects of two different types of tannins, condensed and hydrolysable, on the fungal biomass were investigated and separately tested in two reactors. Fungal and bacterial consortia developed in the long-term operation of bioreactors fed with QT and TT after initial inoculation with *Aspergillus tubingensis*. Reactor fed with QT outlasted the colonization of bacteria and allowed a stable fungal biofilm able to remove QT with RE up to 53%. Reactor fed with TT suffered the detachment of the inoculated fungal biofilm after start-up. Fungi were outcompeted by bacteria as demonstrated through biomolecular analysis leading to a stable culture of suspended biomass in the long run that reached 90% removal of hydrolysable tannins. Additionally, co-treatment of both tannins was demonstrated feasible. Fungi have been shown to be more resistant to the inhibitory effect of QT than bacteria, and the QT concentration therefore allowed for the maintenance of a stable fungal biofilm in non-sterile conditions. The study provided promising results application of the new technology based on fungal biofilms reactors.

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REFERENCES

Aguilar-galvez, A., Noratto, G., Chambi, F., Debaste, F., Campos, D., 2014. Potential of Tara (*Caesalpinia spinosa*) gallotannins and hydrolysates as natural antibacterial compounds. Food Chem. 156, 301–304. https://doi.org/10.1016/j.foodchem.2014.01.110

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

Andreottola, G., Esperia, 2001. Respirometria applicata alla depurazione delle acque: principi e metodi, Collana scientifico-divulgativa Monographia. Università di Trento. Dipartimento di ingegneria civile e ambientale.

APHA-AWWA-WPCF, 2005. Standard Methods for the Examination of Water and Wastewater Part 1000 Standard Methods for the Examination of Water and Wastewater.

Barbehenn, R. V., Peter Constabel, C., 2011. Tannins in plant-herbivore interactions. Phytochemistry 72, 1551–1565. https://doi.org/10.1016/j.phytochem.2011.01.040

Bellotti, N., Deyá, C., Del Amo, B., Romagnoli, R., 2012. "Quebracho" tannin derivative and boosters biocides for new antifouling formulations. J. Coatings Technol. Res. 9, 551–559. https://doi.org/10.1007/s11998-012-9403-0

Belmares, R., Garza, Y., Rodríguez, R., Contreras-esquivel, J.C., Aguilar, C.N., Keywords, C., 2009. Composition and fungal degradation of tannins present in semiarid plants. Electron. J. Environ. Agric. Food Chem. 8, 312–3118.

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., Wolfender, J.L., 2014. Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. Biotechnol. Adv. 32, 1180–1204. https://doi.org/10.1016/j.biotechadv.2014.03.001

Bushnell, B., 2014. Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. BMC Bioinformatics 13, 238. https://doi.org/10.1186/1471-2105-13-238

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, K., Gordon, J.I., Huttley, G. a, Kelley, S.T., Knights, D., Jeremy, E., Ley, R.E., Lozupone, C. a, Mcdonald, D., Muegge, B.D., Reeder, J., Sevinsky, J.R.,

Turnbaugh, P.J., Walters, W. a, 2011. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.QIIME

Casieri, L., Prigione, V.P., Anastasi, A.E., Tigini, V., Varese, G., others, 2008. Metabolism-independent biosorption of industrial dyes by fungal biomasses revealed by batch sorption experiments and confocal microscopy. Int. J. Chem. Eng. 1, 273–287.

Edgar, R., 2016. UCHIME2: improved chimera prediction for amplicon sequencing. bioRxiv 74252. https://doi.org/10.1101/074252

Espinosa-Ortiz, E.J., Rene, E.R., Pakshirajan, K., van Hullebusch, E.D., Lens, P.N.L., 2016. Fungal pelleted reactors in wastewater treatment: Applications and perspectives. Chem. Eng. J. 283, 553–571. https://doi.org/10.1016/j.cej.2015.07.068

Garro Galvez, J.M., Riedl, B., Conner, a. H., 1997. Analytical Studies on Tara tannins. Holzforschung 51, 235–243. https://doi.org/10.1515/hfsg.1997.51.3.235

Giaccherini, F., Munz, G., Dockhorn, T., Lubello, C., Rosso, D., 2017. Carbon and energy footprint analysis of tannery wastewater treatment: a global overview. Water Resour. Ind. 17, 43–52. https://doi.org/10.1016/j.wri.2017.03.001

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. 9, 177–92. https://doi.org/10.1038/nrmicro2519

He, Q., Sun, D., Xin, L., Wei, L., Bi, S., 2008. Evaluation of environmental impact of typical leather chemicals. Part III: Biodegradability of vegetable tannin extracts by activated sludge. J. Soc. Leather Technol. Chem. 92, 103–106.

Khanbabaee, K., Ree, T. van, 2001. Tannins: Classification and definition. Nat. Prod. Rep. 18, 641–649. https://doi.org/10.1039/B101061L

León-Galván, F., Torres-Pacheco, I., Jiménez-Espinoza, F., Romero-Gómez, S., Guevara-Olvera, L., de la Rosa, A.P.B., González-Chavira, M.M., Guevara-González, R.G., 2010. Molecular and biochemical characterization of extracellular tannin acyl hydrolase activity from a mexican isolate of *Aspergillus niger*. J. Sci. Ind. Res. (India). 69, 942–947.

Li, W.W., Li, X.D., Zeng, K.M., 2009. Aerobic biodegradation kinetics of tannic acid in activated sludge system. Biochem. Eng. J. 43, 142–148. https://doi.org/10.1016/j.bej.2008.09.010

Lofrano, G., Meriç, S., Zengin, G.E., Orhon, D., 2013. Chemical and biological treatment technologies for leather tannery chemicals and wastewaters: A review. Sci. Total Environ. 461–462, 265–281. https://doi.org/10.1016/j.scitotenv.2013.05.004

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

Mannucci, A., Munz, G., Mori, G., Lubello, C., 2010. Anaerobic treatment of vegetable tannery wastewaters: A review. Desalination 264, 1–8. https://doi.org/10.1016/j.desal.2010.07.021

Mingshu, L., Kai, Y., Qiang, H., Dongying, J., 2006. Biodegradation of gallotannins and ellagitannins. J. Basic Microbiol. 46, 68–84. https://doi.org/10.1002/jobm.200510600

Mohan, S.K., Viruthagiri, T., Arunkumar, C., 2013. Statistical optimization of process parameters for the production of tannase by *Aspergillus flavus* under submerged fermentation. 3 Biotech 4, 159–166. https://doi.org/10.1007/s13205-013-0139-z

Munz, G., Angelis, D. De, Gori, R., Mori, G., Casarci, M., Lubello, C., 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, 733–739. https://doi.org/10.1016/j.jhazmat.2008.08.070

Murugan, K., Saravanababu, S., Arunachalam, M., 2007. Screening of tannin acyl hydrolase (E.C.3.1.1.20) producing tannery effluent fungal isolates using simple agar plate and SmF process. Bioresour. Technol. 98, 946–949. https://doi.org/10.1016/j.biortech.2006.04.031

Mutabaruka, R., Hairiah, K., Cadisch, G., 2007. Microbial degradation of hydrolysable and condensed tannin polyphenol-protein complexes in soils from different land-use histories. Soil Biol. Biochem. 39, 1479–1492. https://doi.org/10.1016/j.soilbio.2006.12.036

Ni, H., Chen, F., Jiang, Z.D., Cai, M.Y., Yang, Y.F., Xiao, A.F., Cai, H.N., 2015.

Biotransformation of tea catechins using *Aspergillus niger* tannase prepared by solid state fermentation on tea byproduct. LWT - Food Sci. Technol. 60, 1206–1213. https://doi.org/10.1016/j.lwt.2014.09.010

Palli, L., Gullotto, A., Tilli, S., Caniani, D., Gori, R., Scozzafava, A., 2016. Biodegradation of 2-naphthalensulfonic acid polymers by white-rot fungi: Scale-up into non-sterile packed bed bioreactors. Chemosphere 164, 120–127. https://doi.org/10.1016/j.chemosphere.2016.08.071

Patel, B., Kumari, S., Banerjee, R., Samanta, M., Das, S., 2017. Disruption of the quorum sensing regulated pathogenic traits of the biofilm-forming fish pathogen *Aeromonas hydrophila* by tannic acid, a potent quorum quencher. Biofouling 33, 580–590. https://doi.org/10.1080/08927014.2017.1336619

Rene, E.R., Veiga, M.C., Kennes, C., 2010. Biodegradation of gas-phase styrene using the fungus *Sporothrix variecibatus*: impact of pollutant load and transient operation. Chemosphere 79, 221–227. https://doi.org/10.1016/j.chemosphere.2010.01.036

Romer, F.H., Underwood, A.P., Senekal, N.D., Bonnet, S.L., Duer, M.J., Reid, D.G., Van Der Westhuizen, J.H., 2011. Tannin fingerprinting in vegetable tanned leather by solid state NMR spectroscopy and comparison with leathers tanned by other processes. Molecules 16, 1240–1252. https://doi.org/10.3390/molecules16021240

Sciences, M., Safety, F., Kingdom, U., 2007. Profiling and characterization by LC-MSn of the galloylquinic acids of green tea, Tara tannin, and annic acid. J. Agric. Food Chem. 55, 2797–2807. https://doi.org/10.1021/jf0635331

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, 1789–1796.

Svobodová, K., Novotný, C., 2017. Bioreactors based on immobilized fungi: bioremediation under non-sterile conditions. Biotechnol, Appl Microbiol. https://doi.org/10.1007/s00253-017-8575-z

Tigini, V., Munz, G., Spennati, F., Bardi, A., Spina, F., Varese, G.C., 2015. Selection of

autochthonous and allochthonous fungal strains for the treatment of recalcitrant wastewaters. X edition of the Italian Forum on Industrial Biotechnology and Bioeconomy - IFIB Lodi (Italy). 24 – 25 September 2015.

Tilli, S., Mori, G., Mannucci, A., Munz, G., Gori, R., Lubello, C., Scozzafava, A., Varese, G.C., Briganti, F., Chimica, D., Lastruccia, V., 2010. Natural tannins for leather treatments : biodegradation by Penicillium chrysogenum MUT 4444 on a fixed bed bioreactor. Oxidative Enzymes as Sustainable Ind. Biocatalisis. Santiago Compostela (Spain) 14– 15 September 2010.

Van Diepeningen, A.D., Debets, A.J.M., Varga, J., van der Gaag, M., Swart, K., Hoekstra, R.F., 2004. Efficient degradation of tannic acid by black Aspergillus species. Mycol. Res. 108, 919–925. https://doi.org/10.1017/S0953756204000747

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Rómero-Gomez, S. de J., Díaz-Godínez, G., Augur, C., 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem. Eng. J. 13, 157–167. https://doi.org/10.1016/S1369-703X(02)00128-6

Yang, Q., Wang, L., Gao, J., Liu, X., Feng, Y., Wu, Q., Baloch, A.B., Cui, L., Xia, X., 2016. Tannin-Rich Fraction from Pomegranate Rind Inhibits Quorum Sensing in *Chromobacterium violaceum* and Biofilm Formation in *Escherichia coli*. Foodborne Pathog. Dis. 13, 28–35. https://doi.org/10.1089/fpd.2015.2027

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620. https://doi.org/10.1093/bioinformatics/btt593