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Potentials for a continuous flow non-sterile bioreactor

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Fungal removal of humic acids from treated wastewater

Potentials for a continuous flow non-sterile bioreactor

Dissertation

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An electronic copy of this dissertation is available at TUDelft repository.

To my family. Obviously.

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Chapter 1

Introduction

1.1. Background

A literature search reveals that the first reports on wood-rotting basidiomycetous fungi go back to the mid-19th century. The natural growth of these microorganisms on dead wood triggered a vast excitement in studying their metabolic and growth characteristics. Despite the presence of lignin in the woody tissue, which is known as the most recalcitrant natural biopolymer, these fungi can digest woody tissues, utilize its cellulose and hemicellulose as carbon source and grow on it [1,2]. Shortly after the first reports, researchers showed that these fungi can degrade lignin through an enzymatic oxidation process [3]. In the 1970s the main focus was defining laboratory conditions for the growth of WRF and achieving maximum degradation efficiency of lignin, and *Phanerochaete chrysosporium* was the most used species of WRF for research during this period. In the next decade, focus was moved towards the lignin-degrading enzymes of WRF, which initially were called "ligninase" enzymes [4]. In the 90s, another line of research was developed to find more selective lignin degraders. The goal was mainly to find fungal species that mostly degrade lignin and leave the carbohydrates (like cellulose and hemi cellulose) intact. This new line of research resulted in introducing new species of WRF other than *P. chrysosporium* [5]. Later on, following the biochemical research line on the enzymatic system of WRF, the responsible enzymes were identified and metabolic pathways for these lignin-degrading peroxidases were suggested [6]. Consequently, researchers broadened the range of targeted compounds from lignin to other recalcitrant compounds and proved that the enzymatic system of WRF can also be effective in degrading other complex and refractory molecules [7]. In the late 90's, the major subjects of WRF research involved the application of WRF in biochemical pulping and pulp bleaching. In this way, a growing number of researchers started to study the application of WRF in liquid phase, which later on developed to a major line of research focused on degradation of water-soluble organic recalcitrant compounds. Based on the literature, the number of studies on WRF in liquid phase increased at the end of the 20th century. In 2006, it has been estimated that studies on bioremediation using WRF, comprise 30% of all literature on fungal bioremediation [8].

1.2. White rot fungi

Saprotrophic fungi are well known for their important role in utilizing organic matter in natural ecosystems [9]. They facilitate organic matter decomposition and nutrients (re)cycling in favor of own and other organisms growth [10]. Among these fungal species, white rot fungi (WRF) are of particular interest, due to their capability to efficiently mineralize lignin [2,11]. They are the most efficient lignin degraders in nature, which make them the major agents for recycling the carbon from lignified tissues in nature [4]. WRF are a heterogeneous group of fungi classified in the Basidiomycota and are so named because of the bleached appearance they leave on the wood fibers following their growth [12]. Many species of fungi have been researched in the biodegradation of lignin, but the most extensive research has been done on the WRF *P. chrysosporium* [13]. A systematic search in Scopus revealed that about 20% of all published studies on WRF between 2000 and 2015 have been focused on *P. chrysosporium*. However, the physiological conditions for lignin degradation and the enzyme systems expressed by these fungi vary among different species [13]. Recently, however, there has been growing interest in studying the lignin-degrading (also known as lignin-modifying) enzymes of other WRF, including: *Trametes* sp., *Bjerkandera* sp., *Pleurotus* sp., *Phlebia radiata* and *Pycnoporus cinnabarinus* [14,15]. The degradation of lignocellulosic substrates by WRF requires the secretion of a complex set of ligninolytic enzymes and the presence of corresponding metabolites such as H_2O_2. This process entails an oxidative and non-specific process of decreasing the amount of methoxy, phenolic, and aliphatic units of lignin, cleaving aromatic rings, and creating new carbonyl groups [13,16].

The non-specific extracellular enzymes of WRF give them the capability to degrade a wide range of highly recalcitrant organopollutants with molecular structure similar to lignin [17,18], such as azo dyes [12,19], polyphenolic compounds [20,21], pharmaceuticals [22,23] and humics [24,25].

1.2.1. White rot fungi's enzymes

The characteristics of the enzymatic system of WRF could be directly related to the natural substrate of these enzymes, i.e. lignin. The enzymatic system required for the degradation of macromolecular lignin should be able to overcome several challenges. The substrate is a large heterogeneous aromatic polymer, which necessitates an attack by extracellular enzymes [26]. Lignin does not contain

hydrolysable linkages, which means that the linkages should be oxidized in order to break the polymeric structure. Therefore, the degrading enzymes should be oxidative [13,26]. Lignin is stereo-irregular, which also dictates a non-specific characteristic for the degrading enzymes [13].

Lignin peroxidase, manganese peroxidase and laccase are the three major extracellular enzymes produced by white rot fungi, which are responsible for the degradation of lignin and other compounds with similar molecular structure to lignin [13,14].

Lignin peroxidase (LiP), also known as "ligninase", is a hemeprotein. LiP (EC 1.11.1.14) oxidizes non-phenolic lignin substructures and similar compounds by removing one single electron from the aromatic ring and generating cation radicals that are then decomposed chemically. The mediators for this enzyme are veratryl alcohol and H_2O_2 [13,27].

Manganese peroxidase (MnP) is a heme-containing glycoprotein. MnP (EC 1.11.1.13) oxidizes Mn (II) to Mn (III), which then oxidizes phenolic rings to phenoxyl radicals and ultimately results in the decomposition of the phenolic compound. MnP requires the presence of Mn(II) and H_2O_2 as mediators. [13,28,29].

Laccase (benzenediol: oxygen oxidoreductase (EC 1.10.3.2) is a copper containing enzyme that catalyzes the oxidation of various substrates, especially aromatic substrates, with the simultaneous reduction of molecular oxygen to water [15,30]. Laccase can also oxidize non-phenolic compounds in the presence of required mediators such as ABTS [31].

There is another group of peroxidases produced by some species of WRF such as *Pleurotus* and *Bjerkandera*, which are also non-specific and have characteristics similar to both LiP and MnP, called versatile peroxidases (VPs) [32,33]. VP (EC 1.11.1.16) were initially classified as MnP enzymes, but later were recognized as a separate group of peroxidases. They can oxidize Mn^{2+}, similar to MnP, and also can oxidize high redox potential aromatic compounds, similar to LiP [15].

In addition, some accessory enzymes are involved in hydrogen peroxide production by WRF, namely glyoxal oxidase (GLOX)[34–36] and aryl alcohol oxidase (AAO) [13,37].

Table 1 summarizes the main enzymes produced by WRF, and also their mediators and their main reactions.

Table 1. The main enzymes of WRF, their mediators and reactions involved

Enzyme	Main Cofactor/ Mediator	Main reaction	Ref.
Lignin peroxidase (LiP)	H_2O_2, Veratryl alcohol	Oxidizing aromatic non-phenolic ring to cation radical	[38–40]
Manganese peroxidase (MnP)	H_2O_2, Mn (II)	Oxidizing phenolic rings, oxidizing Mn(II) to Mn(III)	[28,29,39]
Laccase (Lac)	O_2	Oxidizing phenol ring	[30, 41, 42]
Glyoxal oxidase (GLOX)	Glyoxal, methyl glyoxal	Oxidizing glyoxal to glyoxylic acid, H_2O_2 production	[34–36]
Aryl alcohol oxidase	Aromatic alcohols (anisyl, veratryl alcohol)	Oxidizing aromatic alcohols to aldehydes, H_2O_2 production	[13, 37]
Versatile peroxidases (VPs)	Mn(II), H_2O_2	Oxidizing phenolic & non-phenolic structures	[32, 33, 43]

1.2.2. Application of WRF under non-sterile conditions: the challenge

Although the application of WRF has shown promising results in removing refractory molecules from water, still the implementation of this technique in (waste)water treatment systems has not yet happened. This is due to some obstacles in using these microorganisms under real conditions, namely non-sterile conditions. Studies on using WRF under non-sterile conditions started in the beginning of the 1990s, but today the number of published studies on WRF under non-sterile conditions only comprises around 1% of the total publications on WRF (based on Scopus database). It is obvious that sterilizing the wastewater prior to treatment with fungi is not a feasible option at the industrial scale. The high inflow rate of wastewater treatment plants is the major reason that makes it economically non-feasible to sterilize the wastewater. Therefore, it is very important to understand the

problems with the application of WRF under non-sterile conditions and to provide new strategies to overcome these problems.

The fungal growth rate and enzyme activity of WRF decrease drastically in reactors under non-sterile conditions, and it is especially noticeable during long-term operations [44–47]. It has been shown that even if fungal activity is high in the start-up phase, it is very hard to maintain a long-term fungal growth and its associated enzyme activity in the reactor [48–50].

The low growth rate and enzyme activity of WRF under non-sterile conditions is mainly due to the fact that WRF are low-grade eukaryotic microorganisms and grow slowly compared to fast growing bacteria. Therefore, bacteria can compete with WRF for the available substrate and nutrients. Bacterial proliferation results in severe competition for available organic substrate, and it negatively affects the WRF metabolism [49,51,52]. There are also reports suggesting that contamination with other microorganisms not only limits the WRF's growth but also can destabilize the secreted fungal enzymes. [46,47]. The mechanisms and details of this inhibition of ligninolytic enzymes under non-sterile conditions are not clear yet.

1.2.3. Recent attempts of applying WRF under non-sterile conditions

Cruz-Morató *et al.* [53] investigated the ability of WRF to degrade pharmaceuticals under non-sterile conditions. This was the first study that used WRF under non-sterile conditions to treat real pharmaceutical wastewater containing a mixture of contaminants in low concentrations. The authors used pellet forms of *Trametes versicolor* in a fluidized bed bioreactor. They started from sterile conditions and continued their work under non-sterile conditions. Under sterile conditions, they could achieve complete (100%) removal of almost half of the targeted pharmaceutical compounds. Interestingly, they reported a high removal percentage of mammalian metabolites of polycyclic aromatic hydrocarbons (PAHCs). Nevertheless, the removal efficiency dropped from sterile batch to non-sterile batch operation. Also, under non-sterile conditions mycelia lysis occurred after 5 days, the turbidity of the broth increased and the degradation efficiency dropped significantly.

In another study, focused on the removal of pharmaceuticals and personal care products (PPCPs) from wastewater using WRF, *Phanerochaete chrysosporium* was applied to degrade naproxen and carbamazepine as targeted compounds [54]. First

the effect of immobilization on bioremediation efficiency of fungi under non-sterile conditions was investigated by immobilizing fungi on wood chips. After one week of incubation, the authors observed 28% higher removal of carbamazepine and 4% higher removal of naproxen in immobilized cultures compared to free cell cultures. Although the small change in naproxen concentration might not be significant (considering the measurement errors), they suggested that the significant increased removal efficiency of carbamazepine is due to the better growth and enzyme production of fungi when they are immobilized [54]. In the next step, the authors applied the immobilized fungi in a fixed-bed up-flow reactor (3 L working volume) under non-sterile conditions with intermediate feeding. The removal efficiency of both targeted compounds in the first periods of reactor operation was 60-80% but then dropped to almost zero removal. At the same time, pH increased from 4.7 to around 8, which usually indicates bacterial contamination. They assigned the malfunction of the fungal reactor to bacterial contamination and therefore investigated the addition of a bactericide to inhibit the bacterial growth. They added sodium hypochlorite (8.25%) to the influent of the reactor (ratio 1:100 v/v) and subsequently observed a recovery of the fungal activity in the reactor. The removal efficiency increased up to about 80% for naproxen in two days, and pH decreased. After 5 days of the first dosing of sodium hypochlorite, they added a second dose and managed to increase the naproxen removal to more than 95% and to keep it stable for another 5 days. However, they did not observe the same recovery for carbamazepine removal. Finally, they concluded that a mild dosage of disinfectant can inhibit the bacterial growth without suppressing the fungal growth [54]. It has been reported before that due to various mechanisms of resistance, fungi can resist to some disinfectants that are effective on bacteria [51].

Olivieri and colleagues [55,56] tested the ability of WRF for dephenolization of olive mill wastewater (OMW). They started their work in sterile batch systems [55] and based on the promising results continued their work using continuous systems under non-sterile conditions [56]. They used *Pleurotus ostreatus* in an airlift bioreactor (5 L) to treat raw OMW without any pre-treatment (except filtering out the particulates). Both their batch and continuous tests with raw OMW were unsuccessful under non-sterile conditions. They attributed this observation to endogenous microorganisms in the OMW, suggesting that these microorganisms compete with fungi for common substrates (or nutrients), and in this case oxygen (as electron acceptor). They also reported that the growth of contaminating microorganisms in the fungal reactor would change the environmental conditions in

a way that is not suitable for fungi, such as increasing the pH. However, after pre-aeration of the OMW prior to feeding it to the fungal reactor to reduce the competition for oxygen between fungi and endogenous microorganisms, they observed a successful fungal growth, laccase activity and dephenolization. In their final attempt, they designed a continuous process with continuous feeding of pre-aerated OMW and retaining the fungal biomass in the reactor (by filtering and recycling the biomass from washout). Their results indicated high dephenolization efficiency for 12 days with high laccase activity. However, they reported a very high oxygen consumption rate in this system, which in the long run (after 12 days) favored the establishment of an anoxic environment (owing to massive bacterial growth), deactivating the fungi. [56].

Gao *et al.* (2008) [52] tested three incubation methods for application of WRF under non-sterile conditions. They used *Phanerochaete chrysosporium* to degrade reactive brilliant red (an azo dye) in water. The degradation of dyes in the wastewater is usually expressed as decolorization. Three incubation methods were tested: (a) incubation of fungi and decolorization under sterile conditions, (b) incubation under sterile conditions but decolorization under non- sterile conditions, and (c) incubation and decolorization under non-sterile conditions. Under non-sterile conditions (b and c) fungal cultures were infected by microzymes, cocci, and bacillus within 3 days and pH also increased. The decolorization efficiency was very poor as well as growth and enzyme activity of the fungi. Subsequently, in order to enhance the growth and enzyme activity the fungi were immobilized on polyurethane foam as carrier. Results showed that immobilized fungi can suppress the bacterial growth effectively and also maintain a stable pH of 4-5 during a period of 10 days, under non-sterile conditions (b and c). The immobilized culture reached five times higher MnP activity than suspended culture, and the activity reached its maximum 4 days earlier than the suspended fungal culture. Also, consumption of carbon and nitrogen was higher in the immobilized culture. The other parameter tested between different incubation methods was decolorization efficiency, which was very poor in suspended culture (15%) because of the high level of bacterial contamination. In contrast, with immobilized fungi decolorization efficiency reached 89% after 1 day of treatment and 95% after 3 days. The decolorization was not significantly different between sterile and non-sterile immobilized cultures. However, the reliability of their method is still questionable since they only tested it in flasks, and also their incubation time was only 10 days. They reported that on the last days of the incubation, growing numbers of coccus and bacillus were detected.

The pre-treatment of wastewater could be another method to inhibit the bacterial growth in the fungal culture. Fujita *et al.* (2000) [57] tested heat pretreatment of a dark liquor wastewater in order to reduce the bacterial contamination prior to fungal treatment using *Coriolus hirsutus*. Dark liquor is the byproduct of heat-treatment of sludge, which is done to increase the dewaterability of sludge in some wastewater treatment plants. This dark liquor, also known as heat-treated liquor contains molecules with chemical structures like melanoidins and humic compounds. They used an up-flow bioreactor with active aeration from the bottom, coupled with an ultra-filtration unit to retain the fungal biomass. Initially they showed that the decolorization efficiency of their fungal reactor dropped drastically after 3 days and continued to decrease for 8 days. They observed airborne or waterborne microbial growth in the fungal reactor and assigned the decrease in the fungal reactor efficiency to this contamination. To reduce the microbial contamination, the wastewater recycled from UF to the fungal reactor was heated to 50°C for 10 min before returning to the fungal reactor. They claimed that this heat pre-treatment of the wastewater lowered the microbial contamination by 80% of CFU (colony forming unit). They noted that the fungal enzyme activities (manganese dependent and independent peroxidase) were not significantly affected. Their fungal reactor achieved 40% decolorization under non-sterile conditions [57].

In another study, ozonation was tested as a pre-treatment method to inhibit bacterial growth in a fungal reactor [49]. In this study, a 2 L fungal reactor with a hydraulic retention time (HRT) of 3 days was applied for the degradation of acid blue (azo dye) by *P. chrisosporium*. They studied the effect of immobilization of fungi on knotted cotton thread along with ozonation of the reactor influent with ozone-oxygen gas mixture, containing 0.12 $mL.L^{-1}$ ozone. The reactor's media was recycled through the ozonation tank, where it was treated with 0.0144 $mg.L^{-1}.min^{-1}$ ozone for 60 min and returned to the reactor. Ozone as a strong oxidant can degrade several xenobiotic compounds and can also inhibit bacteria. The authors reported a 99.4% inhibition of contaminating bacteria by ozone (compared to the reactors operated without ozonation). Moreover, they reported that MnP retained its activity under ozonation. Their results showed that, using immobilized fungi and an ozonation treatment unit, they could achieve relatively stable and continuous performance of the fungal reactor for 25 days under non-sterile conditions, with an average azo dye removal efficiency of 84%. The color removal efficiency with free fungal mycelia and without the ozonation unit was 55%, and decolorization stopped after 12 days. They

also noted that the immobilization of the fungi was important to maintain the fungal reactor in a continuous mode over a long period [49].

Libra *et al* [47] and Borchert and Libra [48] investigated the removal of reactive azo dyes from synthetic wastewater with WRF *Trametes versicolor*. Although they could achieve high enzyme activity and color removal in pure culture (sterile conditions) in sequencing batch reactors, their experiments under non-sterile conditions showed that bacterial contamination occurs easily and decreases the decolorization efficiency [48]. Subsequently, they tested four approaches to suppress bacterial growth in WRF reactors under non-sterile conditions: (a) pH reduction, (b) crude enzyme treatment, (c) nitrogen-limited medium, and (d) immobilization of WRF on selective nutrient containing carriers [47]. The first approach, lowering pH, was tested based on the widely reported high stability (with regard to growth and enzyme activity) of WRF species in acidic media. The aim was to suppress the bacterial growth and preserve the WRF's growth. Results showed that this approach might be effective in short term, but on the long term the bacterial community may adapt to the acidic conditions and bacterial growth would increase again. The second approach was separation of the fungal growth and enzyme production step from the wastewater treatment step. The idea was to produce the enzyme mixture in a separate reactor under sterile conditions, collect it and add it to the wastewater in a second reactor under non-sterile conditions to degrade the dyes. They observed that in the wastewater treatment step, under non-sterile conditions, the enzyme activity decreased substantially as a result of bacterial growth. They concluded that bacterial contamination not only suppresses the fungal growth but can also decrease the activity of the secreted enzymes. The third approach was to use a nitrogen-limited medium to reduce bacterial growth. The idea is to pre-grow fungi in a high carbon and nitrogen media under sterile conditions and then deliver the pre-grown fungal mycelia to a non-sterile reactor fed with wastewater and some nutrients but without nitrogen. Because *T. versicolor* produces enzymes as primary and secondary metabolites, they hypothesized that in nitrogen-limited conditions, the enzymatic system of the fungi would be triggered, whereas bacterial growth would be suppressed. They reported that this approach could be effective in short term, although the noticeable problem with this process was that on the long term, by aging and deactivation of the fungal mycelia, bacteria could start to feed on fungal mycelia and grow again, resulting in bacterial proliferation of the culture. The fourth approach was to immobilize fungi on lignocellulosic carriers such as hay, spelt grains and peanut shell. They pre-grew the fungi on carriers under sterile conditions, and

then moved the immobilized fungi to the treatment unit under non-sterile conditions. They reported a maximum 90% decolorization with *T. versicolor* pre-grown (30 days) on a mixture of rye grain/straw mixture, under non-sterile conditions in 10 days. In order to test the potential of this technique for longer periods (>10 days), they performed a sequential decolorization experiment under non-sterile conditions. In the sequential batch experiment, a 4-L stirred tank glass reactor was used. The wastewater and the media (nitrogen-free) were initially autoclaved only for the first cycle. The following cycles were done with non-sterile feeding of the media. Three cycles were performed, first cycle with a period of 10 days, second cycle for 25 days and the third cycle for 15 days. Each cycle was finished when no significant change in the color was detected. Their results showed that more than 90% decolorization was achieved in the first and second cycle, but in the third cycle they only reached a maximum of 50% decolorization. Furthermore, the enzyme activity was dropped to almost zero in the second cycle (non-sterile) and did not recover from then on. Overall, they concluded that the combination of immobilization of fungi on lignocellulosic carriers and feeding with N-limited media, provided the best results, regarding the decolorization efficiency under non-sterile conditions [47].

Hai *et al.* conducted a study to determine the factors affecting the performance of a continuous WRF bioreactor under non-sterile conditions [46]. They used WRF *Coriolus versicolor* for decolorization of an azo dye (acid orange). In this study, they tested different HRTs and feeding strategies. They showed that the decolorization in their reactor was incomplete and enzymatic activity was very low, due to the bacterial contamination of the culture under non-sterile conditions. They also noted that when operating the reactor in continuous mode, enzyme washout was contributing to the decrease in removal efficiency. Results of this study showed that enzyme washout can be limited by increasing the HRT. Yet under non-sterile conditions, they observed severe and non-recoverable damage to fungal granules, which led to a drastic reduction in enzyme activity and decolorization. They concluded that the bacterial contamination, fungal morphology and enzyme washout are the most important factors affecting the performance of the continuous fungal reactor under non-sterile conditions. Also, they suggested that these factors are interrelated [46]. They continued their work by adding a membrane module (submerged hollow fiber) coated with granular activated carbon (GAC) layers, in the reactor. The idea was to decrease the enzyme wash out from the reactor by adsorbing them to the activated carbon[58]. Results indicated that the GAC layer prevents enzyme washout by adsorbing the enzymes and keeping them in the reactor. The low decolorization

efficiency during the reference operation period of the reactor (without GAC) increased to an average removal of about 85-100%. They further compared the degradation ability of WRF in pellet form and in immobilized (attached) form of the fungi [59]. They showed that pellets and attached growth of fungi have better enzyme activity than dispersed mycelia. Pellets had high removal efficiency initially but were affected by bacterial contamination later on, which was followed by drastic reduction in removal efficiency of the fungal reactor. Eventually, attached growth of fungi showed the best fungal activity and decolorization efficiency under non-sterile conditions, compared to pellets and dispersed mycelia.

It has been previously reported that bacteria can colonize the aged fungal mycelia and use it as nutrient source to grow [47]. In general, keeping the fungal mycelia in the reactor fresh and young will increase the stability of the fungal culture and its enzyme activity [47,60,61]. It can also prevent channeling in the reactor caused by accumulated inactive fungal biomass [61–64].

Blanquez *et al.* [60] studied the application of *T. versicolor* in a continuous mode air-pulsed reactor under sterile conditions to degrade the textile dye Grey Lanaset G. In order to control the fungal biomass age and concentration in the reactor, they purged and replaced the biomass periodically. The strategy was to change 1/3 of the biomass inside the reactor every 7 days. In this way, they managed to achieve more than 80% decolorization over 40 days by maintaining a young WRF culture in the bioreactor under sterile conditions. They continued their work in a 10 L air pulsed bioreactor using synthetic textile wastewater [65]. Since *T. versicolor* can produce the ligninolytic enzymes as secondary metabolites, they decreased the nitrogen concentration in the media in order to both suppress the bacterial growth and push the fungi towards producing the ligninolytic enzymes as secondary metabolites. They combined this strategy with their previous strategy, i.e. partial renewal of the fungal biomass. Under non-sterile conditions, they could achieve an average of 78% (approximately 10% lower than the sterile trial) decolorization for more than 70 days treating the synthetic wastewater. Based on the successful results of N-limited media and partial biomass renewal strategy, they continued their work with a real textile wastewater. Continuous treatment of real textile wastewater under non-sterile conditions resulted in 60% color reduction for the first week, which, however, dropped to 40% in the second week (total run time of 15 days). Based on microbial analysis results of the wastewater during the treatment period in the reactor, they suggested that the decrease in the decolorization efficiency of the reactor was due to

bacterial contamination. The initial bacterial concentration was high in the real textile wastewater, as they observed a high bacterial growth in the feeding tank of the reactor [65].

1.2.4. Summary of current knowledge on application of WRF under non-sterile conditions

Overall, the proposed approaches to operate a fungal bioreactor under non-sterile conditions can be divided into two general categories. One category focuses on the fungi itself, trying to support WRF to grow and stay active in the presence of other microorganisms. The second category focuses on the media (wastewater) that is fed to the fungal bioreactor. In the second category, the growth of other microorganisms is inhibited. The main tested strategies are summarized in Table 2.

Table 2. Main reported methods in the literature to maintain the activity of WRF under non-sterile conditions

Focus object	Factor investigated	Subject/ Means of control	Ref.
Fungi	Fungal morphology	Fungal granules	[50, 59]
	Fungal incubation/growth	Free cells vs. Immobilized cells	[46, 47, 52, 54, 59]
	Biomass Age/concentration	Fungal biomass renewal	[60,65]
		HRT/SRT	[46,56]
Media	Selective media for fungi against bacteria	Low pH	[47]
		Nitrogen limited conditions	[47,65]
	Pre-treatment	Ozone	[49]
		Heat	[57]
		Bactericide	[54]
		Pre-Aeration	[56]

In summary, the application of white rot fungi to degrade some of the most recalcitrant pollutants in wastewaters, have been shown to be effective in pure culture under controlled sterile conditions. However technical issues involved in the implementation under real industrial conditions have hindered the development of application of WRF at full scale. The main bottleneck is to maintain the fungal

activity over a long period under non-sterile conditions. The main issue is contamination by bacteria, which results in substantial decrease in fungal activity. Contamination of liquid culture of WRF with bacteria usually results in an increase in pH, increased turbidity of the media, lysis of fungal mycelia, and subsequent drop in fungal growth and enzyme production.

1.3. Humics

1.3.1. Humic substances in nature

Humic substances (HS) are the products of decomposition of plant and animal tissues, although they are much more stable than their precursors [66]. HS are formed when organic matter is decomposed in a process called humification, which includes degradation of organic matter and also polymerization of degradation products. Humification concurs with decay and decomposing processes [67]. HS are the most widespread natural organic substances that are ubiquitous in the environment, both aquatic and terrestrial. They are found in sediments, peat, lignites, brown coal, sewage, composts and other deposits [67,68]. HS are not well defined. The functional groups of the HS, which determine their physical and chemical properties, vary and depend on the origin and age of the humics [69]. HS are generally divided into three basic groups based on their solubility in acids and alkalis: humic acid (HA) that is soluble in alkali and insoluble in acid; fulvic acid (FA) that is soluble in alkali and acid, and humin that is insoluble in both alkali and acid. HA generally represent the largest fraction of HS, with MW up to 5-6 kDa in water and up to 500 kDa in soil. FA are typically smaller molecules with MW up to 1-2 kDa in water and 5 kDa in soil [70–72]. HS structures consist of alkyl/aromatic units crossed-linked by mainly oxygen and nitrogen groups with the major functional groups being carboxyl acid, phenolic and alcoholic hydroxyls, keton, and quinone groups [73,74]. This structure facilitates the binding of hydrophobic and hydrophilic compounds, hence making HS a major agent in transport of heavy metals and hydrocarbons in the environment [67,75,76]. The details of the HS structure are still being studied and debated and new insights are being developed, which have been reviewed elsewhere [69,74,77].

In nature, humic substances are extremely resistant to biodegradation [67,69,78]. Their half-decay time can amount to thousands of years. For instance, ^{14}C dating analysis on the soil organic matter from volcanic soils [79] or from the North

American Great Plains [78], has yielded an estimation of hundreds of thousands of years old age for these compounds. Under aerobic conditions, very slow degradation of HS by prokaryotic microbial populations has been reported, with decomposition rate of 1-5% per day [69]. However, the ability of some eukaryotic basidyomycota, namely white rot fungi (see 1.4), has been reported to efficiently degrade humics [24,25,69]. The interactions between the HS and microorganisms, the non/hardly-biodegradable characteristics of HS, the biological oxidation and reduction mechanisms and the complexation of enzymes by HS, have been recently summarized in an excellent review paper [69].

Although bacteria dominate the environment and participate in the turnover of humic substances [78,80], their ability to degrade stable macromolecules such as HA is limited [67,69,81].

1.3.2. Humic substances in wastewater

The wastewater (WW) organic matter derives from a variety of plant and animal products in various stages of decomposition, as well as from chemically synthesized organic products [66, 67, 72]. The organic matter can be divided into two main groups: biodegradable and non-biodegradable or refractory. The biodegradable organic matter of the WW is composed of a vast variety of simple compounds of known structures and consists of carbohydrates, proteins, peptides, amino acids, fats and other low molecular weight (MW) organic compounds. These compounds are generally easy to degrade by microorganisms and mostly will not leave the WW treatment system with the effluent. Therefore, most of the dissolved organic matter in the effluent of a WW treatment plant consists of non-degradable organic substances [82,83]. Humic and humic-like substances usually comprise a large portion of dissolved organic matter in the effluent [82].

HS (mostly HA) often cause environmental problems once released as part of a wastewater into the ecosystem, owing to the solubility and high absorptive reactivity of these acids with heavy metals and xenobiotic compounds in aqueous environments [84,85]. For example, humic acids can carry heavy metal ions, insoluble organic materials and xenobiotics, and increase their solubility and motility in soil and water [69,86,87]. In addition, their presence may lead to the formation of trihalomethanes and other carcinogenic and mutagenic substances by reaction with chlorine dosed in water purification processes [85,88,89]. Furthermore, the presence of humic compounds results in colored (yellow or brown) effluents leading to

esthetic constraints when these effluents are discharged to the environment [90,91]. In wastewater treatment plants, the presence of HS can cause serious technical problems in biological and physico-chemical units. HA can cause severe membrane fouling and clogging in the filtration units [86,92]. Also, it has been reported that their prolonged contact with adsorbents in wastewater treatment units could induce the deterioration of the adsorbents [93]. In anaerobic treatment systems, severe inhibition (>75%) of hydrolysis [94] and methanogens [95,96] by HA was reported, resulting in a reduced yield in methane production [95].

1.4. Humic acids vs. white rot fungi

1.4.1. Biodegradation of humic acids by white rot fungi

HS and especially HA are resistant to bio-degradation by bacteria [69,97,98]. The involvement of bacteria in the degradation of humic substances is mostly limited to utilization of low molecular weight substances like FA, or HA's building blocks and metabolites [25,99]. White rot fungi, on the other hand, as the most efficient lignin degraders, have been implicated in the transformation of HA to lower molecular weight compounds and even mineralization of HA [25,69].

The capability of WRF to degrade HA has probably developed during the evolution of these microorganisms in nature giving them the ability to produce strong oxitative enzymes (known as lignin-degrading enzymes), which defines the natural role of these species in the ecosystem. In general, fungi involved in the decomposition processes in nature include mainly ascomycetes and basidiomycetes, which are abundant in the upper layer of forest and grassland soils. Basidiomycetes, including WRF, traditionally were considered less common in habitats such as agricultural soils. However, recent studies have revealed much greater diversity than was anticipated in this habitat [9,100]. There are about 8500 species of basidiomycetes that are described as lignocellulose-degrading saprotrophs, and about half of them occur in soil and fallen plant litter [100], which shows the natural growth of these species in humic-associated environments. Although it is known that they are effective in the degradation of HA, their role and mechanisms of their involvement in the turnover of humics is not fully elucidated [101,102]. Interesting results of a study [103] related to the global warming, showed that over a 6 years period under elevated CO_2 levels, 50% reduction in the soil carbon content was detected. This reduction in carbon content of the soil was driven by the high activity of the

microbial/fungal community of the soil. Interestingly, soils exposed to elevated CO_2 had higher relative abundance of fungi (to bacteria) and higher activity of soil carbon-degrading enzymes. The increased fungal abundance in soils exposed to elevated CO_2 has been reported before. [104-106]. Fungi generally have higher carbon/nitrogen ratios than bacteria, which lessen their demand for nitrogen [107]. On the other hand, Soil exposed to elevated CO_2 levels, have less available nitrogen [103], probably due to increased photosynthesis and nitrogen fixation by plants [103,107]. The lower nitrogen dependency in fungi (compared to bacteria), together with reduced nitrogen availability in soils exposed to elevated CO_2, could explain the increased relative abundance of fungi, in these soils[107,103]. Overal, these observations emphasize the natural role of fungi in humic turnover. Among the basidiomycetes, WRF have been reported to be the most efficient species capable of degradation, transformation and even mineralization of HA [98,108–110].

1.4.2. Ambiguities and knowledge gaps

Although the degradation of HA by WRF have been studied and demonstrated before, some serious ambiguities still exist regarding the mechanisms involved. It has been reported that the enzymes (making up the so-called ligninolytic system) of WRF are the main role players in HA degradation by WRF [108,109]. However, there are some contrasting reports on condensation, polymerization and further humification of humic substances by WRF enzymes [111,112]. The ambiguities in the interaction between WRF and HA even goes deeper and involve certain enzymes and their interaction with HA molecules. For example, it has been reported that there is a direct correlation between the laccase activity and HA degradation [109,113], suggesting the direct involvement of laccase in HA degradation. In contrast, it has been reported that laccase can polymerize HA, resulting in an increase in its MW or even its concentration [111]. Furthermore, there are contradicting reports on stimulation of laccase activity by HA [113–115], and also inhibition of laccase by HA [25,111].

Since HA are not well defined and their measurement could be complicated and time consuming, there are some ambiguities about the measurement methods and the analytical techniques applied in studying HA degradation by WRF. For example, accepting that the decolorization of HA is an indication of its removal, still it could be due to biodegradation, conversion or biosorption of HA. For instance, in some

studies the decolorization of HA has been used to assess the biodegradation of HA, whereas bio-sorption was not checked [25,116].

The decolorization of HA, measured at 400-500 nm, has been accepted as an indication of the decrease in HA concentration in that solution [25,109]. However, increase in color of HA solution as a result of the degradation by WRF enzymes, and decrease in color as a result of the polymerization of humics also has been reported [111]. Also, it is known that the growth of microbial species could result in a change in the color of their culture media, which perhaps could interfere with the color of HA.

The change in the average molecular weight (MW) of HA before and after the treatment has been used previously to investigate polymerization/depolymerization of HA [117,118], although the reliability of this method is questionable. The reason is that the MW could be a fair indication of HA degradation if there is no biosorption of HA occurring in the process. As mentioned before, HA constitute a wide range of molecules with different MW. If larger HA molecules are absorbed to the biomass more than the smaller HA, the MW of HA content of the wastewater decreases, without any degradation of HA occurring.

Most of the previous studies on the application of WRF for removal of HA from wastewater, have been conducted using synthetic wastewater, using HA isolated from soil, coal or compost, as it has been summarized elsewhere [25]. It was reported that the origin, environmental conditions and 3D structure of HA could significantly affect its biodegradability by WRF [25,69,111]. Therefore, the results that have been achieved by application of WRF in synthetic wastewater could be challenged when real wastewater is used.

1.5. Research outline

The main objective of this thesis is to develop a fungal reactor to treat HA-rich industrial wastewater under non-sterile conditions. To this end, sub-objectives were set as follows:

- Screen and select WRF strains capable of removing HA from water (chapter 2)
- Explore the mechanisms involved in HA removal by fungi and clarify the current ambiguities (chapter 2, 3)

- Use WRF for treatment of real industrial HA-rich wastewater (chapter 3)
- Apply WRF under non-sterile conditions (Chapter 4,5,6)
- Design a suitable bioreactor and bioprocess for application of WRF under non-sterile conditions (chapter 5,6)

The outline of this thesis can be divided into two parts. The first part describes the proof of principles, focusing on the HA removal by WRF and the mechanisms involved. In chapters 2 and 3, several different strains of WRF were screened for their ability to remove HA in solid and liquid phase. The mechanism of HA removal by selected WRF strains were studied, and also ambiguities and knowledge gaps mentioned in the introduction (1.4.2) were addressed. Chapter 2 is focused on synthetic wastewater and chapter 3 deals with real industrial wastewater, both under sterile conditions.

The second part of the thesis is focused on the non-sterile application of WRF. In Chapter 4, a novel approach for the application of WRF under non-sterile conditions is proposed. The highlight of this chapter is immobilization of WRF on sorghum as the main nutrient source to facilitate the application of fungi under non-sterile conditions. Furthermore, in chapter 5 and 6, the application of WRF in bioreactors under non-sterile conditions was studied. In chapter 5, a process that was recently developed to apply pellets of WRF under non-sterile conditions, was adopted from literature and applied for HA removal from synthetic and real wastewater. The highlight of this process is the periodic partial renewal of fungal biomass. In chapter 6, the new technique that was developed and reported in chapter 4, i.e. immobilization of WRF on sorghum, was combined with the process that was tested in chapter 5, i.e. partial renewal of fungal biomass, and applied for continuous treatment of synthetic and real humic-rich wastewater under non-sterile conditions. Fig 1, shows the schematic outline of the research, which could be instrumental in understanding the flow of the research and the relationship between the chapters.

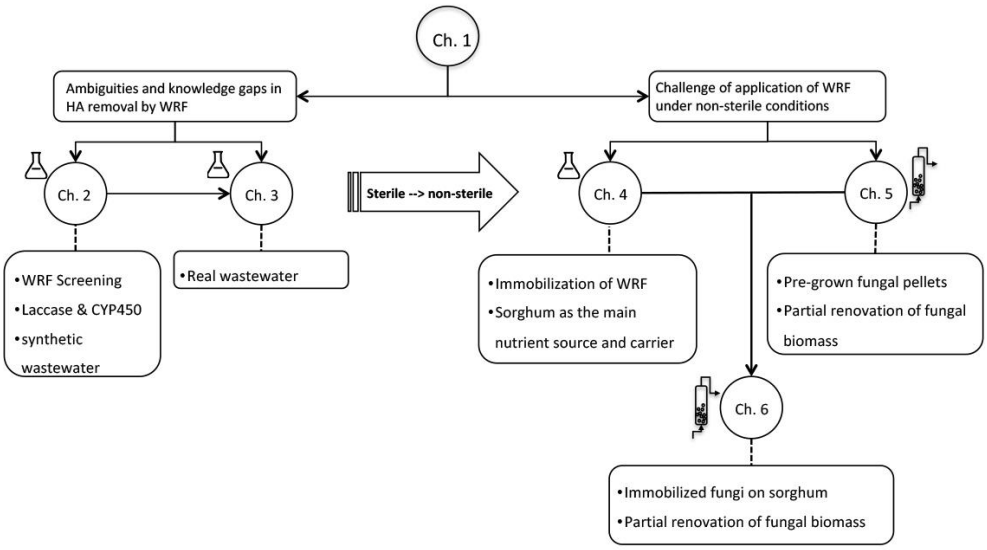

Fig 1. Schematic of the thesis outline. Arrows indicate the information flow between the chapters. The symbols (flask/reactor) beside each chapter show whether the experiments were performed in flasks or reactors.

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Chapter 2

Bioremoval of humic acid from water by white rot fungi: exploring the removal mechanisms

Abstract

Twelve white rot fungi (WRF) strains were screened on agar plates for their ability to bleach humic acid (HA). Four fungal strains were selected and tested in liquid media for removal of HA. Bioremediation was investigated by HA color removal and changes in the concentration and molecular size distribution of HA by size exclusion chromatography. *Trametes versicolor* and *Phanerochaete chrysosporium* showed the highest HA removal efficiency, reaching about 80%. Laccase and manganese peroxidase were measured as extracellular enzymes and their relation to the HA removal by WRF was investigated. Results indicated that nitrogen limitation could enhance the WRF extracellular enzyme activity, but did not necessarily increase the HA removal by WRF. The mechanism of bioremediation by WRF was shown to involve biosorption of HA by fungal biomass and degradation of HA to smaller molecules. Also, contradicting previous reports, it was shown that the decolorization of HA by WRF could not necessarily be interpreted as degradation of HA. Biosorption experiments revealed that HA removal by fungal biomass is dependent not only on the amount of biomass as the sorbent, but also on the fungal species. The involvement of cytochrome P450 (CYP) enzymes was confirmed by comparing the HA removal capability of fungi with and without the presence of a CYP inhibitor. The ability of purified laccase from WRF to solely degrade HA was proven and the importance of mediators was also demonstrated.

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2.1. Introduction

2.1.1. Humic substances

Humic substances are the most widespread natural organic substances that are ubiquitous in the environment, both aquatic and terrestrial. They are found in sediments, peat, lignites, brown coal, sewage, composts and other deposits [1,2]. Humic substances are not well defined, but are generally divided into three fractions based on their solubility in acids and alkalis: humic acid (HA) that is soluble in alkali and insoluble in acid; fulvic acid (FA) that is soluble in alkali and acid, and humin that is insoluble in both alkali and acid. Humic substances are comprised mainly of aromatic, aliphatic, phenolic and quinonic components, which are covalently bound through C-C, C-O-C and N-C bonds [1]. In nature, humic substances are extremely resistant to biodegradation [3]. HA can absorb heavy metals and xenobiotic compounds, hence increase their solubility and mobility in water. Also during chlorination of treated water, HA can cause the formation of trihalomethanes and other carcinogenic and mutagenic substances. Therefore their presence in industrial effluents can cause damage to the ecosystem. [4,5]. Furthermore, the presence of humic compounds results in colored effluents leading to esthetic constraints when these effluents are discharged to the environment [6,7].

Microorganisms are the driving force behind the formation, transformation, degradation and mineralization of humic substances. Although bacteria dominate the environment and participate in turnover of humic substances [3,8], their ability to degrade stable macromolecules such as HA is limited [9].

2.1.2. White rot fungi

White rot fungi (WRF) are the most abundant wood degraders in nature, which possess the unique ability of efficiently degrading lignin to CO_2 [10,11]. They are also able to decompose several aromatic pollutants or xenobiotics and thus can be used in (waste) water treatment [12,13]. The non-specific nature of WRF enzymes has been reported to be the key factor in their ability to degrade some complex aromatic polymers with molecular structures similar to lignin [14,15]. Extracellular Peroxidases and laccase have been reported to be the key enzymes involved in the degradation of recalcitrant aromatic polymers by WRF [15–17]. Cytochrome P450 (CYP) enzymes comprise another group of enzymes of which their involvement in

the degradation of some organic compounds by WRF has been demonstrated [18–20]. CYPs are membrane-bound hemoproteins that catalyze hydroxylation, epoxidation and monooxygenation reactions [21,22]. In fungi, CYPs are involved in the biosynthesis of secondary metabolites and ergosterol, and also in catabolic reactions that lead to the degradation of xenobiotic compounds [22].

2.1.3. Ambiguities and knowledge gaps

The removal of HA by WRF has been studied before, and it was shown that WRF can degrade humic acid [23,24]. However, there are still ambiguities regarding the level of biosorption and degradation/transformation of HA by WRF. The decolorization of an HA solution has been accepted as an indication of the decrease in HA concentration in that solution [1] , which could be due to degradation, conversion or sorption of HA. Yet the decolorization has also been used to estimate the degradation of HA [24,25]. The ability of laccase to degrade HA has been studied before. However, results are not in agreement. Fakoussa *et al.*[26] and Steffen *et al.* [27] observed a correlation between laccase activity and the degradation of HA in a WRF culture and concluded that laccase is involved in the degradation of HA by WRF. On the contrary, Zavarzina *et al.* [28] reported that laccase could polymerize HA. They also reported that the degradation of HA by WRF's laccase could result in an increase in color, whereas polymerization of HA was leading to a decrease in the color. Besides, it was reported that HA have an inhibitory effect on laccase activity, which is in contrast with results that show a stimulation of laccase activity in the presence of HA [29].

In this study, the ability of 12 different strains of WRF to remove HA from solid and liquid media was investigated. HA color removal was compared with changes in concentration and molecular size distribution measured by size exclusion chromatography (SEC) to clarify the ambiguity about the interpretation of decolorization of HA, whether it is indicating degradation of HA or only decrease in its concentration. The SEC results were analyzed numerically and the reliability of average molecular weight (calculated based on the SEC results) to conclude the degradation of HA was investigated. The correlation between the SEC results and color measurement was studied to clarify whether the degradation of HA result in a decrease or an increase in the color. Mechanism of HA removal by WRF was explained by distinguishing the sorped HA from degraded/transformed HA. The extracellular enzyme activities were measured, and the relationship between

extracellular enzyme activities of WRF and HA removal was investigated. The ability of laccase to degrade HA was studied using pure laccase, and also the effect of different mediators on the performance of laccase was tested. Additionally, the contribution of cytochrome enzymes to HA removal by WRF was studied.

2.2. Material and Methods

2.2.1. Fungal strains and chemicals

Twelve WRF strains (Table 1) were obtained from the fungal stock culture collection of Wageningen University and Research (Wageningen, the Netherlands) and DSMZ (Germany). The fungal strains were pre-cultivated on 2% malt extract agar, and subcultures were made periodically every 40 days to keep the cultures fresh. All the chemicals including coal humic acid powder and pure fungal laccase (from *Trametes versicolor*) were purchased from Sigma-Aldrich (Germany), unless otherwise stated.

2.2.2. Media

2.2.2.1. Preparation of HA stock solution

Humic acid powder (4 g) was dissolved in 200 mL of NaOH solution (0.1 M) and mixed for 30 min. The solution was centrifuged (7000 rpm, 20 min) to remove the particulates. Then 100 mL of phthalate buffer (0.5 M) was added to the particulate-free HA solution, and pH was adjusted to 4.5 with HCl. The buffered solution was centrifuged again (7000 rpm, 20 min) and the supernatant was used as the HA stock solution. For each set of experiments a fresh batch of the stock solution was prepared. The concentration of humics in the stock solution was determined by drying (48 hr, 100 °C) 30 mL of the stock solution (triplicate) and deducting the weight of the buffer from the dry weight. The humics concentration of the stock solution was 8 $g.L^{-1}$ (± 0.4 $g.L^{-1}$). For all the experiments HA stock solution was filtered (0.45 μm) prior to use.

2.2.2.2. Liquid media

Liquid media was adapted and simplified from the defined culture media for growth and enzyme production of WRF as described by Kirk *et al.* [30]. Defined media was prepared as defined nitrogen limited (NL) and nitrogen sufficient (NS) media

including basal media, minerals (trace elements) and vitamins along with carbon and nitrogen sources. In all cases, media contained glucose as carbon source (56 mM) unless otherwise stated. Ammonium tartrate was used as the nitrogen source in a final concentration of 2 mM for NL media and 20 mM for NS media. Media was supplemented with sodium phthalate buffer (10 mM, pH 4.5). The defined media were spiked (50 $mL.L^{-1}$) with HA from HA stock solution when needed (final concentration of HA in media: ≈ 400 $mg.L^{-1}$).

2.2.2.3. Solid media

Two types of media were prepared to evaluate the ability of the WRF to grow in the presence of HA. The media Type "A" contained only HA (~ 250 $mg.L^{-1}$) as the carbon source, along with the other elements of NS medium. Media Type "B" was basically the Kirk medium (NS) with glucose as the carbon source, dosed with HA (~ 250 $mg.L^{-1}$). Both media were supplemented with 10 $g.L^{-1}$ Agar for solidification.

2.2.3. Experimental procedures

2.2.3.1. Pre-screening on agar plates

Plastic petri dishes (90 mm inner diameter) containing about 35-40 mL solid media were used in pre-screening experiments. All petri dishes were inoculated with an agar piece (5x 5 mm) of the respective pre-cultivated fungi. Plates were made in triplicates for each media type and fungal strain and incubated at 25 °C for 15 days in the dark. The fungal growth and decolorization of HA were both estimated qualitatively by observing the diameter and density of the mycelia on the agar plate and the bleaching of the agar medium.

2.2.3.2. HA removal by fungi from water

Selected fungal strains from the pre-screening experiment were used in the defined NL and NS liquid media. Experiments were done in 500 mL flasks filled with 150 mL defined media, inoculated with five pieces of fungal agar. Bioremediation flasks were prepared in quadruplicate, two of which were subjected to sampling during the incubation period for the analysis. The other two were kept intact and only used at the end of the incubation for the recovery process. Flasks were closed with cotton stoppers and incubated in a shaker incubator (26 ± 1 °C, 150 rpm).

Three different sets of controls were prepared to ensure that the observations of the experiments were linked to the fungi. The first set of controls were uninoculated NL and NS media supplemented with HA (uninoculated controls), to reveal any chemical interaction between the defined media and HA, verifying the stability of HA in the media. The second set of controls was NL and NS media inoculated with the respective fungal strains, in the absence of HA (HA-free control). This was done to monitor any changes in the color of the media as a result of the fungal growth, as well as a possible production of metabolites that can interfere with HA analysis via SEC. The third control was uninoculated HA solution without defined media, to test the stability of HA solution itself with regard to color and MW distribution.

2.2.3.3. Recovery of sorped HA from fungal mycelia

To recover the sorped HA from mycelia, a weighted amount of NaOH was added to each jar to a final concentration of 1 M (pH >12), and then the fungal mycelia were disrupted by means of vigorous mixing for 2 hours, followed by 2 min of sonication. At the end, samples were withdrawn and filtered through 0.45 μm filters following the procedure of Ralph and Catcheside [31,32].

2.2.3.4. HA sorption by deactivated fungal mycelia

In order to investigate the capability of fungal mycelia for biosorption of HA, four fungal strains were grown separately in potato dextrose broth (PDB) in a shaker incubator for 10 days (26 ± 1°C, 150 rpm). Fungal biomass, grown in the form of pellets, were collected and washed five times with distilled water. Afterwards the jars containing the fungal pellets in distilled water were autoclaved (121°C, 20 min) to deactivate the fungi. The deactivated (heat-killed) fungal pellets were then used for the biosorption experiment. Different amounts of biomass for each fungal strain were used to study the HA sorption on fungal biomass. The fungal biomass was transferred to 4 flasks containing 100 mL HA solution (~ 200 mg.L^{-1}, pH 5) in 4 levels. The same amount of pellets were also filtered and dried (100°C, 24 hours) and weighted separately to estimate the dry biomass weight (DBW) of each experiment. Flasks were kept in a shaker incubator for 48 hours (26 ± 1°C, 130 rpm). At the end of the incubation period, 1 mL samples were withdrawn from each flask to be analyzed for color. Then the HA was recovered to estimate the efficiency of the recovery procedure via SEC analysis. Experiments were done in duplicate under sterile conditions.

2.2.3.5. HA degradation by purified laccase

The degradation of HA by laccase was performed in triplicate in 150 mL flasks containing 50 mL of laccase solution at 500 $U.L^{-1}$ in malonate buffer (pH 4.5). The effect of mediators was investigated by the addition of 1-hydroxybenzotriazol hydrate (HOBt), 2,2-azino-bis-(3- ethylbenzthiazoline-6-sulfonic acid (ABTS) and violuric acid (VA) at the final concentration of 1 mM (Mir-Tutusaus *et al.* 2014). Flasks were spiked with 2 mL of HA stock solution and kept in a shaker incubator for 48 hours (26 ± 1 °C, 130 rpm) under sterile conditions. 2 mL samples at designated times were withdrawn and analyzed by SEC.

2.2.3.6. Cytochrome P450 inhibition

To study the involvement of CYP enzymes, the fungal pellets were incubated in the presence of 1-aminobenzotriazole (ABT) as the CYP enzymes inhibitor to hinder the activity of these enzymes [33]. Fungal pellets (~ 8 g wet weight) were incubated in NL and NS media (100 mL flasks containing 25 mL media) for 7 days and 1 mL samples were withdrawn at designated times for analysis. All flasks were prepared in triplicate.

2.2.4. Analytical methods

2.2.4.1. Size exclusion chromatography (SEC)

Samples for SEC analysis were prepared by separating humic acid- like molecules from the media. Each sample (2mL) was acidified (pH <2) by adding 20 µl HCl (37%), and centrifuged (14000 rpm, 20 min). The acid supernatant was separated as FA, and the precipitants were re-suspended by adding 2 mL NaOH (0.1 M) and used as HA portion of the sample (Hofrichter and Fritsche 1996). Both FA and HA portions of the samples were analyzed by SEC. The SEC was conducted using Phenomenex column (Yarra™ 3 µm SEC-2000, LC Column 300 × 7.8 mm, Ea) connected to an ultra fast liquid chromatography (UFLC) system (Shimadzu, Prominence) to detect changes in the concentration and MW of HA (and FA) molecules during the incubation with WRF. The method was adapted from a protocol that has already been developed for molecular size fractionation of HA [34], with slight modification. The mobile phase was 25% acetonitrile in ultra pure water supplemented with 10 mM sodium phosphate buffer (pH 7). The flow rate of the mobile phase was 1 $mL.min^{-1}$ and the injection volume was 10 µl. Polystyrene sulfonate standards (Polymer Standard Service, Germany) were used for the

calibration of the column. Separation was done at 25°C for 16 min and eluted substances were detected at 254 nm.

2.2.4.2. Other analysis

Decolorization of HA was assessed by measuring light absorbance at 450 nm [35].

Extracellular enzyme activities were determined spectrophotometrically in culture supernatant obtained by filtering through 0.45 µm syringe filters. Lignin peroxidase (LiP) was assessed at 30°C using veratryl alcohol as substrate [36]. MnP activity was assayed using Mn(II) as the substrate [37]. Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxiphenol (DMP) as described before [38]. The enzyme activities were expressed in units (U: micromoles.min^{-1}).

Fungal growth was assessed by measuring the dry biomass weight (DBW) of the fungal mycelia. The fungal biomass was harvested through filtration (10 µm paper filters, pre-weighted) and dried in pre-weighted aluminum cups (100°C, 48 hours). The net weight of the cups with and without the fungal biomass was calculated as the DBW.

2.3. Results

2.3.1. Pre-screening on agar plates

A total of 12 WRF strains were tested during the pre-screening experiment using HA agar medium, with and without the additional carbon source. The fungal growth on the media was evaluated qualitatively based on observation of the diameter and density of growth around the inocula. Bleaching was also evaluated qualitatively by observing the horizontal and vertical bleaching of HA agar around the inocula. The typical growth of fungi in agar plate containing humic acid as well as bleaching of HA agar is shown in Fig1.

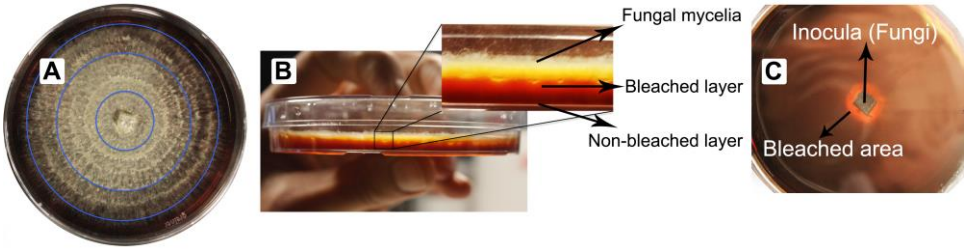

Fig 1. A: Fungal growth on the humic-agar plate. B: Vertical Bleaching of humic acid agar media by WRF, C: Horizontal bleaching

The results of the growth of the WRF strains and their bleaching effects on HA-agar medium are presented in Table 1. The levels of growth and bleaching are specified with numbers, from 0 for no growth (no bleaching) to 4 for excessive growth (bleaching).

Table 1. Qualitative results of pre-screening on agar plates. Media "A": HA as sole carbon source, Media "B": with additional carbon source (PDB)

Fungal strain	Growth		Bleaching	
	Media A	Media B	Media A	Media B
Clitocybula dusenni MES11937	0	1	0	0
Trametes suaveolens MES12281	0	2	0	0
Trametes suaveolens MES11922	0	1	0	1
Trametes versicolor DSMZ 3086	0	4	0	3
Trametes versicolor MES02055	0	2	0	1
Ceriporiopsis subvermispora MES13094	0	1	0	1
Pleurotus sajor-caju MES03464	0	4	0	2
Pleurotus ostreatus MES00036	0	4	0	2
Pleurotus ostreatus MES00050	0	3	0	1
Pleurotus ostreatus MES01475	0	1	0	1
Pleurotus ostreatus MES03772	0	3	0	1
Phanerochaete chrysosporium DSMZ 1556	0	3	0	2

In the media type "A", using HA as the sole carbon source, no significant growth was observed for any of the WRF strains. In media type "B" on the other hand, growth was observed. This shows that WRF strains could not utilize HA as the sole carbon source and needed additional carbon sources to grow.

Based on growth and bleaching results of the pre-screening experiments (Table 1), 4 strains of WRF, *Pleurotus sajor-caju* MES03464, *Pleurotus ostreatus* MES00036, *Trametes versicolor* DSMZ 3086 and *Phanerochaete chrysosporium* DSMZ 1556 were selected for screening in the liquid phase.

2.3.2. Screening in liquid media (water)

The SEC analysis of the uninoculated HA solution showed that HA is a complex of molecules with a broad range of MW from 6.5 kDa to less than 0.5 kDa. In order to facilitate the comparison of SEC results during the fungal treatment experiments, each SEC chromatogram was sliced into three separate areas based on the main peaks, as it is shown in Fig 2. The large (high molecular weight) HA molecules, having the molecular weight of 1-6.5 kDa (blue), comprised most (54%) of the HA complex. The medium size HA and building blocks, weighing between 1 and 0.5 kDa (red) covered around 26%, and small (low molecular size) HA weighing less than 0.5 kDa (green) covered the rest of the HA complex (20%). The total height of HA column (Fig 2) representing the total area under the curve of HA chromatogram, can be used to qualitatively monitor variations in the concentration of HA. Also changes in the ratio between different portions of the HA (blue, red and green areas) indicate changes in the MW distribution of the HA complex. The average MW of HA was 1.41 kDa (± 0.05). The FA portion of the HA solution was also subjected to SEC analysis. Although the HA solution was made using HA powder, still a relatively narrow peak was observed in the SEC chromatogram of the acid soluble portion of the HA solution (data not shown). The average MW of these fulvic-like molecules was 0.2 kDa (± 0.03).

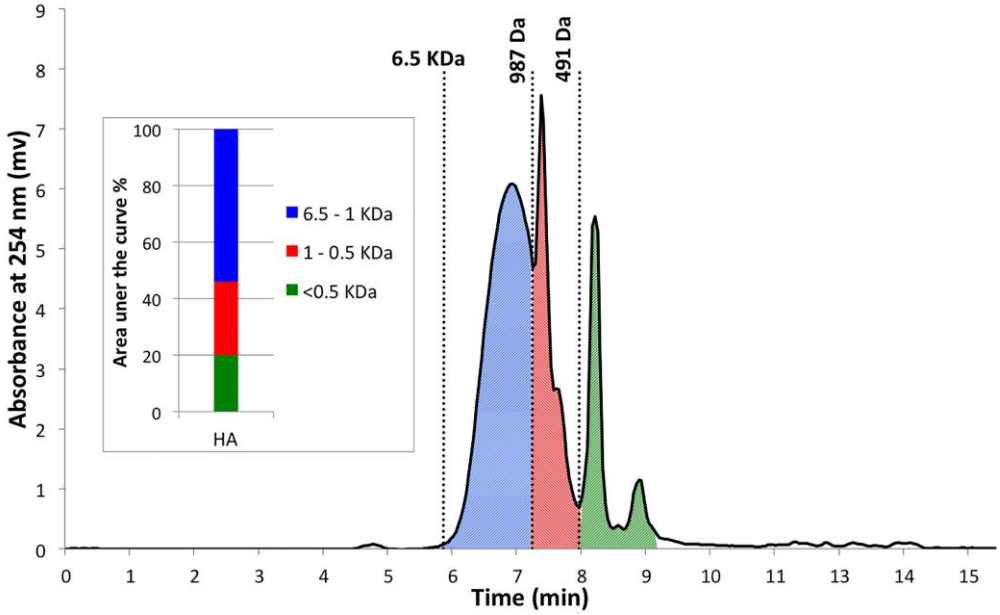

Fig 2. SEC chromatogram of HA and its stacked column presentation

The results of the HA color removal along with the results of the SEC analysis are shown in Fig 3. The presented results of color measurement in Fig 3 were corrected for the HA-free controls. Performing the recovery procedure on HA-free controls revealed that the color of fungal media even in the absence of HA, increased significantly during the recovery process (data not shown). Therefore it was not reasonable to use color as an indicator of HA concentration after the recovery process. The SEC results of the HA-free controls showed that no metabolites were produced during the incubation nor during the recovery process, that can interfere significantly with the SEC analysis of HA. Therefore, the SEC results can be used for analysis of HA during the incubation and after the recovery procedure. The uninoculated controls did not show any significant change in the color (<5%) nor in MW distribution, indicating the high stability of the HA solution.

T. versicolor and *P. chrysosporium* had the largest bleaching effect on the HA media of about 80% color removal. *P. chrysosporium* removed 80% of the color after 18 days of incubation in the NS media, and 40% in the NL media. *T. versicolor* showed a different behavior, removing 50% of the color after 18 days in NS media and 75-80% in NL media.

SEC results clearly indicated that *P. chrysosporium* could completely remove the large HA (blue) after 18 days of incubation in NS media. The removal of large HA by *T. versicolor* was incomplete in the NS media, although complete removal of large and medium size HA was achieved in the NL media. Looking at the different portions of humic acids (blue, red and green areas) after 18 days, for all the experiments except *P. chrysosporium* in NL media, a decrease in the concentration of large HA molecules (blue zones) coincided with an increase in the concentration of smaller HA molecules (red or green zones), suggesting incomplete degradation of large HA molecules to smaller HA molecules.

The changes in the concentration of FA were less significant than for HA molecules. For *T. versicolor*, 15% increase in FA concentration was observed after 18 days of incubation in NL media. However, in NS media, the FA concentration slightly decreased. *P. chrysosporium* and *Pleurotus* species showed a slight increase in FA concentration (5-10%). The correlation between the HA removal and increase in FA concentration suggests the conversion of HA molecules to FA molecules, which is in line with some previous reports [39].

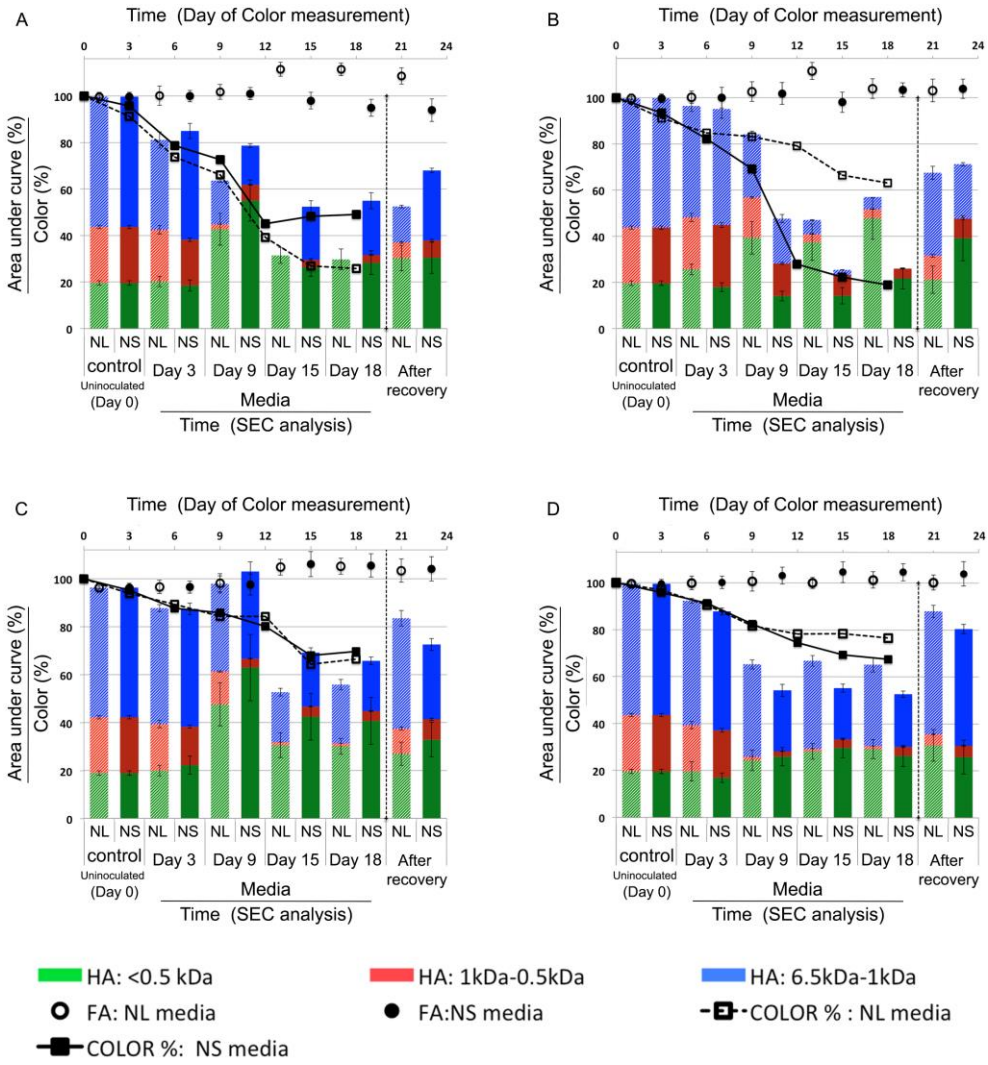

Fig 3. Decolorization of HA by four fungal strains and SEC results (area under the curve) of HA and FA content of the media. A: *T. versicolor*, B: *P. chrysosporium*, C: *P.sajor-caju*, D: *P.ostreatus*

The results of the SEC analysis after desorption of HA from fungal mycelia (recovery) are most important to understand the involvement of biosorption in the removal of HA by WRF. In case of *T. versicolor* in NL media, after the recovery (desorption), almost 50% of the initial HA was detected in the media via SEC, which means that from the 70% reduction in HA concentration after 18 days, at least 20% was due to the biosorption of HA molecules to fungal mycelia. It is obvious that for

both NL and NS media, the ratio between the three portions of HA (blue, red, green areas) was shifted towards the smaller molecules (Fig 3, A, after recovery). The MW distribution was changed from 54%: 26%: 20% (large: medium: small) to (when normalized to 100%) 29%: 13%: 58% for NL media and 44%: 11%: 45% for NS media. The HA average MW after the recovery was reduced from 1.41 kDa to 0.8 kDa in the NL media and 1.1 kDa in the NS media. Another important observation is the total area under the SEC curve, as an indication of HA concentration. In the culture of *P. chrysosporium*, the ratio between the three different portions of HA after recovery in NL media was 53%: 16%: 31% (large: medium: small) and in NS media it was 33%: 12%: 55%. Interestingly, the ratio (MW distribution) in the NL media (after the recovery) is close to the initial composition of HA, although about 30% degradation/transformation of HA was observed from total area under the curve. The average MW of HA in the NL media was 1.33 kDa and in the NS media it was 0.9 kDa. The results of the SEC analysis of *P.ostreatus* in the NS media shows that after fungal treatment (Fig 3, D, after recovery) the HA was comprised of 62% large HA (blue), 6% medium size (red) and 32% small HA substances (green). The average MW of the HA was 1.49 kDa, which indicates polymerization of HA when compared to the initial average MW of (1.41 kDa). Although from the total area under the SEC curve (height of the column), it is apparent that around 20% of HA were either degraded to smaller non-aromatic molecules or degraded/transformed to FA. Therefore monitoring the average MW of the HA complex is not a reliable way to draw conclusions about depolymerization or polymerization of HA. The reason is that depolymerization of HA could result in non-aromatic products or FA-like substances, which obviously will not be detected during the SEC analysis of HA.

The recovery results did not show any significant increase in the FA concentrations. This suggests that FA molecules have low affinity to biosorption by fungal mycelia.

As it can be seen in Fig 3, there is a correlation between the total area under the HA chromatogram (height of the column) and the color of the media during the incubation period, which confirms the reliability of color measurement to monitor the HA concentration in the media during the incubation period.

Extracellular enzyme activities of the tested WRF strains were measured to investigate the possible role of these enzymes in the degradation of the HA molecules.

The extracellular enzyme activities are shown in Fig 4. Laccase, MnP, and LiP were measured during the incubation period. None of the fungal strains showed LiP

activity except for *P. chrysosporium* that showed low and not verifiable (large differences in repetitions) LiP activities, on days 6 and 9 of the incubation, although for the rest of the incubation period no significant LiP activity was detected. This may be due to the several reasons that have been suggested previously, such as inhibition of LiP by humic compounds and certain difficulties of the assay method [32,35,40], inhibition of LiP activity of WRF due to the agitation (shaking) of the culture [41,42] or the absence of veratryl alcohol in the culture media used in this study, since it can induce and mediate the LiP activity [43,44]. The lack of LiP activity in the culture of the specific strain of *P. chrysosporium* that we used (DSMZ 1556), is not unprecedented [45]. Therefore LiP activity was excluded from the graphs in Fig 4.

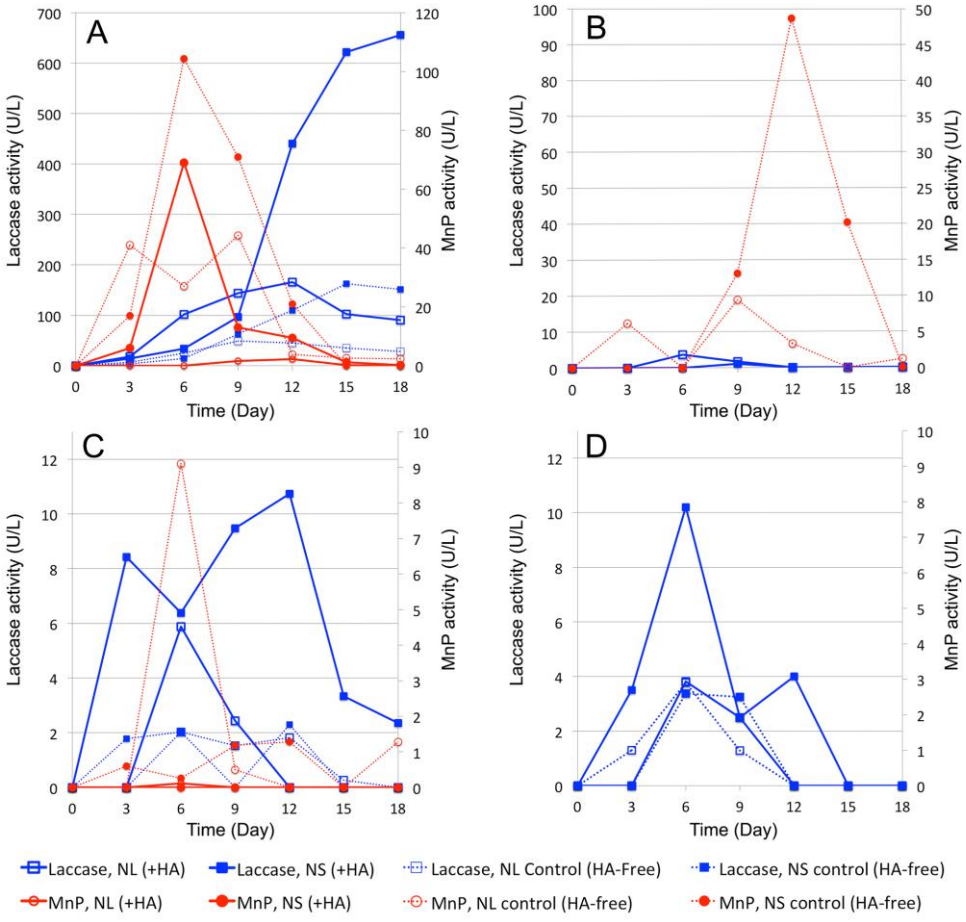

Fig 4. Extracellular enzymes activities of four tested fungal strains in NL and NS

media with and without the presence of HA. A: *T. versicolor*, B: *P. chrysosporium*, C: *P.sajor-caju*, D: *P.ostreatus*. Note the difference in Y-axis scale.

The measured MnP activity was much lower in the media containing HA compared to the control jars (media without HA). It has been reported before that the presence of HA in the media, interfere with the measurement of MnP, and results in underestimation of MnP activity [32,35]. Therefore, it is not realistic to compare the level of MnP activities between fungal species in the presence of HA. However, the MnP activity in HA-free controls could be used qualitatively to prove the ability of fungal strains to produce MnP. When comparing the results of the laccase activity in media with and without HA in Fig 4, it can be seen that in the presence of HA higher laccase activity was detected. The results suggest that HA could induce the laccase activity of WRF, which is in agreement with previous studies [27,46], and in contrast with some reports on inhibition of laccase by HA [28].

P. chrysosporium did not show any enzyme activity in the NS media during the first week but then started to produce MnP at a high rate. It is known that the MnP production by *P. chrysosporium* is part of a secondary metabolism that is triggered by scarcity in nutrients, namely nitrogen [10,47]. The difference in the MnP production by *P. chrysosporium* could be explained by secondary metabolism conditions. *P. chrysosporium* in NL media enter the secondary mechanism conditions sooner than in NS media, so the MnP activity was detected sooner. In the second week, when *P. chrysosporium* enters the secondary metabolism phase in NS media, higher fungal biomass concentration in NS media results in higher MnP activity compared to NL media. For *Pleurotus* species, it seems that the production of extracellular enzymes was not part of a secondary metabolism, since enzyme activity was always higher when growing in NS media. *T. versicolor* showed high extracellular enzyme activities both in NL and NS media. It is known that *T. versicolor* can produce the extracellular enzymes both under limited nitrogen concentration (as secondary metabolites), and also in the presence of high nitrogen concentration [48,49].

T. versicolor showed higher laccase and MnP activity when growing in NS media, but showed more humic removal in NL media. When comparing the final concentration of HA after recovery in NL and NS media of *T. versicolor*, the HA concentration is higher in NS media, suggesting higher HA degradation in NL media, regardless of the higher enzyme activity in NS media. *P. chrysosporium* did not show any significant laccase activity, although it showed high MnP activity

especially after 12 days of incubation in NS media. When comparing Fig 3 and Fig 4, for *P. chrysosporium*, it seems that the humic acid removal correlates with the MnP activity. Also, the SEC results after the recovery of sorped humics show higher recovery of large HA molecules (blue) in NL media than in NS media, suggesting higher degradation of HA in NS media. This correlates with the higher enzyme activity of *P. chrysosporium* growing in NS media.

In order to study the effect of HA on the growth of WRF, the growth of WRF biomass was measured with and without the presence of HA. The results are shown in Fig 5 as dry biomass weight (DBW) at the end of the incubation period.

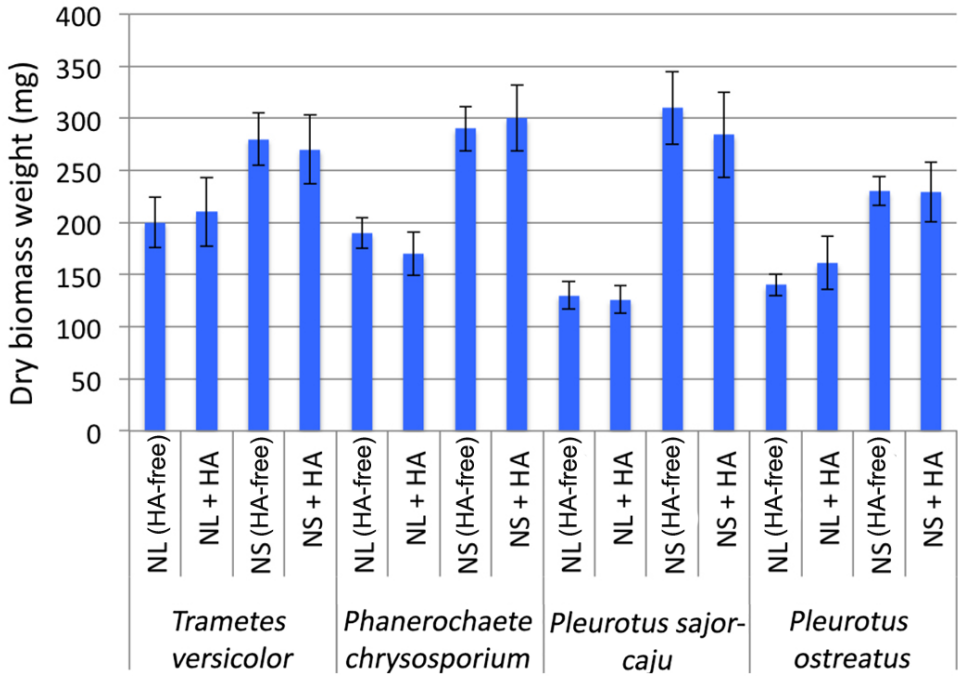

Fig 5. Weight of dry biomass produced after 18 days for four WRF strains

The presence of humic acid in some cases enhanced the fungal growth and in some cases hindered the growth, especially for *Pleurotus sajor-caju*. These results can be compared with previous studies on the effect of humic and humic-like compounds on WRF's growth [50,51]. When comparing the results of DBW in Fig 5 with the results of humic removal in Fig 3, there is no clear correlation between the increase or decrease of fungal biomass and humic removal. *T. versicolor* produced less biomass in NL media than in NS media (Fig 5), but it removed more humics from

water (Fig 3). This suggests that biosorption is not necessarily the main mechanism of humic acid removal by WRF.

2.3.3. Biosorption of HA by deactivated fungi

The biosorption of HA by deactivated WRF (shown by the decrease in color) is apparent from the results shown in Fig 6, and it seemed to be dependent on fungal species as well as the amount of biomass as sorbent.

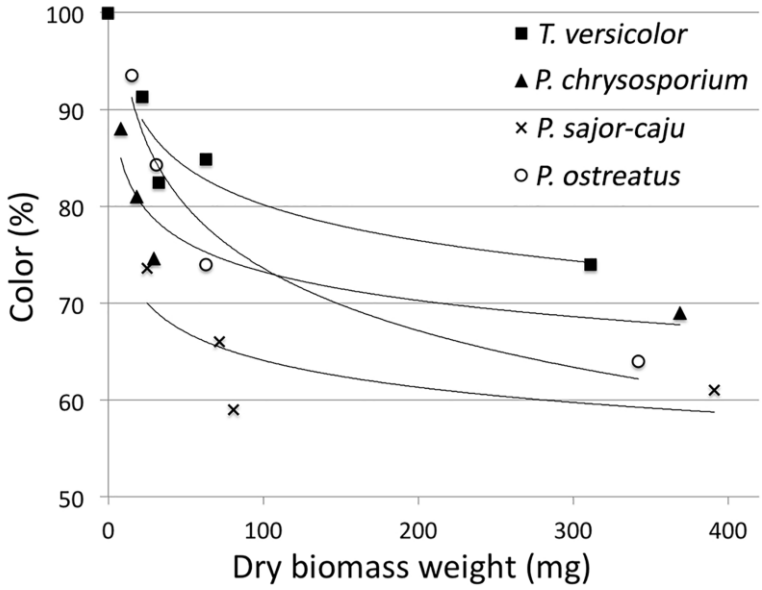

Fig 6. Biosorption efficiency as Color removal by 4 different deactivated WRF

P. sajor-caju showed the highest affinity for the biosorption of HA and *T. versicolor* showed the lowest affinity. The biosorption of HA (showed by the decrease in color) increased by increasing the fungal mycelia, although it showed a logarithmic trend, meaning that by increasing the fungal biomass the effect of the amount of biomass on biosorption of HA decreased. *T. versicolor* showed a maximum of about 25% biosorption with about 300 mg of biomass and *P. sajor-caju* showed about 40% biosorption with 400 mg of mycelia.

The other important parameter to consider, was the efficiency of the HA recovery process, to see what fraction of the humics could be recovered (desorped) from the fungal mycelia. Results are shown in Fig 7.

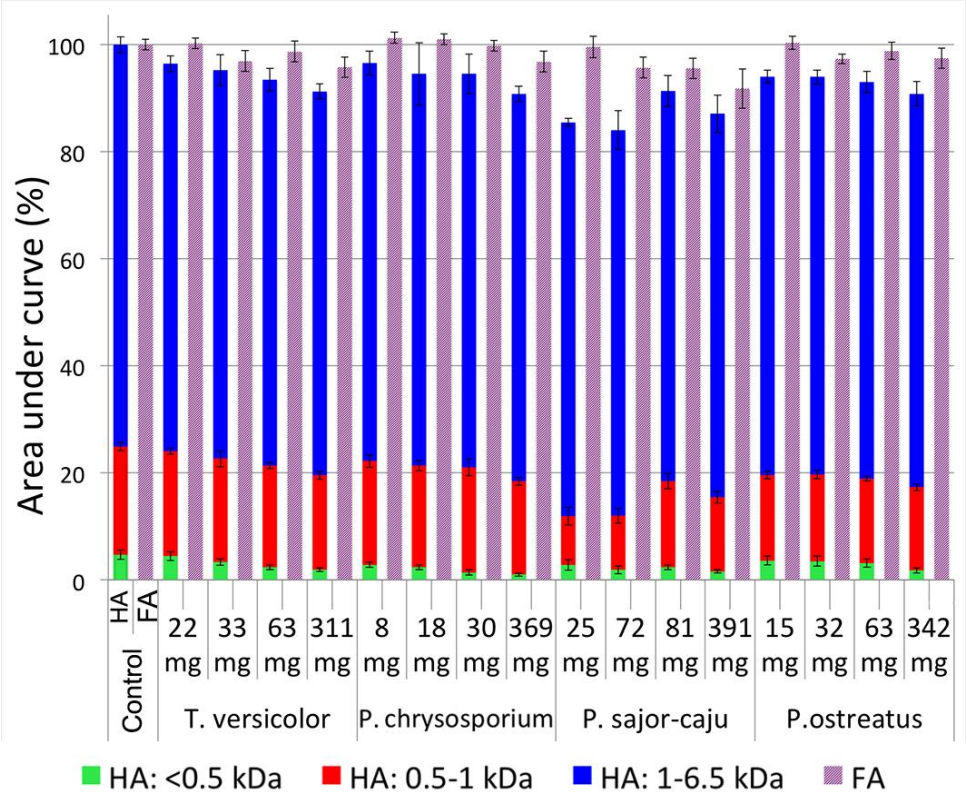

Fig 7. SEC results of the humic acid and fulvic acid portion of the humic compounds recovered from deactivated fungal mycelia after 48 hours

As it can be seen in Fig 7, the efficiency of the humic recovery slightly varied among the fungal strains; it was not 100% efficient, and it decreased with the increase in fungal biomass. Nevertheless, the average efficiency of the recovery protocol was more than 85%. The deficiency of the recovery was mostly due to the smaller HA molecules (red and green) rather than the large HA molecules (blue). Almost in all cases large HA were fully (>96%) recovered from fungal mycelia, but in most cases, the smaller molecules (red and green) were not recovered completely, especially in case of *P. sajor-caju*.

Knowing the high efficiency of the recovery process, and taking the SEC results of the recovered HA in Fig 3 into account, the role of enzymatic degradation of HA becomes more clear.

2.3.4. Purified laccase

In order to study the effect of laccase on HA degradation, experiments were carried out using purified laccase in absence and presence of mediators.

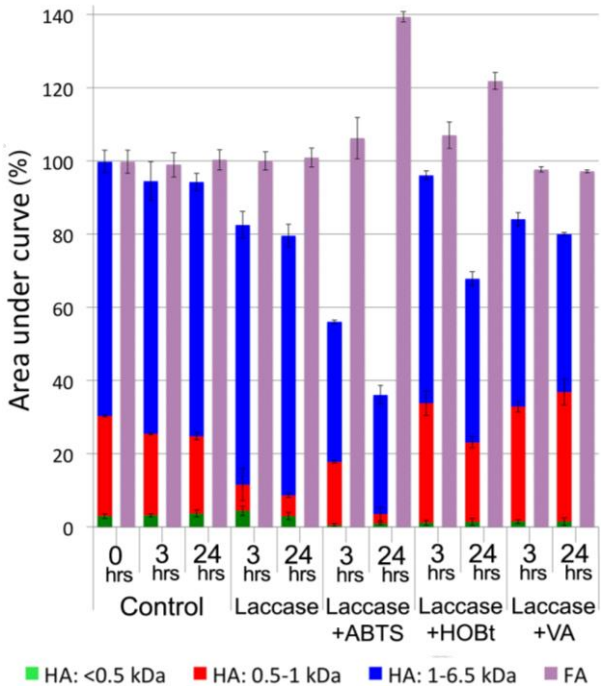

Fig 8. Degradation of HA by laccase, with and without the presence of mediators, expressed as SEC results (area under curve %) of HA and FA (Control: humic acid without enzyme and mediators)

It is clear from Fig 8 that laccase can degrade HA. Although, when comparing the results after 3 hrs and 24 hrs, it becomes apparent that the degradation is very slow when there is no mediator present. In the absence of mediators, only 20% reduction in the concentration of HA was observed after 24 hours. ABTS proved to be the best mediator among the tested mediators. In the presence of ABTS, more than 60% of the humic acid was degraded after 24 hours. The changes in the FA concentration was not as significant as in HA concentration. Although, in ABTS samples, a 40% increase in the FA concentration was observed. This simultaneous decrease in HA and increase in FA concentrations clearly shows the conversion of HA to FA by laccase in the presence of ABTS as the mediator. When using VA as the mediator, 20% of HA was degraded after 24 hours, without significant change in

FA concentration. Therefore it can be concluded that different mediators have different effects on the mechanism of HA degradation by laccase. The analysis of the MW distribution of HA confirms that the measurement of average MW is not necessarily representing the polymerization or depolymerization of HA. The concentration of HA was reduced to 36% of its initial value after 24 hours of treatment with laccase in the presence of ABTS. This reduction is clearly due to the degradation of HA to non-aromatic compounds or conversion to FA-like substances, since there was no fungal biomass present, hence no biosorption could occur. However, the average MW of HA after 24 hours was increased to 2 kDa, which implies polymerization of HA by laccase. The composition of the remaining HA in the media consisted of (after normalization to 100%) 90% large, 7% medium and 3% small HA molecules. In comparison with the initial composition of HA, shows a shift towards the larger molecules. The reason for this false implication is that when comparing the MW distribution and average MW of HA before and after the treatment, the non-aromatic products of the degradation of HA were not considered, since they cannot be detected via the UV detector during the SEC analysis.

2.3.5. Role of Cytochrome P450 enzymes

In order to investigate the involvement of the CYP enzymes in the degradation of humic acid, *T. versicolor* was incubated with and without the presence of CYP inhibitor. The results of the HA removal is presented as color removal in Fig 9.

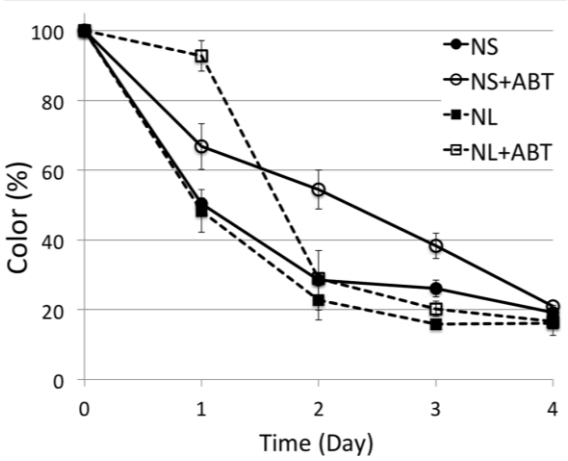

Fig 9. Role of CYP enzymes in HA bleaching by *T. versicolor*. ABT was used as the inhibitor of CYP enzymes.

The presence of the CYP enzyme inhibitor decreased the rate of the humic acid removal significantly for the first 2 days. Although after 4 days, all samples reached almost 80% color removal. This observation suggests the contribution of CYP enzymes to the humic acid degradation. It seems that the CYP enzymes contribute most importantly to the rate of the degradation. It has been suggested that CYP enzymes mainly catalyze the reactions involving epoxidation of C=C double bonds and hydroxylation of aromatic compounds [19,52].

2.4. Discussion

The HA removal was assessed by measuring the decolorization of HA and confirmed by SEC analysis of the HA content of the media, and it was shown that the color (measured at 450 nm) correlated with the concentration of HA. Some previous studies used decolorization as a measurement of degradation of HA [25,46], based on research were decolorization of HA was observed when degradation of HA was also proven [24]. The co-occurrence of the decolorization and degradation of HA was also observed in our study; however, decolorization was not necessarily representing the degradation of HA. For example, in the case of *P.sajor-caju* (Fig 3, C), similar decolorization was measured at the end of the incubation period in the NL and NS media, but after desorption of HA, the SEC analysis revealed different degradation levels in the two media. Also, results of the HA removal by deactivated fungi proved that biosorption of HA (measured as decolorization) by fungi is not similar among fungal species. Therefore decolorization of HA is not necessarily representing the rate or extent of degradation.

In none of the experiments resulting in the degradation of HA, any increase in the color was observed. This is in line with previous reports on the decolorization of HA in the result of its degradation [1] and in contrast with others reporting that the depolymerization of HA results in an increase in color [28].

The measurement of average MW before and after the treatment was shown not to be a valid method to conclude on degradation and/or depolymerization or polymerization of HA. The reason is that the depolymerization and/or degradation of HA could basically result in three products, smaller HA molecules, smaller non-aromatic molecules, and FA. Except for the first group, the other products from the

degradation of HA could not be detected in the SEC analysis (at 254 nm), and are therefore not considered in the calculation of the average MW.

These findings could clarify some confusion in the degradation [27] or polymerization of HA [28] by laccase. Looking closely at the method used in the polymerization studies, the average MW or MW distribution measured with SEC has been used for these claims, without considering the total area under the SEC curve which links to the concentration.

It was shown that different WRF species have different capabilities for biosorption of HA (Fig 6). This might be due to the different structural properties of different fungal mycelia. The mechanism of biosorption of humic compounds by fungal biomass has been studied elsewhere [53,54]. Also, It was shown that the large HA molecules were being recovered almost completely. Therefore any significant difference between the concentrations of large HA molecules (blue zone) at the beginning and the end of incubation (after the recovery), could be interpreted as degradation or conversion of these molecules. The observations of this study cannot necessarily prove the mineralization of HA by WRF, i.e. complete degradation to CO_2 and water. However, it has been shown before that the degradation of HA by WRF enzymes could be associated with mineralization of HA molecules [55,56].

From the results presented in this study, there is no clear and general correlation between extracellular enzyme activity (Fig 4) and HA degradation (Fig 3), which is in line with previous reports [57]. This might be due to the involvement of other extracellular enzymes such as versatile peroxidases [58].

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Chapter 3

Fungal treatment of humic-rich industrial wastewater: application of white rot fungi in remediation of food processing wastewater

Abstract

Humic substances (HS) comprise the major content of hardly or non-biodegradable organic matters in the industrial wastewaters. Although the application of fungi has been reported before as a novel and sustainable technology for the treatment of humics, yet most of the previous studies have been performed using synthetic wastewaters. This paper presents the results of fungal treatment of a real industrial wastewater, providing insight into the main mechanisms involved and clarifying some ambiguities and uncertainties in the previous reports. In this regard, the mycoremediation potentials of four strains of white rot fungi (WRF); *Phanerochaete chrysosporium*, T*rametes versicolor, Pleurotus ostreatus* and *Pleurotus pulmonarius* were tested to remove humic acids (HA) from a real humic-rich industrial treated wastewater of a food processing plant. The HA removal was assessed by color measurement and size exclusion chromatography (SEC) analysis. *T. versicolor* showed the best decolorization efficiency of 90% and yielded more than 45% degradation of HA, which was the highest among the tested fungal strains. The nitrogen limitation was studied and results showed that it affected the fungal extracellular laccase and manganese peroxidase (MnP) activities. The results of the SEC analysis revealed that the mechanism of HA removal by WRF involves degradation of large HA molecules to smaller molecules, conversion of HA to fulvic acid-like molecules and also biosorption of HA by fungal mycelia. The effect of HS on the growth of WRF was investigated and results showed that the inhibition or stimulation of growth differs among the fungal strains.

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3.1. Introduction

The wastewater (WW) organic matter derives from a variety of plant and animal products in various stages of decomposition as well as from chemically synthesized organic products [1]. The organic matter can be divided into two main groups: biodegradable and non-biodegradable or refractory. The biodegradable organic matter of the WW is composed of a vast variety of simple compounds of known structures and consists of carbohydrates, proteins, peptides, amino acids, fats and other low molecular weight (MW) organic compounds. These compounds are generally easy to degrade by microorganisms, and therefore will be mostly degraded during the biological treatment in WW treatment plants. Therefore most of the dissolved organic matter in the effluent of a WW treatment plant consists of non-degradable organic substances [2,3]. Humic and humic-like substances usually comprise a large portion of dissolved organic matter in the effluent [2].

Humic substances (HS) are the products of decomposition of plants and animal tissues, although they are much more stable than their precursors [1,4]. HS are formed when organic matter is decomposed in a process called humification. Humification concurs with decay and decomposing processes [5]. The presence of HS in water is often undesirable in water-based industries because they can pose health and environmental risk and also reduce the organic matter removal efficiency of the treatment plant [6,7]. In addition, HS contribute to the chemical oxygen demand (COD), which based on the regional or national environmental legislations, could cause additional discharge fees for the WW treatment plants. During chlorination, HA can cause the formation of potentially carcinogenic compounds such as trihalomethanes [8,9]. In the WW treatment plants, HS can cause membrane fouling [10] and may induce the deterioration of adsorbents, hence reducing the efficiency of the WW treatment plants [7]. HS are not well defined, but are generally divided into three fractions based on their solubility in acids and alkalis: humic acid (HA) is soluble in alkali and insoluble in acid, fulvic acid (FA) is soluble in alkali and acid, and humin is insoluble in both alkali and acid [5].

White rot fungi (WRF) are abundant in nature, especially in forest ecosystems, degrading dead wood, which involves the degradation of lignin in cell walls [11]. The non-specific enzymes of WRF have been reported to be capable of degrading some complex aromatic polymers with the molecular structure similar to lignin [12,13], such as HA [14].

Most of the previous studies on application of WRF for removal of HA from wastewater, have been conducted using synthetic wastewater, using HA isolated from soil, coal or compost, as it has been summarized elsewhere [15]. It was reported that the origin, environmental conditions and 3D structure of HA could significantly affects its biodegradability by WRF [16]. Therefore, the results that have been achieved by application of WRF in synthetic wastewater could be challenged when real wastewater is used. Although several studies have been conducted before on the bioremoval of HA by WRF, there are still ambiguities about the mechanisms involved. It is accepted that the decolorization of HA solution is an indication of a reduction in the concentration of HA [5], yet there are uncertainties regarding the relationship between decolorization and degradation/depolymerization of HA. The decrease in HA concentration in water, could be due to HA degradation by fungal enzymes [17] as well as biosorption of HA by fungal mycelia [18]. Some researchers have used the HA decolorization as an indication of the degradation of HA by WRF [19]. However biosorption of HA by fungal mycelia could also contribute to the decolorization of HA solution [18, 20]. The change in the average molecular weight (MW) of HA before and after the treatment has been used previously to investigate polymerization/depolymerization of HA [21,22], although the reliability of this method is questionable.

The goal of this study was to examine the ability of four WRF strains to remove HA from a real industrial effluent. The industrial effluent was taken after the treatment of wastewater from a food processing company. Therefore, the HA in this study was naturally originated from animal and agricultural waste. Decolorization of the wastewater during the fungal treatment was compared with the results of SEC analysis to clarify the ambiguities about the interpretation of decolorization of HA, regarding its relation to the degradation of HA. The reliability of average MW calculation based on SEC results to conclude degradation or polymerization of HA was tested. The extracellular enzyme activities were monitored to study their possible correlations with HA removal. Also the effect of the nitrogen content of the media on the mycoremediation of HA was demonstrated. The mechanism of HA removal by WRF was discussed by distinguishing biodegradation, bioconversion and biosorption of HA.

3.2. Material and methods

3.2.1. Fungal strains and chemicals

Four WRF strains were used to test their ability to treat the HS-rich WW. These fungal strains were selected as a result of a pre-screening experiment on HA-rich agar media, which has been done previously [20]. *Trametes versicolor* DSMZ 3086 and *Phanerochaete chrysosporium* DSMZ 1556 were obtained from DSMZ (Germany) and *Pleurotus pulmonarius* (obtained as "*Pleurotus sajor-caju* MES03464") and *Pleurotus ostreatus* MES00036 were obtained from the fungal stock culture collection of Plant breeding group, Wageningen UR (Wageningen, The Netherlands). The fungal strains were pre-cultivated on 3% malt extract agar and subcultures were made periodically every 40 days to keep the cultures fresh. All the chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated.

3.2.2. Media

3.2.2.1. Industrial wastewater

Industrial wastewater was collected from the effluent of a WW treatment plant of a food processing company (Eindhoven, The Netherlands). The main characteristics of this wastewater were as follow: soluble chemical oxygen demand (COD): 260 (± 6) $mg.L^{-1}$, Total COD: 262 (±8) $mg.L^{-1}$, biochemical oxygen demand (BOD_5): < 8 $mg.L^{-1}$. The ammonium concentration (NH_4^+-N) and total suspended solid (TSS) were not quantifiable (negligible values). The SEC chromatograms of the HS content (HA and FA) of the WW are shown in Fig 1. The WW was kept at 4°C for a week after the collection and autoclaved (120 °C, 15 min) before starting the experiments.

3.2.2.2. Defined media

Defined media was adapted from the media for growth and enzyme production of WRF as described previously [23]. In all cases, media contained glucose as carbon source (56 mM). Defined media consisted of KH_2PO_4, 0.2 $g.L^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.05 $g.L^{-1}$; $CaCl_2$, 0.01 $g.L^{-1}$ and trace element solution, 1 $mL.L^{-1}$ [18]. Glucose was used as the main carbon source in a final concentration of 10 $g.L^{-1}$ and ammonium tartrate was used as the main nitrogen source. For nitrogen limited media (NL) the final concentration of ammonium tartrate was 20mM and for the nitrogen sufficient

media (NS) it was 20 mM. The defined media were made in the industrial WW for mycoremediation experiments and in tap water for HS-free controls. All media were sterilized prior to use.

3.2.3. Experimental procedures

3.2.3.1. Mycoremediation of HA from HS-rich wastewater by WRF

Four WRF strains were used to grow in HS-rich WW containing defined NL and NS media. Experiments were done in 500 mL flasks (glass bottles, Duran) filled with 150 mL defined media, inoculated with 5 pieces (0.5 x 0.5 cm^2) of pre-cultivated fungal agar (see 2.1). Mycoremediation flasks were prepared in quadruplicate, two of which were subjected to sampling during the 15 days of incubation period for color measurement, enzyme activity, and SEC analysis. The other two were kept intact and only used at the end of the incubation for the recovery procedure (see 3.2.3.2). Flasks were closed with cotton stoppers and incubated in a shaker incubator (25 °C, 150 rpm) under sterile conditions.

Three different controls were prepared to certify that the observations of the experiments could completely be linked to the fungi. The first control was HS-free NL and NS media inoculated with the respective fungal strains. This was done to monitor any changes in the color of the media as a result of the fungal growth, as well as production of metabolites that can possibly interfere with the HA and FA analysis via SEC. The second control was the HS-rich WW (without adding the defined media), inoculated with the respective fungal strains to investigate the ability of WRF to utilize HS as the carbon source for growth. The third control was uninoculated HS-rich WW in the absence of the defined media, to test the stability of the WW with regard to color and molecular weight (MW) distribution of HA and FA substances. All controls were made in duplicate and incubated identical to mycoremediation flasks.

3.2.3.2. Recovery of sorped HA from fungal mycelia

In order to recover the sorped HA from mycelia, a weighed amount of NaOH was added to each jar to a final concentration of 0.1 M (pH >12) and then the fungal mycelia were disrupted by means of vigorous mixing for 2 hours. At the end, samples were withdrawn and filtered through 0.45 μm filters [24].

3.2.4. Analytical methods

3.2.4.1. Size exclusion chromatography (SEC)

Samples for SEC analysis were prepared by separating humic acid-like molecules from the media. Each sample (2 mL) was acidified (pH <2) by adding 20µl HCl (37%), and centrifuged (14000, 20 min). The acid supernatant was separated as FA-like molecules and the precipitants were re-suspended by adding 2 mL NaOH (0.1 M) and used as HA content of the sample [25]. Both FA and HA content of the samples were analyzed by SEC. The SEC was conducted using Phenomenex column (Yarra™ 3 µm SEC-2000, LC Column 300 × 7.8 mm, Ea) connected to an ultra fast liquid chromatography (UFLC) system (Prominence, Shimadzu) to detect changes in the concentration and MW of HA (and FA) molecules during the incubation with WRF. The method was adapted from a protocol that has already been developed for molecular size fractionation of HA [26], with slight modification. The mobile phase was 25% acetonitrile in ultra pure water supplemented with 10 mM sodium phosphate buffer (pH 7). The flow rate of the mobile phase was 1 $mL.min^{-1}$ and the injection volume was 10 µL. Polystyrene sulfonate standards (Polymer standard service, Germany) were used for the calibration of the column. Separation was achieved at 25 °C for 16 min and eluted substances were detected at 254 nm.

3.2.4.2. Other analysis

Decolorization of HA was determined by measuring the light absorbance at 450 nm [17].

Extracellular enzyme activities were determined spectrophotometrically in the culture supernatant obtained by filtering through 0.45 µm syringe filters. Lignin peroxidase (LiP) was assayed at 30°C by the method of Tien and Kirk (1988), using veratryl alcohol as substrate [27]. MnP activity was assayed, using Mn(II) as the substrate [28]. Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxiphenol (DMP) as described before [29]. The enzyme activities were expressed in enzyme units (U: $micromoles.min^{-1}$).

Fungal growth was estimated by measuring the dry biomass weight (DBW) of the fungal mycelia. The fungal biomass was harvested by means of filtration (pre-weighed filter papers) and dried in pre-weighed aluminium cups (100 °C, 48 hours).

The net weight of the cups with and without the fungal biomass was calculated as the DBW.

All the results are presented as the average of the measurements of duplicate experiments (flasks). Bars present the range between the maximum and minimum measurements.

3.3. Results

3.3.1. Mycoremediation

The HS content of the WW was divided into HA and FA substances. The HA portion consisted of a complex of substances of a broad range of molecule sizes as shown in Fig 1. To facilitate the comparison of SEC results, each SEC chromatogram of HA was divided into three regions based on major detected peaks, and their relative areas (calculated by Labsolution software, Shimadzu, Japan) were presented as stacked columns, as shown in Fig 1.

The large HA molecules weighing in the range of 1.1-6.5 kDa (Blue) comprised 37% of the HA complex. Medium size HA molecules and building blocks weighing 0.3-1.1 kDa (red) covered about 31%, and small acids (low MW HA) weighing less than 0.3 kDa (green) comprised the remaining 32% of the HA complex. The SEC results, as they are shown in stacked columns, help to not only qualitatively monitor the concentration of the HA as the area under the curve, but also to observe the possible changes in the ratio between different portions of HA complex, which indicate changes in the MW distribution of HA. The avg. MW of the HA content of the WW was 1.3 (± 0.09) kDa. The FA content of the WW was also analyzed via SEC. The FA chromatogram showed a lower MW range compared to HA and was shown as a single column in Fig 2. SEC results of FA content of the WW showed that the FA-like molecules weighed less than 3 kDa with an average MW of 0.5 kDa (± 0.1).

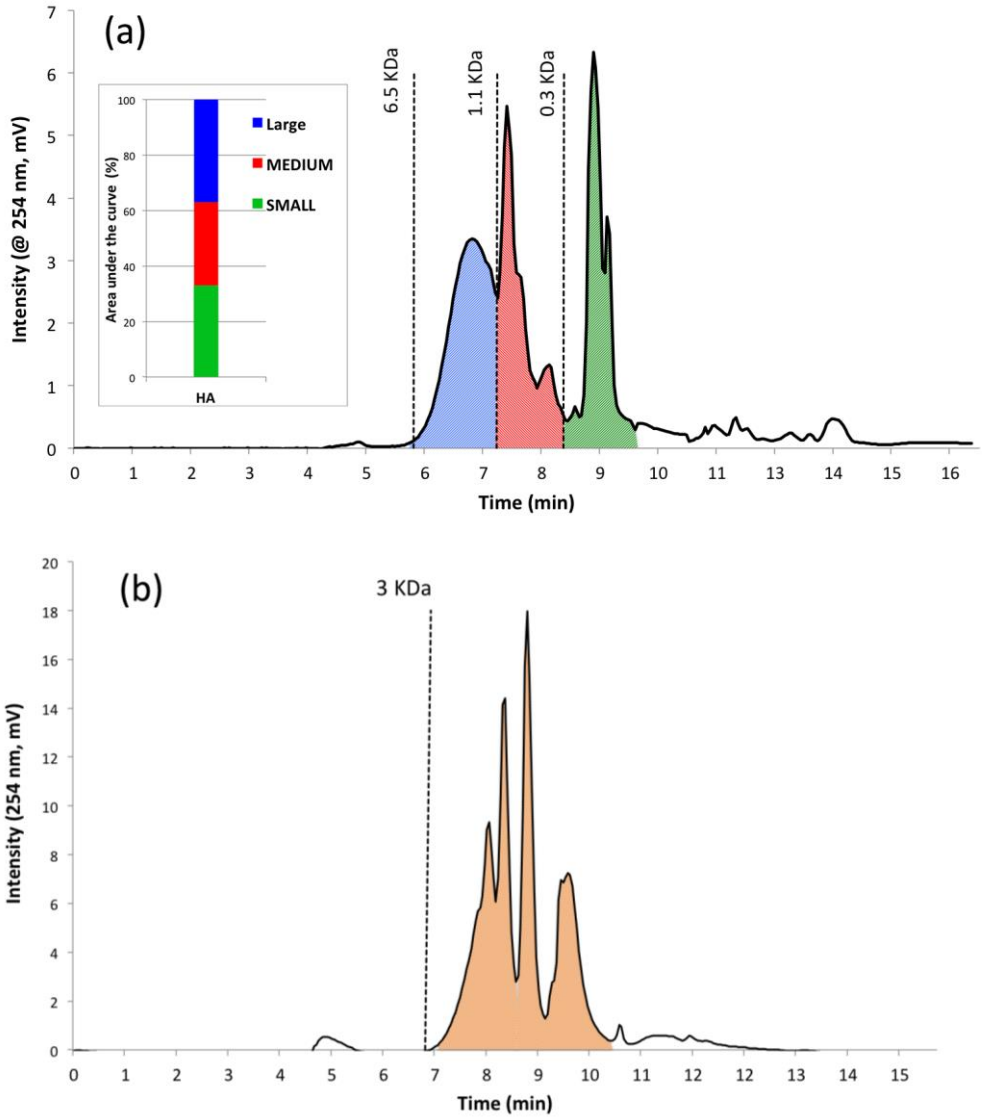

Fig 1. SEC chromatogram of HA (a) and FA (b) content of the wastewater

The results of the fungal treatment of the humic-rich WW are shown in Fig 2. The color removal during the incubation period is already corrected for the changes in the color of the HS-free controls. The color removal during the fungal treatment of the HS-rich WW, indicates the overall mycoremediation potential, which could be

due to the biosorption of HA to fungal mycelia, degradation or conversion of HA. The SEC results presented as "control" in Fig 2, were calculated as the average of the SEC results of the uninoculated samples at the beginning and the end of the incubation period, and the error bars represent the stability of the HA and FA content of the WW during this period. The results of SEC analysis of the HA and FA content of the NL and NS media at the end of the mycoremediation period (15 days) and after the recovery of sorped HA from fungal mycelia, are also presented in each graph in Fig 2. The comparison between the SEC results of uninoculated control and the SEC results of the mycoremediation experiments after the recovery of sorped HA from fungal mycelia (Fig 2) reveals the changes in the HA content of the WW as a result of degradation (or conversion) of HA by WRF. The uninoculated controls showed a stable color (<3% variation) during the incubation period, suggesting the high stability of HA molecules. However, the SEC results of the HA content of the uninoculated controls showed some instability in the concentration (area under the curve) of small HA molecules (green) during the incubation period (15 days), which can be seen by a relatively high error bar in Fig 2 (uninoculated controls, green column). The large and medium size HA molecules (blue and red zones) were stable, as well as FA-like molecules (<6% change in the area under the curve). Since the small HA molecules comprise a large portion of HA complex (33% of the area under the curve) in this study, the relatively high variation in the concentration of this portion of the HA complex in the uninoculated controls, mystifies the contribution of the fungal treatment to the reduction of this portion of the HA. Therefore, in order to determine the effect of the fungal treatment on the HA, only changes in the large (blue) and medium (red) size HA are discussed.

The SEC analysis of the HS-free controls showed that fungi did not produce metabolites that could interfere with HA analysis, since no peak was detected during the SEC analysis.

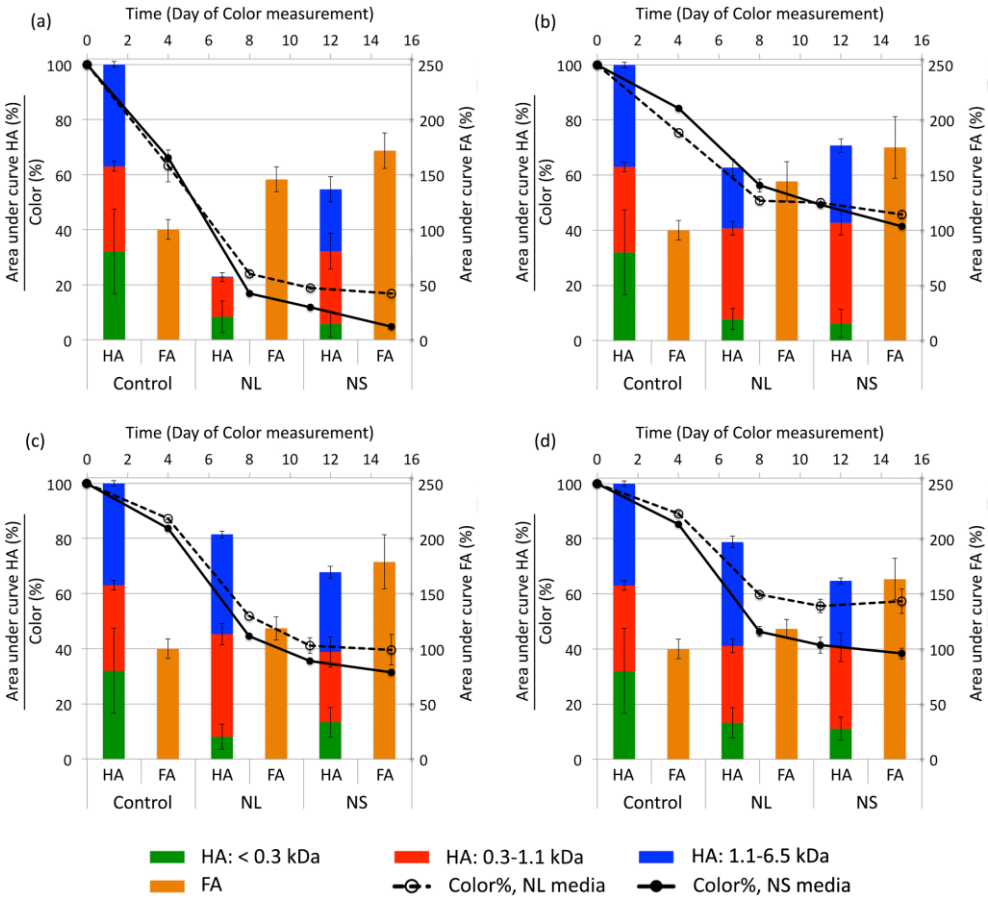

Fig 2. HA color removal during the fungal treatment and SEC results of the HA and FA content of the wastewater (area under the curve) before (uninoculated control) and after the fungal treatment (after recovery of sorped HA) in NL and NS media. a: *T. versicolor*, b: *P. chrysosporium*, c: *P. pulmonarius* and d: *P. ostreatus*

According to the results of color removal in Fig 2, all four WRF strains were capable of removing HA from the media. *T. versicolor* showed the best color removal efficiency of 80-90%. Although it showed a better color removal in NS media, but the difference with NL media was only 10%. However, when looking at the SEC results, the difference between the performance of *T. versicolor* in NS and NL media is more noticeable. The large HA molecules (blue zone) were completely degraded in NL media. The complete degradation of large HA molecules was concluded from the SEC results of the HA content of the NL media (after the recovery of sorped HA from fungal mycelia), where no blue zone (large HA

molecules) was observed. In the NS media, the area under the curve of large HA was reduced from 37% in uninoculated controls to 22% after the fungal treatment, suggesting 40% degradation of the large HA molecules. The area under the curve of the medium size HA was reduced from 30% to 14.5% in the NL media, and to 25.5% in the NS media, indicating almost 50% degradation of medium size HA in NL media and 15% in the NS media. The avg. MW of the recovered HA after the treatment with *T. versicolor* was 0.5 kDa in NL media and 1.3 KDa in NS media. The avg. MW of the HA content in the NS media appeared to be unchanged (even slightly increased) after the fungal treatment, regardless of the clear degradation (40%) of large HA molecules. This can be explained by an almost 80% decrease in the concentration of small HA molecules after the fungal treatment, which contributed to the increase in the overall avg. MW of the total HA complex. Therefore, monitoring the avg. MW of the HA content is not necessarily a valid way to conclude the degradation or even polymerization of HA. The SEC analysis of the FA content of the media after the fungal treatment revealed that in both NL and NS media, the area under the curve of FA-like molecules increased. However, this increase was higher in the NS media, suggesting a higher conversion of HA molecules to FA-like molecules. The increase in the concentration of FA-like molecules was observed for all four tested fungal strains (Fig 2).

In the case of *P. chrysosporium*, the color removal in NL and NS media were very close (60% for NS media and 55% for NL media), although NL media showed a slightly higher decolorization for the first 8 days of incubation. Looking at the SEC results, almost 60% of the initial amount of large HA molecules were detected after the recovery procedure in the NL media, suggesting 40% degradation of the large HA molecules (blue zone). In the NS media, the co-incubation of *P. chrysosporium* and HS resulted in a 25% reduction in the large HA (blue) fraction and around 20% increase in the medium size HA fraction (from 37% in the uninoculated controls to 28% after the fungal treatment), which suggests the incomplete degradation of large HA to medium size HA molecules. Both *Pleurotus* species showed higher color removal in NS media than in NL media. Looking at the SEC results, both *Pleurotus* species showed higher degradation of large HA molecules in NS media, which coincided with an increase in the concentration of FA-like molecules. Looking at the SEC results in fig 2, the effect of nitrogen limitation on the degradation pattern of WRF becomes clear. *P. pulmonarius* degraded around 20% of the large HA and 20% of medium size HA (red) in NS media, when in NL media the concentration of the large HA was reduced only 5% and the concentration of medium size HA

increased 20% (from 30% in the uninoculated controls to 36% after the recovery). The concentration of large HA molecules was not changed in the NL culture of *P.ostreatus,* but a 10% decrease in the concentration of medium size HA was observed. In the NS media, 35% decrease in the concentration of large HA was detected and the concentration of medium size HA showed only 5% decrease. It is known that the nitrogen concentration could affect the extracellular enzyme activity of the WRF [30], which along with our observations signifies the role of WRF's extracellular enzymes in the degradation of HA.

3.3.2. Extracellular enzyme activities

In order to investigate the possible role of extracellular enzymes of WRF in the degradation (or conversion) of HA, the laccase, MnP and LiP activities of the WRF were measured during the incubation period. No significant LiP activity was detected for any of the strains, therefore it is excluded from the presented results. The absence of a significant LiP activity in the fungal cultures in these experiments might be due to the inhibition of the enzyme by HA [17], or the absence of veratryl alcohol in the culture [31]. Also, the agitation caused by shaking the cultures could have contributed to the inhibition of LiP activity [32,33]. The absence of LiP activity in the culture of the specific strain of *P. chrysosporium* that we used (DSMZ 1556), is not unprecedented [34]. The laccase and MnP activities for all four WRF strains are shown in Fig 3.

T. versicolor showed the highest Laccase and MnP activities among the tested strains. It is known that the presence of HA interferes with the measurement of MnP activity, resulting in an underestimation of MnP [17,35]. Our results also demonstrate this, since the MnP activities in the HS-free controls were always higher than in the media containing HS. Therefore, the MnP activities in the HS-free controls were used to qualitatively show the general ability of fungal strains to produce MnP.

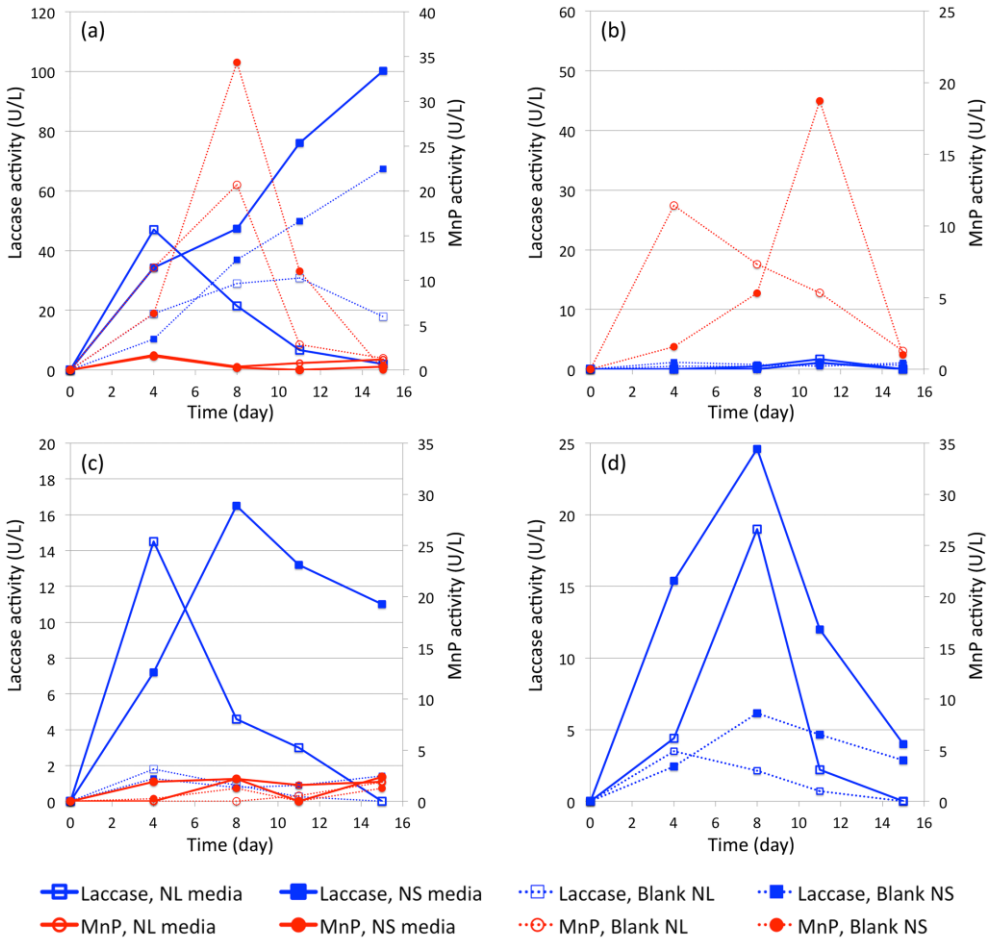

Fig 3. Laccase and MnP activity during the incubation period with (solid lines) and without (blank, dot lines) the presence of HS. a: *T. versicolor*, b: *P. chrysosporium*, c: *P. pulmonarius* and d: *P. ostreatus*. Note: differences in Y-axis scales

As it can be seen in Fig 3, *Pleurotus* species did not show any significant MnP activity even in the HS-free controls, but *T. versicolor* and *P. chrysosporium* expressed MnP activity in both NL and NS media. The NS culture of *T. versicolor* expressed a maximum of 34 $U.L^{-1}$ MnP activity after 8 days (in HS-free controls) and maximum laccase activity of 100 $U.L^{-1}$ after 15 days (in presence of HS). In the NL media both laccase and MnP activities were lower than in the NS media. It is known that *T. versicolor* can produce the extracellular enzymes as a secondary metabolite under limited nitrogen concentration, and also in the presence of high nitrogen concentration [34,36]. When comparing the results of the enzyme activities of *T.*

versicolor in NL and NS media with the SEC results in Fig 2, there is no clear correlation between extracellular enzyme activities and the degradation of HA. *T. versicolor* growing in the NL media degraded all the large HA molecules (blue), but in the NS media it degraded only 40% of the large HA molecules, regardless of higher laccase and MnP activities in NS media.

P. chrysosporium did not show any significant laccase activity. *P. chrysosporium* has been widely quoted before as an example of WRF that does not produce laccase [37,38]. When growing in NS media, *P. chrysosporium* showed a maximum of 19 $U.L^{-1}$ MnP activity after 12 days, but in NL media the maximum enzyme activity reached a lower and sooner maximum of 12 $U.L^{-1}$ after 4 days. It is known that the MnP activity of *P. chrysosporium* is part of a secondary metabolism, which is triggered by scarcity in nutrients, namely nitrogen [30]. The difference in the MnP production in NL and NS media by *P. chrysosporium* could be explained by secondary metabolism conditions caused by nitrogen limitation in the media. *P. chrysosporium* in NL media enters the secondary metabolism conditions sooner than in NS media due to the nitrogen limitation in NL media; hence the MnP activity was detected sooner in NL media. In NS media, fungi enter the secondary metabolism later than in NL media, due to a higher initial nitrogen concentration. Although when it enters the secondary metabolism phase in NS media, there is more fungal biomass grown compared to NL media, therefore more MnP was produced. *P. pulmonarius* showed higher laccase activity in NS media than in NL media. Also, the laccase activity in NS media lasted longer than it did in NL media. This correlated with a higher degradation of HA molecules in the NS media, as it can be seen from the SEC results shown in Fig 2. For *P.ostreatus* also laccase activity was higher in NS media than in NL media, and this also correlates with the higher degradation of large HA molecules in NS media.

Overall, by comparing the results of *T. versicolor* and the other tested fungal strains in Fig 2 and 3, it seems that when the MnP and Laccase are both being produced, higher degradation of HA was achieved compared to when only one of them were detected.

For all four tested WRF strains, the laccase activity was higher in the media containing HS, which indicate that HS stimulates the production of laccase by WRF. This observation was in line with some previous reports on stimulation of WRF's laccase activity by aromatic compounds [39,40].

3.3.3. Effect of HS on the growth of WRF

The effect of HS on WRF's growth was assessed by measuring the dry weight of fungal biomass after incubation in HS-rich WW and in tap water (HS-free), both supplemented with defined media. Results are shown in Fig 4.

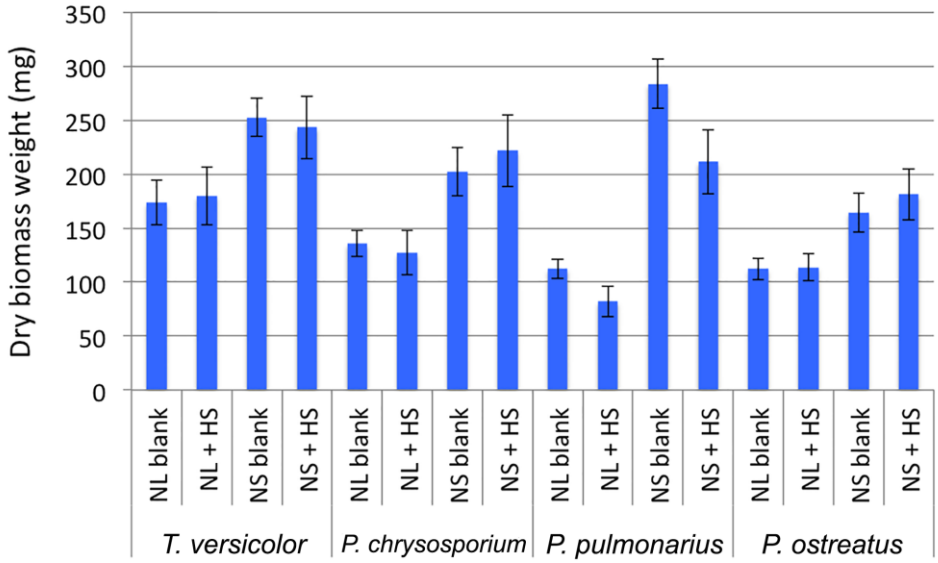

Fig 4. Growth of WRF after 15 days of incubation in NL and NS media, with (+HS) and without (blank) the presence of HS

From the results shown in Fig 4, it is not possible to draw an overall conclusion on the co-incubation of WRF and HS, regarding any general positive or negative effect of HS on WRF growth. In the case of *T. versicolor*, the effect of HS in the media is not significant. *P. chrysosporium* showed slight increase in growth in the presence of HS when growing in NS media, but it did not show the same pattern in NL media. The presence of HS significantly hindered the growth of *P. pulmonarius*, which can be seen especially in NS media. *P.ostreatus* produced slightly more biomass in the presence of HS in the NS media. But in the NL media it seems that the presence of HS had no effect on the fungal growth.

None of the tested fungal strains could grow in the HS-rich WW without the presence of the defined media (see 2.3.1, second set of controls). This shows that the fungal strains could not utilize the HS as the sole carbon source to grow. This

observation is in agreement with previous reports, stating that the degradation of humics by WRF occurs under co-metabolic conditions (i.e., in presence of an assimilable carbon source) [20,39].

3.4. Discussion

From the results of HA removal by *T. versicolor* and *P. chrysosporium*, the uncertainty of whether the decolorization of HA is representing the degradation of HA could be elucidated. *T. versicolor* could degrade more HA in NL media comparing to NS media, concluding from complete degradation of large HA in NL media and in-complete degradation of large HA in the NS media. However, when looking at the color removal, decolorization was higher in NS media than the NL media. Biosorption of HA by WRF's biomass (mycelia) could explain this. Looking at the results in Fig 4, *T. versicolor* produced significantly more biomass in the NS media than in the NL media (due to nitrogen limitation in NL media). Consequently, more biosorption of HA was achieved during the incubation in the NS media. Similar observation could be made in case of *P. chrysosporium*. Clearly higher HA degradation occurred in the NL media, judging by higher reduction in the area under the curve of HA in the NL media. However, the decolorization of HA in both media was almost the same, even slightly higher in the NS media. Therefore, it could be concluded that the decolorization of HA is not necessarily representing the degradation of HA.

The reliability of average MW, calculated based on the SEC analysis of HA could be challenged based on results of this study. *T. versicolor* was able to degrade HA in the NS media. However, The average MW calculated based on its SEC analysis was not changed much (even slightly increased), comparing to its value before the treatment. It is noteworthy that during the SEC analysis of HA, the eluted substances are being detected by a UV detector (at 254 or 280 nm), which could detect the aromatic compounds [26]. Therefore the non-aromatic products of the degradation of HA could not be detected during the SEC, hence were not included in the average MW calculations. Overall, it could be deducted that monitoring the changes in the average MW of HA, is not necessarily a valid way to conclude degradation/depolymerization or polymerization of HA.

The mechanism of HA removal different among the WRF species. Although the biosorption of HA by WRF was observed for all tested species, the degradation of

HA showed different patterns. In all cases, the concentration of FA-like molecules was increased after the fungal treatment. This suggests the conversion of HA to FA, which is in agreement with some previous studies [20,25]. In some cases the reduction in the concentration of large HA molecules resulted in an increase in the concentration of smaller HA molecule, which shows the incomplete degradation of larger HA to smaller HA substances. This can be seen in the NS culture of *P. chrysosporium* and the NL culture of *P.pulmonarius*. However, it was also observed that the degradation of large HA molecules could concur with a slight reduction in the concentration of smaller molecules, such as observed in the NS culture of *T. versicolor*. This observation suggests the degradation of HA molecules to non-aromatic molecules that were not detectable via the SEC analysis. The complete mineralization of HA molecules, i.e. complete degradation to H_2O and CO_2, could not be concluded from the results presented in this study, although the ability of WRF to mineralize HA has been demonstrated before [41-43].

The significant effect of extracellular enzymes of WRF on the degradation of HA has been studied before and it has been shown that MnP and laccase can degrade HA [20,44]. However, the results of this study, in accordance with a previous report [45], show that the degradation of HA by WRF could not always be explained only by the presence of MnP and laccase activity. This probably is due to the involvement of other extracellular enzymes like versatile peroxidases [46] and also membrane bound fungal enzymes like Cytochrome P450 [47].

3.5. Conclusions

WRF could remove HA in real industrial treated wastewater originating from animal and agricultural waste. The mechanism of mycoremediation of HA contaminated waters by WRF included biosorption, biodegradation and bioconversion. The decolorization of HA-rich water by WRF indicated the decrease in the concentration of HA in water. However, it did not necessarily indicate the rate or extent of the degradation of HA. Although it seemed that laccase and MnP are effective in the degradation of HA, the involvement of other fungal enzymes in the degradation of HA could not be excluded. The HA content of the used wastewater, is a complex of vast variety of substances with different molecular sizes. The degradation of HA molecules, resulted in a decrease in the concentration of some portions of HA (large, medium or small molecules), but did not necessarily result in a decrease in the average MW of the HA complex.

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Chapter 4

A novel approach for application of White rot fungi in wastewater treatment under non-sterile conditions: immobilization of fungi on sorghum

Abstract

In this study we tested a new approach to facilitate the application of white rot fungi (WRF) under non-sterile conditions, by introducing grain sorghum as carrier and sole carbon and nutrient source for WRF. To this end, *Trametes versicolor* was immobilized on sorghum, and its ability to remove humic acid (HA) from synthetic and real industrial wastewater was studied. HA removal was measured as colour reduction and also analysed via size exclusion chromatography (SEC). Under sterile conditions, 80% colour removal was achieved for both synthetic and real wastewater using immobilized WRF on sorghum, without adding any additional carbon or nutrient sources. Under non-sterile conditions, immobilized fungi could again remove 80% of the colour and reached a maximum of 40 U/L laccase activity. In contrast, non-immobilized fungi cultivated in non-sterile wastewater supplemented with additional nutrients, reached only 10% decolourization and maximum 5 U/L laccase activity. SEC analysis showed that bioremoval of HA by WRF was associated with degradation of HA. Finally, immobilized fungi were used to treat real wastewater, under non-sterile conditions, in a sequential batch order without renewing the immobilized fungi. Four batch feedings were conducted and 80%, 70%, 50% and 40% colour removal was achieved for each batch, respectively, over a total incubation period of 19 days.

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4.1. Introduction

Saprotrophic fungi are well known for their important role in utilizing organic matter in natural ecosystems [1]. They facilitate organic matter decomposition and nutrient recycling in favour of own and other organisms growth [2]. Among these fungal species, white rot fungi (WRF) are of particular interest, due to their capability to efficiently mineralize lignin [3,4]. The extracellular enzymes of WRF have been reported to be responsible for the degradation of lignin [5,6]. WRF typically secrete one or more of the three principal ligninolytic enzymes i.e. lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac)[5,7]. These enzymes are highly non-specific with regard to their substrate [5,8], which gives them the capability to degrade a wide range of highly recalcitrant organopollutants with molecular structure similar to lignin [7,8], such as humics [9–11].

Humic substances (HS) result from plant and animal tissue decomposition, but they are much more stable than their precursors [12]. Typically, HS can be divided into three groups, based on their solubility in water: humic acids (HA) that are insoluble at acidic pH (<2) and soluble at higher pH, fulvic acids (FA) that are soluble at all pH values and humins that are generally insoluble in water [13]. HA generally represent the largest fraction of HS, with MW up to 5-6 kDa in water and up to 500 kDa in soil. FA are typically smaller molecules with MW up to 1-2 kDa in water and 5 kDa in soil [14,15]. Due to their low bio-degradability, HS comprise a major part of the organic content of the effluent of water treatment plants [16,17]. Humic substances can cause serious technical problems in water treatment plants, such as membrane fouling [18,19] and the deterioration of adsorbents [20]. The presence of humic substances in water can also pose serious environmental and health problems. They form strong complexes with heavy metals and can increase their transportation in waters [21]. Also, humic substances can react with chlorine during water treatment, and produce carcinogenic compounds such as trihalomethanes [22,23]. Moreover, a high residual HS concentration in treated water leads to a yellow or brown color, which is undesirable [24].

Most of the mycoremediation studies using WRF have been conducted under sterile conditions [25,26]. However, The sterilization of wastewater on industrial scale is not feasible. The main problem with the application of WRF under non-sterile conditions is the bacterial contamination. Bacterial proliferation results in severe competition for available organic substrate, and it negatively affects the WRF

metabolism [26]. Therefore, in order to maintain the WRF's growth and enzyme production, it is required to develop selective strategies to support WRF against bacteria and to suppress bacterial growth without inhibiting the fungal growth. It is known that immobilization of WRF increases their stability and growth rate [27]. Recently various materials including Ca-alginate [28,29], polyurethane [30], wood chips [31] and glass beads [32] were used for immobilization of WRF. In all these cases, immobilized fungi were provided with defined or general nutrient media for fungal growth and enzyme production. Most of the previous studies on the application of WRF for treatment of recalcitrant organopollutants have been conducted using defined media, based on the recommendations by Tien and Kirk [33]. Although the composition of this media has been slightly modified by some researchers in order to reduce the cost [34] or increase the enzyme activities [35,36], still easily degradable soluble carbon sources were used, which can be easily assimilated by bacteria and other microorganisms.

We recently reported on the ability of WRF to remove humics from water, and also provided some insight into the enzymes and mechanisms involved [10,11]. These previous studies have been done under sterile conditions using defined media for fungal growth and enzyme production. The goal of our present study was to investigate the application of immobilized WRF on grain sorghum to remove HA from synthetic and real humic-rich wastewater under non-sterile conditions. Grain sorghum (sorghum) is a grain, forage, or cereal crop consisting of white, yellow, red, brown or black endosperms. Their main components are starch (≈ 75%), protein (≈12%), lipids (≈4%), fiber (≈3%) and ash (≈ 2%) along with several minerals, vitamins and amino acids [37]. The compositional profiles of sorghum along with some of its applications were summarized elsewhere [38]. Also the nitrogen, amino acids, soluble sugar, protein and mineral contents of sorghum have been studied and reported before [39,40,41].

In our present study, sorghum was used as carrier material (for immobilization of fungi) as the sole carbon and nutrient source (instead of defined media). Therefore, it is expected that the use of sorghum will give an advantage to fungi over bacteria in the access to carbon and nutrients. Mycoremediation experiments were firstly performed under sterile conditions, to confirm the ability of sorghum to act as the sole carbon and nutrient source for fungal growth and enzyme production during the HA removal from synthetic and real wastewater. Then, experiments were continued under non-sterile condition, to evaluate the ability of immobilized WRF to remove

HA from real wastewater under non-sterile conditions. Finally, a sequential batch experiment was conducted to treat several batches of real wastewater with the same fungal biomass, to test the durability of the fungi immobilized on sorghum and to evaluate the potential of the approach for future applications in bioreactors.

4.2. Material and methods

4.2.1. Fungal strain and chemicals

Trametes versicolor DSMZ 3086 was obtained from DSMZ (Germany). *T. versicolor* was pre-cultivated on 3% malt extract agar and subcultures were made periodically every 40 days to keep the cultures fresh. All the chemicals, including coal HA, were purchased from Sigma-Aldrich (Germany), unless stated otherwise.

1.1.Sorghum and immobilization of fungi

The sterilized sorghum was provided by Wageningen University (department of Plant Breeding, The Netherlands). Four pieces (~2 cm^2) of pre-cultivated colonized agar culture were added to the sterilized sorghum grains (~300 grains) and incubated at 25°C until all grains were colonized by fungal mycelium. The immobilized fungal granules were then kept at 4°C (for maximum 3 days) until further use.

4.2.2. Defined media

Defined media was prepared according to the defined culture media for growth and enzyme production of WRF as described before [42]. The defined media contained glucose as the main carbon source and ammonium tartrate as the main nitrogen source along with minerals and vitamins. Defined media was only used in non-sterile experiment (see 4.2.4.1 and 4.3.5), to compare the results of immobilized fungi on sorghum as the nutrient source with the results of free fungal pellets with defined media as the nutrient source.

4.2.3. HS-rich wastewater

4.2.3.1. Synthetic wastewater

Synthetic wastewater was made by adding 50 mL HA from a stock solution to 950 mL of tap water. The stock solution of HA was prepared using coal HA powder

(Sigma-Aldrich). HA powder (4 g) was dissolved in 200 mL of NaOH solution (0.1 M) and mixed for 30 min. The solution was centrifuged (7000 rpm, 20 min) to remove the particulates. Then 100 mL of phthalate buffer (0.5 M) was added to the particulate-free HA solution and pH was adjusted to 5.5 with HCl. The buffered solution was centrifuged again (7000 rpm, 20 min) and the supernatant was used as HA stock solution. For all the experiments using synthetic wastewater, HA stock solution was filtered (0.45 μm pore size, Millipore, Germany) prior to use.

4.2.3.2. Industrial wastewater

Industrial wastewater was collected from the effluent of a wastewater treatment plant of a food processing company (Eindhoven, The Netherlands). The main characteristics of this wastewater, hereafter called "real wastewater", were as follow: soluble chemical oxygen demand (COD): 282 (± 3) $mg.L^{-1}$, Total COD: 283 (±8) $mg.L^{-1}$, biochemical oxygen demand (BOD5): < 10 $mg.L^{-1}$. The ammonium concentration (NH_4^+-N) and total suspended solid (TSS) were not quantifiable (negligible values). The treated wastewater was kept at 4°C for a week after the collection and before starting the experiments.

4.2.4. Experimental procedure

4.2.4.1. HA removal by WRF

Experiments were done in 500 mL flasks (glass bottles, Duran) filled with 150 mL media (synthetic or real wastewater), and inoculated with immobilized fungal granules (~10 granules). Bioremediation flasks were divided in two sets. The flasks in the first set were subjected to sampling during the incubation period for color measurement, enzyme activity, and SEC analysis. The other set of flasks was kept intact and only used at the end of the incubation for the recovery procedure (see 4.2.4.2). Flasks were closed with cotton stoppers and incubated in a shaker incubator (25°C, 150 rpm). For sterile experiments, wastewater (both real and synthetic) was autoclaved (121°C, 15 min) prior to the inoculation. Non-sterile experiments were conducted using real wastewater. In order to compare the HA removal efficiency of immobilized fungi on sorghum with that of free fungal pellets (non-immobilized), in addition to flasks containing immobilized fungi, a set of flasks was prepared using real wastewater supplemented with defined media (See 4.2.2) and inoculated with five pieces of fungal agar (~ 1 cm^2). The flask containing free fungal pellets were prepared identical to what was described and reported before [11] under sterile

conditions, with the only difference being that in this study, the media was not sterilized.

In order to ensure the correct interpretation of the results, two different sets of controls were prepared for each set of experiments. The first control was the HS-free control, which was prepared by using tap water instead of wastewater and inoculated with immobilized fungi as described above. The HS-free control served to distinguish any change in the media that was due to the fungal growth or release of metabolites from fungal mycelia or sorghum, which is not related to the HS (HA or FA) in the wastewater. The second set of controls was simply the uninoculated real or synthetic wastewater, which was incubated under the same conditions as the bioremediation flasks, to evaluate the stability of HA during the incubation period.

4.2.4.2. Recovery of sorped HA from immobilized fungi

In order to recover the sorped HA from fungal granules, a weighted amount of NaOH was added to each jar to a final concentration of 0.1 M (pH >12) and then the fungal granules were disrupted by means of vigorous mixing for 2 hours. At the end, samples were withdrawn and filtered through 0.45 μm filters [10,43].

4.2.4.3. Biosorption of HA by deactivated immobilized fungi

Biosorption experiment was performed in triplicate under sterile conditions using 500 mL flasks containing 150 mL tap water (sterile). Each flask was inoculated with of immobilized fungal granules (~10 granules) and incubated for two weeks in a shaker incubator (25°C, 150 rpm). Then the flasks were autoclaved (121°C, 20 min) in order to deactivate the fungi. The deactivated fungal granules were washed 3 times with sterilized water and were added to flasks containing synthetic wastewater. The flasks were incubated for 48 hours and monitored for changes in color. After 48 hours the recovery procedure (4.2.4.2) was performed and samples were analysed via SEC.

4.2.4.4. Sequential batch experiment

A sequential batch experiment was conducted in duplicate using immobilized fungi and real wastewater under non-sterile conditions. The preparation and inoculation with immobilized fungi was performed as explained before (see 4.2.1). After each incubation period, the immobilized fungi were kept in the jar and the treated wastewater was decanted, and replaced with the same volume of fresh wastewater.

4.2.5. Analysis

The details of the SEC analysis of humic content of the media have been explained before [10]. Briefly, each sample (2 mL) was acidified (pH <2) by adding 20 µL HCl (37%), and centrifuged (14000, 20 min). The acid supernatant was separated as FA and the precipitants were re-suspended by adding 2 mL NaOH (0.1 M) and used as HA portion of the sample. Both FA and HA portions of the samples were analyzed by SEC. The SEC was conducted using Phenomenex column (Yarra™ 3 µm SEC-2000, LC Column 300 × 7.8 mm, Ea) connected to an ultra fast liquid chromatograph (UFLC) (Shimadzu, Prominence) to detect changes in the concentration (area under chromatogram) and MW of HA (and FA) molecules during the incubation with WRF. The areas under the curves, as well as the (weighted) average molecular weights of the eluted substances, were calculated by Labsolution software (Shimadzu).

Decolorization of HA was assessed by measuring light absorbance at 450 nm [43]. The color of HA (commonly measured at 400-600 nm) is considered an indication of HA concentration [10,13].

Laccase activity was determined spectro-photometrically in the culture supernatant obtained by filtering through 0.45 µm syringe filters and measured by monitoring the oxidation of 2,6-dimethoxiphenol (DMP) as described before [44]. The enzyme activity was expressed in enzyme units (U: micromoles.min^{-1}).

All the experiments were done at least in triplicates unless otherwise is stated. The results are presented as the average of the measurements.

4.3. Results

4.3.1. Growth of fungi on sorghum as the sole carbon and nutrient source

The growth of *T. versicolor* immobilized on sorghum as carrier and nutrient source is shown in Fig 1. The observations clearly showed that fungi could grow on sorghum as the sole carbon and nutrient source. Also, it is clear that the granular shape of the carrier with fungi growing on it was maintained during the incubation period.

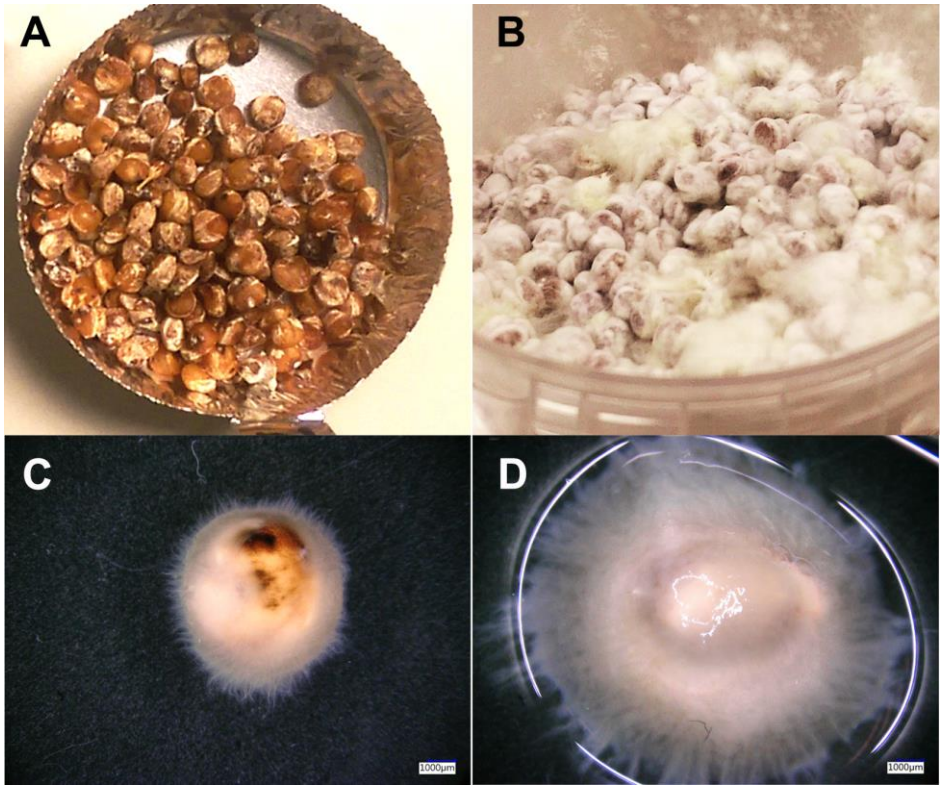

Fig 1. Immobilized fungi. A: Sorghum (as carrier), B: Pre-grown fungi on Sorghum, i.e. immobilized fungi (after 3 weeks of incubation in solid phase), C: Immobilized fungal granule after 2 days incubation in liquid phase (water), D: Immobilized fungal granule after 14 days of incubation in liquid phase (water).

4.3.2. HA content of the synthetic and real wastewater

The HA content of both real wastewater and synthetic wastewater comprised a broad range of molecular sizes. A sample SEC chromatogram of the HA extracted from the synthetic wastewater is shown in Fig 2. This chromatogram shows a complex of HA molecules with MW of 0.1-6.5 kDa. Each SEC chromatogram of HA complex was divided into three regions based on the major detected peaks, and their areas (calculated by Labsolution software, Shimadzu) were normalized (% area under the curve) and presented as stacked columns to facilitate the comparison of SEC results [10].

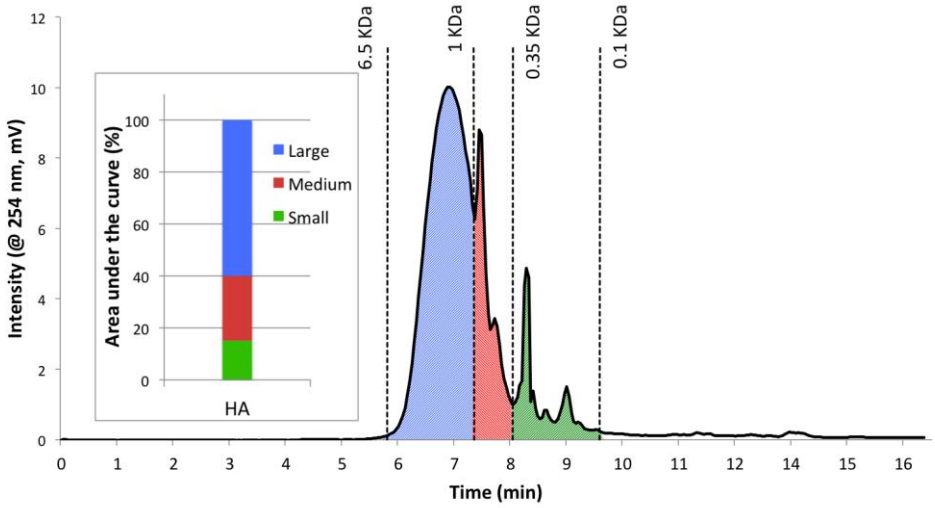

Fig 2. SEC chromatogram of HA complex (synthetic wastewater). The chromatogram is divided into three zones based on the main peaks: Large HA molecules (Blue), medium size HA molecules (Red) and small HA molecules (Green). The area under the curve was normalized and presented in the stacked column graph.

The HA extracted from the real wastewater was in a similar molecular range of synthetic wastewater (results not shown). However, the ratio between large, medium and small molecules was different. The average molecular weight of the HA from synthetic wastewater was 1.6 (± 0.11) kDa, and for real wastewater it was 1.4 kDa (± 0.05). The average MW of HA in the real wastewater was slightly reduced to 1.3 (± 0.15) kDa after sterilization (autoclave). The FA-like molecules extracted from the media were also analyzed by SEC. The synthetic wastewater showed a relatively narrow FA peak starting from 0.5 kDa to 0.2 kDa, with an average MW of 0.3 (± 0.06) kDa. However, the real wastewater showed a broader range of FA-like molecules starting from 3 kDa to 0.1 kDa with an average molecular weight of 0.45 (± 0.11) kDa. The SEC analysis of the HS-free controls revealed that the growth of the immobilized fungi did not produce metabolites that can interfere with HA analysis (no peak was detected), but they could produce metabolites that could be detected as FA-like molecules. However, the concentration of these molecules (area under the curve) was negligible compared to the FA concentration of the wastewater (data not shown). The results of the recovery (desorption) procedure performed on

HS-free controls showed that there were no metabolites released from the immobilized fungal granules that could be detected as HA. However, a significant amount of metabolites was detected as FA-like molecules, making up a concentration equal to 5-10% of the FA content of the real wastewater (data not shown). The FA results shown from this point onwards are corrected for the HS-free controls.

4.3.3. Removal of HA from synthetic wastewater by immobilized fungi under sterile conditions

The HA color removal along with the results of the SEC analysis and the enzyme activities are shown in Fig 3. The MW distribution analysis showed that the composition of HA complex is made up by 66% large HA, 20% medium size and 14% small HA molecules. The analysis of uninoculated control samples at the beginning and at the end of the incubation period showed less than 6% variation in color and less than 10% in the area under SEC curve for HA and FA, indicating the high stability of HA molecules (data not shown).

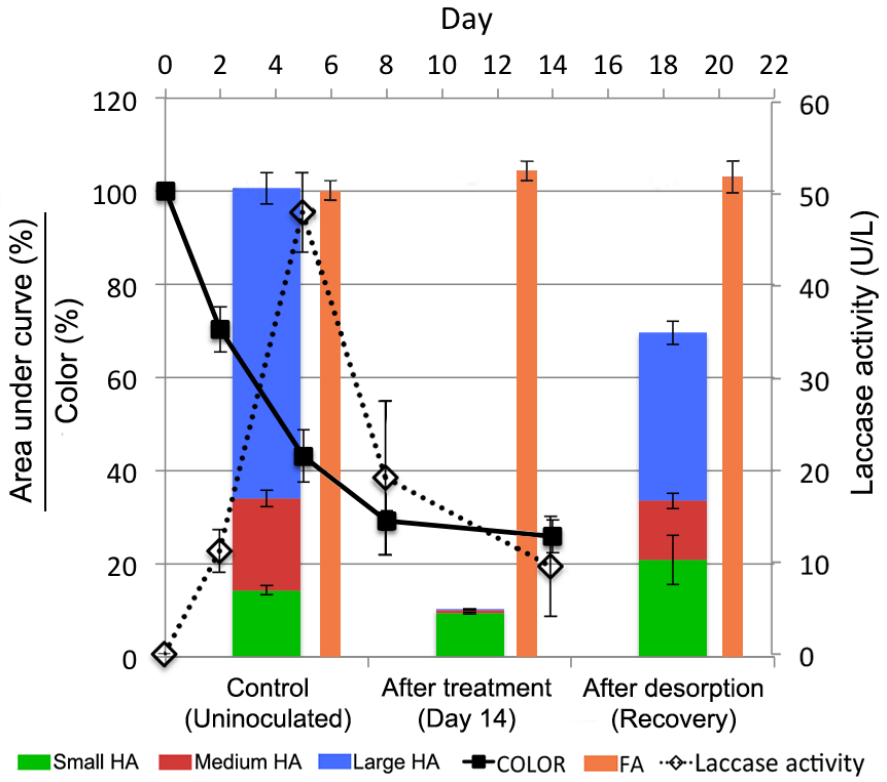

Fig 3. Fungal treatment of the synthetic wastewater under sterile conditions. SEC results of HA and FA are presented as area under the curve (left Y-axis and lower X-axis). Colour removal (%) and enzyme activity (laccase) were monitored during the incubation period (upper X axis). Results are presented as average of triplicates ±SD.

During the fungal treatment, the humic concentration was significantly reduced, as it is shown by the color reduction in Fig 3. The results of the color removal are already corrected for the changes in the color of the HS-free control flasks. Most of the color was removed after 8 days of incubation when 70% color removal was achieved (75% after 14 days). The SEC analysis at the end of the incubation period (day 14) showed a complete removal of large and medium size HA molecules from the media and slight increase in the concentration (area under curve) of FA-like molecules. After performing the recovery (desorption), SEC analysis revealed that almost 60% of the HA were recovered from the mycelia, suggesting biosorption of 60% of the initial HA content to the fungal mycelia during the incubation. The MW distribution analysis showed a change in the composition of HA complex with regard to the ratio of large, medium and small size molecules. When normalized to

100%, the composition of recovered HA was made up by 52% large, 18% medium and 30% small HA molecules. By comparing these ratios with the initial composition of the HA complex (66% Large, 20% medium, 14% small), it is apparent that there is a shift towards the lower size molecules in the HA complex. The avg. MW of the HA after the recovery procedure was about 1.14 kDa. After the recovery of sorped HA, the SEC results (Fig 3) showed 46% reduction in the concentration of large HA molecules compared to their initial concentration (from 66% to 36%). Also it showed 35% reduction in the medium size HA molecules (from 20% to 12%) and 46% increase in the concentration of small HA molecules (from 14% to 20%), as a result of the fungal treatment. These observations suggest the degradation of large and medium size HA molecules to smaller molecules.

Laccase activity reached a maximum of 48 $U.L^{-1}$ after 5 days and then decreased to 10 $U.L^{-1}$ after 14 days. The increase in the laccase activity during the first 5 days correlated with the high color removal during that period, suggesting involvement of laccase in the mycoremediation of HA. This is in agreement with previous studies under sterile conditions reporting on the degradation of humics by laccase [10,45].

4.3.4. HA removal from real industrial wastewater by Immobilized fungi under sterile conditions

The results of the fungal treatment of real wastewater are shown in Fig 4. The molecular size distribution of HA was different from the HA in the synthetic wastewater (Fig 3). Large HA molecules, medium size molecules and small molecules comprised 51%, 32% and 16% of the HA complex of the real wastewater, respectively. The analysis of the uninoculated controls during the incubation period showed less than 4% variation in color and less than 7% in the area under SEC curve for HA and around 14% for FA (data not shown).

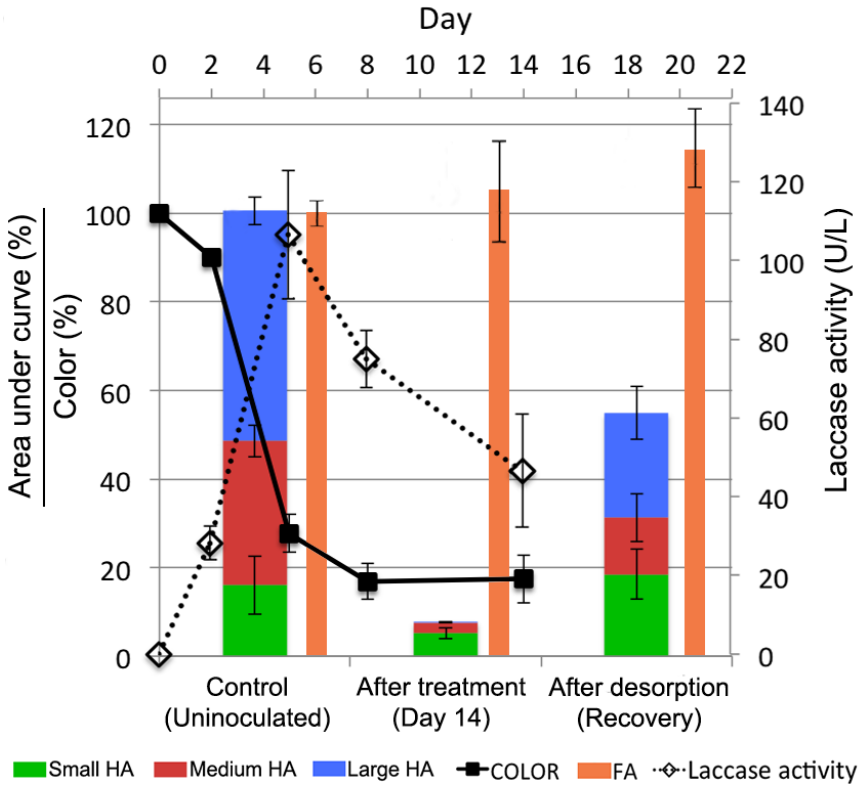

Fig 4. Fungal treatment of the real wastewater under sterile conditions. SEC results of HA and FA are presented as area under the curve (left Y-axis and lower X-axis). Color removal (%) and enzyme activity (laccase) were monitored during the incubation period (upper X axis). Results are presented as average of triplicates ±SD.

Colour of the industrial wastewater reached its minimum of 18% (82% color removal) after 8 days of fungal treatment, and then remained steady. The SEC analysis of the HA content of the wastewater at the end of the treatment (day 14) revealed that large and medium size HA molecules were removed almost completely, along with 68% of the small HA molecules. SEC analysis of the HA content after the recovery of sorped HA from fungal mycelia, revealed that about 50% of the HA were recovered from the fungal granules.

At the end of the incubation period and after the recovery of the sorped HA, the composition of HA complex is comprised of 43% large HA, 23% medium size and 34% small HA. The average MW of the HA complex was reduced to 0.96 kDa.

The FA content of the media showed a 25% increase, which may suggest conversion of HA molecules to FA-like molecules, as reported before [11,46].

The increase in the laccase activity during the first 5 days of the incubation coincided with a steep reduction in color, and when the enzyme activity started to decrease after 5 days, the rate of color removal was also reduced. This correlation between laccase activity and color removal, suggests the involvement of laccase in the degradation or conversion of HA, although concomitant absorption of HA cannot be excluded.

4.3.5. HA removal from real industrial wastewater by immobilized fungi under non-sterile conditions

The HA content of the wastewater showed to be stable during the incubation period under non-sterile conditions, as it was observed by less than 5% change in color, around 10% change in area under the SEC curve of HA and less than 15% for FA in the uninoculated controls (data not shown).

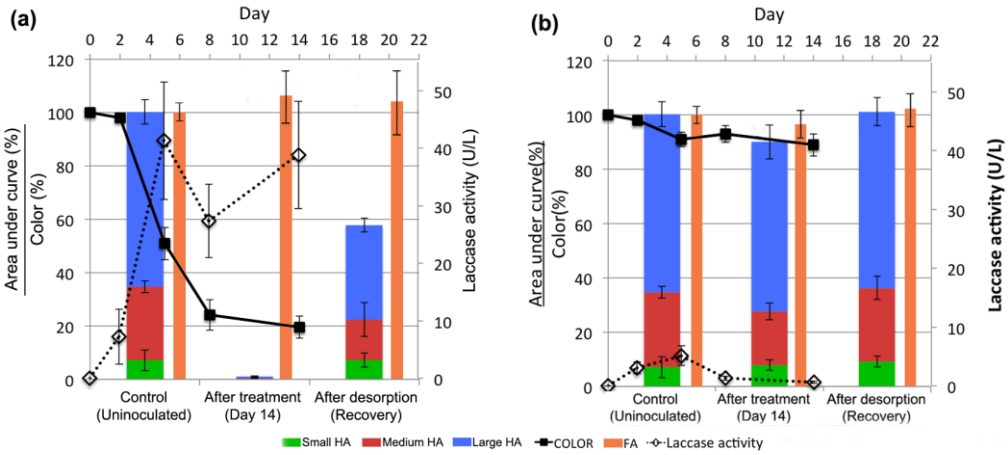

Fig 5. Fungal treatment of real wastewater under non-sterile conditions with immobilized fungi (a) and with free (non-immobilized) fungal cells (b). SEC results of HA and FA portions of the media are presented as area under the curve (left Y-axis and lower X-axis). Color removal (%) and enzyme activity (laccase) were monitored during the incubation period (upper X axis). Results are presented as the average of duplicates and error bars indicate the min and max values.

The results of the HA removal by immobilized fungi as well as free fungal cells (non-immobilized) are presented in Fig 5. It is clear that under non-sterile conditions, immobilized fungi showed higher HA removal and enzyme activity than free fungal cells. Previously, we have reported a successful humic removal from this (real) wastewater, supplemented with the same defined media, using free cells of *T. versicolor* under sterile conditions [11]. However, under non-sterile conditions, free fungal cells incubated in real wastewater supplemented with defined media showed very low enzyme activity (maximum 7 $U.L^{-1}$) and maximum 10% color removal. The SEC results showed only a small reduction in medium size HA after 14 days of incubation. After the recovery procedure almost all the initial HA was recovered back to the media, suggesting no significant degradation or conversion of HA. It was observed that the media of free fungal cells became turbid and cloudy after 4 days, suggesting high bacterial growth in the media [47].

In the case of immobilized fungi, 75% color removal was achieved after 8 days of incubation, which was further increased to 80% after 14 days. The SEC results indicated that almost complete removal of HA molecules was achieved after 14 days, which was accompanied by a slight increase in the concentration of FA-like molecules. After the recovery procedure, about 60% of the total HA content of the wastewater was recovered from the fungal mycelia, suggesting about 40% degradation of HA. The composition of the recovered HA was slightly different from what it was before the fungal treatment. The initial composition of HA complex (shown as uninoculated control) was 65% large, 28% medium and 7% small HA, and (after normalizing to 100%) it changed to 61% Large, 26% medium and 13% small HA, after the fungal treatment. The average MW of the HA in the wastewater was slightly reduced from 1.4 kDa to 1.3 (± 0.04) kDa. Laccase activity reached its maximum of 41 $U.L^{-1}$ after 5 days, and then decreased to 27 $U.L^{-1}$, although it was recovered to 38 $U.L^{-1}$ on day 14.

4.3.6. Deactivated fungi; biosorption

Treatment of HS-rich wastewater with deactivated immobilized fungi is important to study the efficiency of the sorption of HA to fungal mycelia. Deactivated fungi cannot produce enzymes hence, no degradation can occur. The efficiency of sorption of HA to deactivated fungal mycelia was measured by color removal and presented in Fig 6.

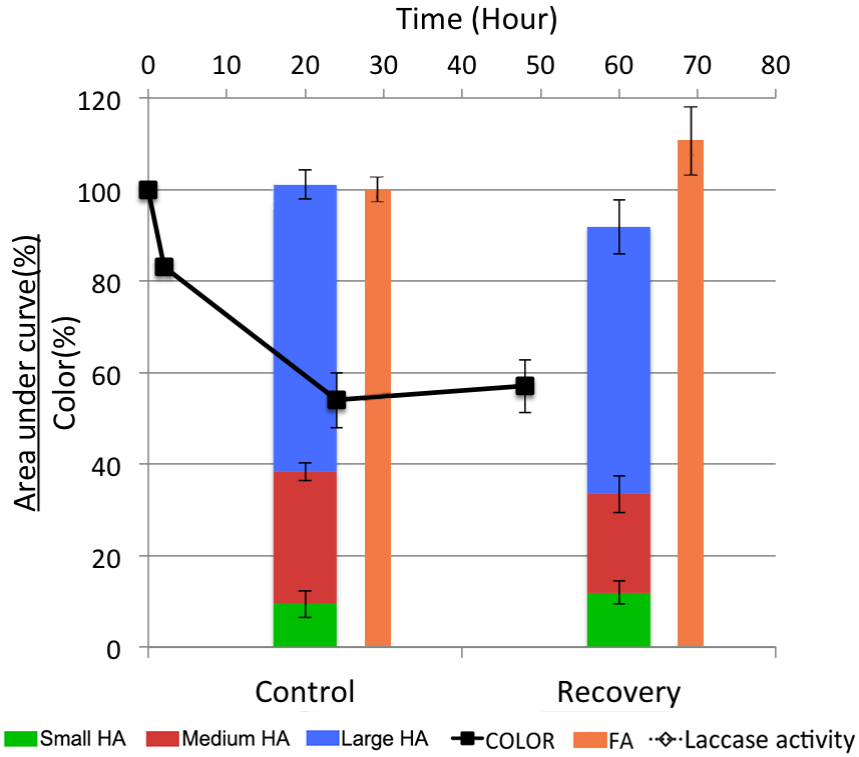

Fig 6. HA removal by deactivated immobilized fungi. SEC results of HA and FA are presented as area under the curve (lower X-axis). Color removal (%) was monitored during the incubation period (upper X axis). Results are presented as the average of triplicates ± SD.

Deactivated fungal mycelia could remove 55% of the color after 24 hours, although some of the color was recovered later on, resulting in about 40% color removal after 48 hours. The increase in color after the second day of incubation, was possibly a result of desorption of some of the HA from the fungal mycelia. The SEC results after the recovery of the sorped HA, revealed that the large HA molecules were recovered almost completely. However, only 75% of the medium size HA molecules were recovered. On the other hand, the concentration of the recovered small HA molecules were slightly higher than the control. The area under the curve of FA showed 12% increase compared to control. The latter might be due to the release of FA-like molecules from the carriers, as well as the fungal mycelia as a result of vigorous mixing and dispersion during the recovery procedure. Also, the decrease in

the fraction medium size HA molecules and the increase in the fraction small size HA molecules might be due to chemical reactions in the media during the 48 hours of incubation. Overall, the total recovery efficiency was more than 95%.

4.3.7. Sequential batch experiment

The real wastewater was subjected to treatment by immobilized WRF under non-sterile conditions, in four sequential batches, without renewing the inoculum (immobilized fungi). Results are shown in Fig 7.

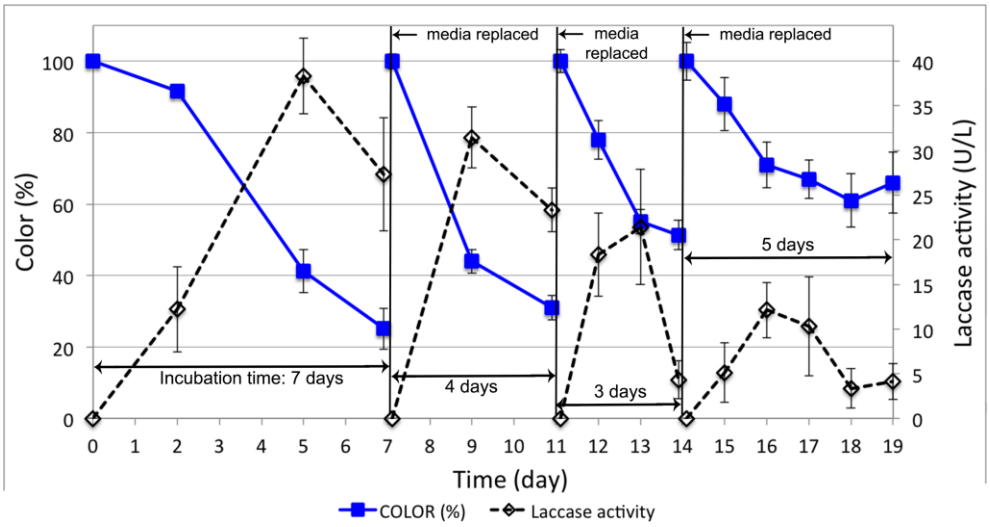

Fig 7. Color removal and laccase activity during the sequential batch experiment using immobilized fungi under non-sterile conditions. Results are presented as the average of duplicates and error bars indicate the min and max values.

The first batch lasted 7 days, and when about 78% decolorization was achieved, the media (treated wastewater) was removed, while the immobilized fungi were kept in the jar. Then fresh wastewater was added and the incubation was continued. After 2 days of the second batch (9 days in total) about 60% decolorization was detected, also a recovery of laccase activity in the media to 33 $U.L^{-1}$ was observed. After the reduction in the enzyme activity in the last 2 days of the first batch, the recovery of laccase activity in the second batch suggests that the decrease in the laccase activity was probably due to growth of heterotrophic bacteria in the media, and when replacing the old media with a fresh one, the enzyme activity was recovered. However laccase activity decreased to 23 $U.L^{-1}$ after 4 days in the second batch feeding, when about 70% decolorization was measured. When the reduction in the

laccase activity and decrease in the rate (slope) of decolorization were observed, the incubation was stopped and the media was replaced again. After 2 days of incubation in the third batch, enzyme activity dropped drastically from 20 ($U.L^{-1}$) to less than 5 ($U.L^{-1}$). This might be due to the depletion of carbon and nutrients available for fungi as a result of exhaustion of sorghum grains, which was supported by the observation that loose mycelia appeared in the media and the granular shape immobilized fungi started to disperse. Maximum 50% decolorization was achieved after 3 days incubation in the third batch. In the fourth batch 40% decolorization was achieved after 4 days, and after that color slightly increased. The increase in color was probably due to the release of sorped HA from fungal mycelia. The enzyme activity in the fourth batch reached a maximum of 13 ($U.L^{-1}$) and then started to decrease to less than 5 ($U.L^{-1}$) in day four.

4.4. Discussion

Application of WRF in wastewater treatment has been put off due to the challenges associated by the growth of these fungi under non-sterile conditions. To address this important issue, it was hypothesized in this study that the immobilization of WRF on a nutrient source could facilitate the growth of WRF under non-sterile conditions. Results showed that sorghum could act as the sole nutrient source for growth and laccase production of *T. versicolor*.

Under non-sterile conditions, immobilized fungi could degrade HA in real industrial wastewater, when fungal free cells could not grow. Nonetheless, the laccase activity was much lower (almost 50%) of that observed in the sterile experiments using real industrial wastewater. This may be related to the deactivation or inhibition of laccase by other microorganisms that were presented in the wastewater. Also, heterotrophic bacteria could simply use the protein laccase as substrate [48], which might be another reason for the decrease in laccase activity in the real wastewater. It is noteworthy that the compounds which are usually used in the defined WRF media to induce laccase activity, such as Cu^{2+} [49] , Mn^{2+} [50] or veratryl alcohol [51] were not used in this study.

Although only laccase was assayed as the fungal enzyme in this study, the involvement of other enzymes like MnP should not be neglected. The involvement of MnP in the degradation of humics has been reported before [11,52]. However,

the presence of humics in the media could result in misestimating MnP activity [10,43,53], hence it was not assayed in this study.

Immobilized fungal granules could remove HA from wastewater in the sequential batch operation, without renewing the fungal inoculum. However, the HA removal efficiency deteriorated in each batch. Therefore, More studies are needed to increase and maintain the extracellular enzyme activities of the WRF for long term treatments and likely, sorghum should then be added periodically as the substratum for WRF.

Overall, the immobilization of WRF on the nutrient source could be considered as a promising strategy to facilitate the application of WRF under non-sterile conditions.

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Chapter 5

Continuous fungal treatment of humic-rich wastewaters under non-sterile conditions: application of a fluidized bed bioreactor with partial renewal of fungal biomass

Abstract

The application of White rot fungi (WRF) under non-sterile conditions is considered as a major challenge in applying WRF in wastewater treatment. In this study, a fluidized bed bioreactor was inoculated with pellets of T.versicolor to remove HA from synthetic and real humic-rich wastewaters. To maintain a young and active fungal culture, half of the fungal biomass was periodically renewed. Results showed that 90% color removal was achieved when treating synthetic wastewater for more than 30 days of continuous treatment. In this period, an average of 47 UL-1 laccase activity was detected with a peak after each fungal biomass renewal. Results of size exclusion chromatography (SEC) confirmed that more than 80% of the large HA molecules were removed from the wastewater during fungal treatment. When treating real wastewater, the color removal results were not meaningful, due to the low initial color of the wastewater. However, the SEC results revealed that more than 85% removal of large HA molecules was achieved within the first 3 weeks of the treatment. In this study, for the first time, the continuous treatment of humic-rich wastewaters with a fungal reactor under non-sterile conditions was successfully demonstrated.

5.1. Introduction

Humic substances (HS) comprise the most abundant portion of the natural organic matter (NOM) in the aqueous system and soil [1,2]. HS are produced during the decomposition of animal and plant tissue and are extremely resistant to biodegradation [3]. The HS involve a physically and chemically heterogeneous mixture of biogenic molecules with a wide range of molecular masses, composed of mixed aliphatic and aromatic units [4,5].

Three main fractions of HS can be separated based on their solubility in acids or alkalis: humic acid (HA) (the major fraction of HS), which are soluble in alkali and insoluble in acid; fulvic acids (FA), which are lower-molecular-mass compounds with a smaller number of total aromatic carbons compared to HA and are soluble in both alkali and acid; and humins, which are insoluble in both acid or alkali [2,6].

Humic acids may cause environmental problems once released from man-made environments into the ecosystem. HA can carry heavy metals ions and other insoluble xenobiotics, increasing their solubility and motility in soil and water [7]. In addition, HA can become precursors of trihalomethanes, which are carcinogenic compounds formed during disinfection and chlorination of drinking water [8,9]. In wastewater treatment plants, HA can cause membrane fouling [10,11].Additionally, the presence of HA results in colored effluents [12]. Therefore, it is important to promote the degradation of humic acids in the wastewater before discharging the effluent.

HA are highly recalcitrant with respect to biodegradation and the ability of bacteria to degrade HA is limited [13]. The removal of HA from waters by White Rot Fungi (WRF) has been demonstrated before [4,14]. The mechanism of HA removal by WRF has been reported to involve enzymatic degradation of HA, conversion of HA to FA and also biosorption of HA by fungal mycelia [14,15].

Most of the studies on degradation of HA by WRF have been conducted under sterile conditions in Erlenmeyer-scale. Under non-sterile conditions, fungal growth and enzyme activity are inhibited severely, resulting in the failure of HA removal [16]. The application of WRF under non-sterile conditions has been reported to be the major hurdle in industrial application of these organisms. This is mostly attributed to bacterial proliferation, which results in severe competition for nutrients.

WRF are low-grade eukaryotes, and competition with fast growing bacteria, usually result in bacteria's favor [17,18].

The partial renewal of the fungal biomass has been reported to be effective in maintaining the fungal activity under non-sterile conditions[19,20], with a cellular retention time of 21 days [19]. This process was initially developed for textile dye removal from water in a fungal bioreactor, and later on was applied to remove pharmaceuticals in a hospital wastewater [21]. In both cases, the fungal biomass renovation facilitates the maintenance of a young fungal culture, which promotes the fungal activity and avoids operational problems, and therefore allow for continuous long-term operation of a fungal reactor under non-sterile conditions [19–21].

The goal of this study is to apply a fungal reactor for continuous HA removal from wastewater under non-sterile conditions. To this end, a pulse-aerated fluidized bed fungal reactor was operated continuously under non-sterile conditions with periodic partial fungal biomass renovation. The fungal reactor treated both synthetic and real industrial HA-rich wastewaters.

5.2. Materials and methods

5.2.1. Fungi and chemicals

Trametes versicolor DSMZ 3086 was obtained from DSMZ (Germany) and was maintained by sub-culturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25°C (pH 4.5).

Fungal pellets production was done by growing fungi in shaking flasks under sterile conditions as described previously [22]. Pellets were washed with deionized water prior to use for inoculation of the reactors.

All the chemicals were purchased from Sigma-Aldrich (Barcelona-Spain) and were of analytical grade unless otherwise stated.

5.2.2. Synthetic and real industrial wastewater

Synthetic wastewater was prepared by diluting 50 mL of HA stock solution in 950 mL tap water. Stock solution was prepared with humic acid powder (Sigma-Aldrich) as previously described [15].

Industrial real wastewater was collected from the effluent of a wastewater treatment plant of a food-processing company (Eindhoven, The Netherlands) and stored at 4°C. The main characteristics of this wastewater were as follows: pH: 7, conductivity: 4.5 mS.cm-1, COD: 294 mgO2.L-1, sCOD: 288 mgO2.L-1, TSS: 6.6 mg.L^{-1} and VSS: 5.3 mg.L^{-1} and ammonium: 0.51 NH_4^+-N.L^{-1}.

5.2.3. Bioreactors and operating conditions

The experiments were performed in identical glass air fluidized bed bioreactors (1.5 L) [19]. The temperature was maintained at 25°C and pH was controlled at 4.5 by HCl (1M) or NaOH (1M) addition. Hydraulic retention time (HRT) was 3 days. Fluidized conditions in the reactor were maintained by air pulses generated by an electro valve. The electro valve was controlled by a cycling timer (1 s open, 3 s close) and the airflow was 10 L h^{-1}.

Nutrients for maintenance, glucose and ammonium tartrate, were added continuously from their sterile stock solutions (100 g L^{-1} glucose and 26.3 g L^{-1}, ammonium tartrate) at a flow rate of 1 mL h-1. The nutrients were added at the *T. versicolor* consumption rate.

Two different sets of experiments were carried out with synthetic wastewater (SW) and real wastewater (RW). For each type of water, two bioreactors were set up in parallel, one inoculated with pellets of *T. versicolor* and the other one remained non-inoculated as a control. Pellets of *T. versicolor* were added at 2.54 and 2.20 g L^{-1} dry cell weight (DCW) concentration for SW and RW, respectively. The partial biomass renovation was performed by replacing 1/3 of the biomass with fresh biomass every 7-8 days [19]. Samples were collected for laccase measurement, color measurement and size exclusion analysis (SEC). The samples were filtered through 0.45 μm, prior to analysis.

5.2.4. Analytical methods

5.2.4.1. Size exclusion chromatography (SEC)

The SEC analysis was performed as described before [15]. Each sample (2 mL) was acidified (pH <2) by adding 20μL HCl (37%), and centrifuged (14000 rpm, 20 min). The acid supernatant was separated as FA and the precipitants were re-suspended by adding 2 mL NaOH (0.1 M) and used as HA portion of the sample. Both FA and

HA portions of the samples were subjected to SEC analysis. The SEC was conducted using a Phenomenex column (Yarra™ 3 μm SEC-2000, LC Column 300×7.8 mm, Ea) connected to an ultrafast liquid chromatograph (UFLC) (Shimadzu, Prominence) to detect changes in the concentration (area under chromatogram) and MW of HA (and FA) molecules during the incubation with WRF. The areas under the curves, as well as the (weighted) average molecular weights of the eluted substances, were calculated by Labsolution software (Shimadzu). The results of the SEC analysis are presented as area under the SEC curve, indicating the concentration of the detected substances. SEC results of HA are color coded for large (2-1.2 kDa), medium (1.2-0.2 kDa) and small size (< 0.2 kDa) HA molecules, to clearly reflect the changes in the MW distribution of the HA molecules during the treatment. The MW range for each group was selected based on the main peaks detected during the SEC analysis [14,15].

5.2.4.2. Other analysis

Color of the HA-rich wastewater was assessed by measuring the light absorbance at 450 nm [23]. The color is presented as percentage and the initial color of the wastewater, which was set as 100%. Laccase activity was measured through the oxidation of 2,6-dymetoxyphenol (DMP) by the enzyme laccase [24]. Activity units per liter (U L-1) are defined as the amount of DMP in micromoles per liter, which is oxidized per minute (μmol DMP. L-1. min-1).

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighted glass-fiber filter (Whatman, Spain) when reaching constant weight at 100°C.

5.3. Results and Discussion

5.3.1. Synthetic wastewater

The results of the color removal and laccase activity of the fungal reactor treating synthetic wastewater are presented in Fig. 1. Also the color of the wastewater in the feeding tank and the control reactor (without fungi, fed with nutrient media and wastewater) are presented.

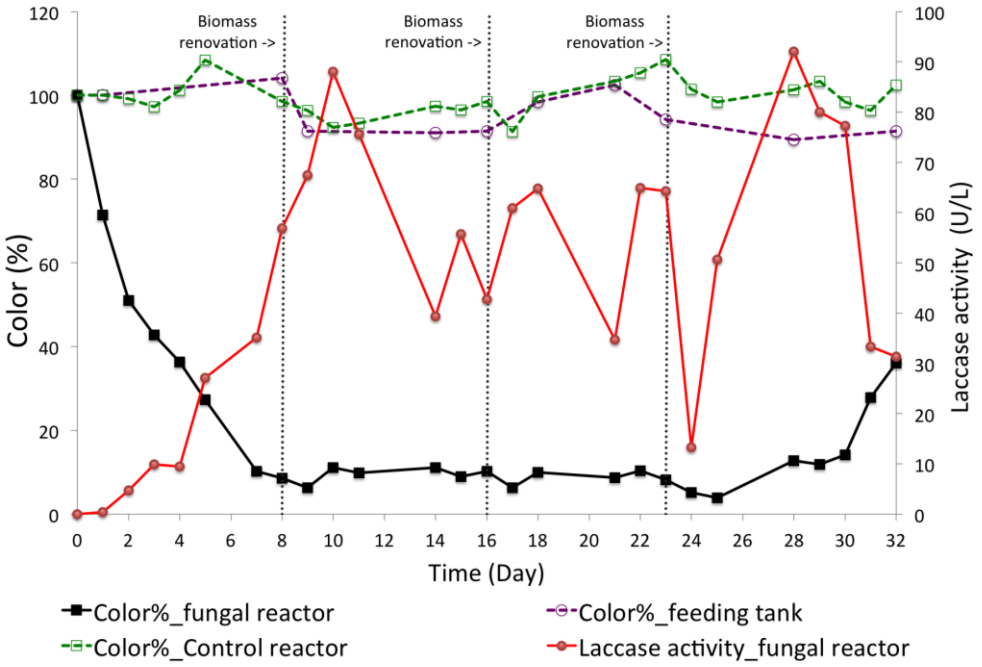

Fig 1. Evolution of the color in the fungal reactor (effluent), control reactor (without fungi) and the feeding tank (wastewater) in the treatment of synthetic wastewater. Laccase activity in the fungal reactor is represented with rhombus. Vertical dotted lines mark the instances of partial fungal biomass renewal. Time 0, is the inoculation time. The results are presented as the average of triplicate measurements, with standard error of less than 2% for color and less than 9% for laccase activity.

The stable color of the feeding tank (10% change) reveals the stability of the color of the synthetic wastewater. Also the color of the control reactor (without fungi) only changed less than 10%, which might be due to the bacterial growth inside the control reactor. Therefore it can be concluded that any change in the color of the wastewater in the fungal reactor is related to the fungi.

One week after inoculation, almost 90% color removal was achieved in the fungal reactor. The first renewal of the fungal biomass was performed on day 8. Following the biomass renewal, the color remained at 10% (90% color removal) for another week. Taking into account that the reactor was performed with HRT of 3 days, on day 8 the stationary state (with regards to color) was achieved. The second fungal biomass renewal was performed on day 16, and the third one on day 23. The 4th fungal biomass renewal was scheduled for day 30. However, in order to emphasize

the importance of biomass renewal on color removal and enzyme activity, the 4th renewal was not performed. As it can be seen, an apparent increase in the color occurred after day 30. It was also observed that fungal pellets started lysing and fragments of mycelia were floating inside the reactor. These observations emphasize the importance of the fungal biomass renewal for maintaining the color removal.

The average of laccase activity obtained along the treatment was 47 UL-1 with a peak of laccase activity after each biomass renewal. The average laccase activity between days 8 and 30, when the color was stable at 10% (90% color removal), was 61 UL-1.The last steep decline in the laccase activity on day 30 and the lack of the recovery, correlating with the color increase from day 30, suggest the crucial role of fungal biomass renewal on maintaining the fungal enzyme activity and color removal in the reactor.

Overall a constant 90% color removal was achieved for more than three weeks in the fungal reactor treating the synthetic wastewater under non-sterile conditions.

In order to have a more in-depth observation on the HA molecules during the fungal treatment, SEC analysis was performed. Results are presented in Fig 2.

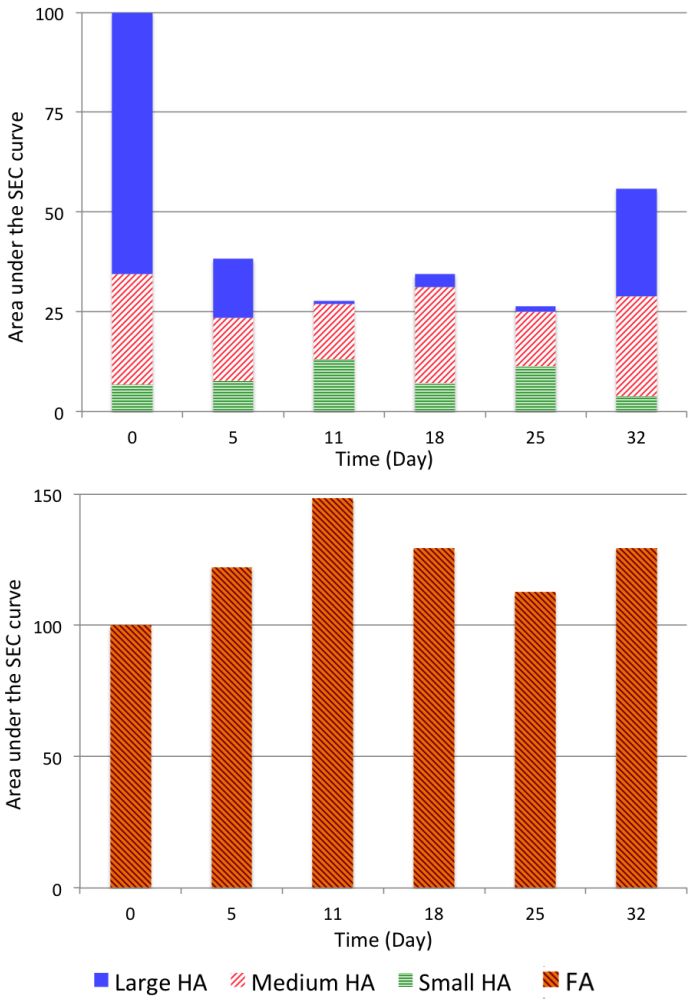

Fig 2. SEC analysis of the synthetic wastewater during the fungal treatment. Top: HA, Bottom: FA. Results are presented as the average of at least duplicate measurements with standard error of less than 2% for HA and less than 6% for FA.

The synthetic wastewater was comprised of 65% large HA, 28% medium size HA and 7% small HA molecules (day 0). The average MW of HA was 2.06 kDa. The SEC analysis was also performed on the feeding tank and the control reactor and no significant change was observed during the experimental period (<10% for HA and <20% for FA), indicating the stability of the HA and FA of the wastewater in the feeding tank and the control reactor (data not shown). On day 5, more than 60% of the total HA content (area under the SEC curve) was removed in the fungal reactor.

However, the MW distribution did not remain the same. Most of the HA removal on day 5 was due to the removal of large HA molecules, i.e. almost 80% of the large HA was removed (from 65% to 15%). The medium size HA were decreased by almost 40% (from 28% to 16%). The average MW of HA content of the wastewater was reduced to 1.4 kDa. On day 11, 18 and 25 the total concentration of HA (area under the SEC curve) stayed at approximately 30% (70% HA removal). Large HA molecules were almost completely removed. In result, the average MW of HA was further reduced to 0.85 kDa. However, on day 32, the large HA molecules were increased to almost 40% of its initial value. The total area under the curve of HA content of the wastewater was equal to 56% of its initial value (44% HA removal). When comparing to the results of the color removal (fig. 1), there is a clear correlation between the color removal and HA removal. Also, results of the day 32, once again indicated the importance of biomass renewal in HA removal. When the 4th biomass renewal was not performed on day 30 (7 days after the last renewal), the HA concentration started to significantly increase on day 32, as can be seen in fig. 1.

The fungal reactor presented high removal values (>72%) during 25 days with a regular biomass renewal treating synthetic water in continuous mode under non-sterile conditions. Other authors also achieved well HA removals values working in batch reactor with synthetic medium. Barışçı (2017) [25] obtained 60% degradation applying electro-synthesized ferrate (VI) and Asghari et al. (2017) [26] reported 96.5% removal efficiency by electrocoagulation.

Comparison of the SEC results of the HA and FA content of the synthetic wastewater during the fungal treatment, suggests that there is an inverse correlation between HA and FA concentrations. The decrease in HA concentration until day 25 corresponded with an increase in FA concentration, and the increase in the HA concentration on day 32, corresponded with a decrease in FA concentration. This observation is in line with some previous reports [15,23], and suggests a formation of FA molecules as a result of the in-complete degradation of HA molecules.

5.3.2. Real Wastewater

Compared to the synthetic wastewater, the real wastewater had a lower color (absorbance at 450 nm, raw data not shown). This difference was probably due to a lower HA concentration in the real wastewater compared to the synthetic wastewater.

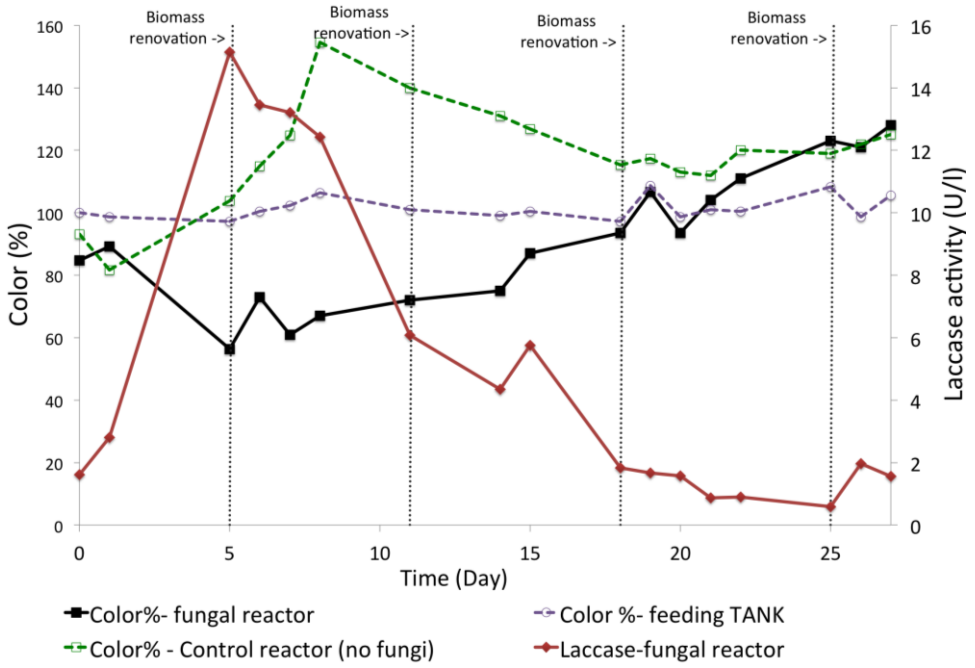

Fig 3. Evolution of the color in the fungal reactor (effluent), control reactor (without fungi) and the feeding tank (wastewater) in the treatment of real wastewater. Laccase activity in the fungal reactor is represented with rhombus. Vertical dotted lines mark instances of partial fungal biomass renewal. Time 0, is the inoculation time. The results are presented as the average of triplicate measurements, with standard error of less than 2% for color and less than 9% for laccase activity.

The color of the wastewater in the feeding tank was stable (<10% change during the experimental period). However, the color of the control reactor (fungi free) was not stable, and increased to 55% higher than the initial color of the wastewater in the second week of the incubation. Since the control reactor was also fed with the nutrient media, identical to the fungal reactor, this increase in the color could be due to growth of bacteria in the media. The relatively low initial color of the real wastewater, makes it hard to interpret changes in media's color as changes in the HA concentration. The reason is that the growth of bacteria, as well as fungi, can change the color of the media, usually resulting in an increase in the color (yellow to brown) of the media [14,15,27]. Therefore, the changes in color of the media, resulting from fungal or bacterial growth could very well have masked the changes in color of the media resulted from HA removal.

As can be seen in Fig. 3, the color of the wastewater in the fungal reactor was reduced to 60% (40% color removal) on day 5, but it started to increase slowly since then and reached 130% (30% higher than the initial color of the HA-rich wastewater) in the last week. Also in the last two weeks of the treatment, it was visually observed that the turbidity of the media was increased. Similar observation was made in the control reactor, but from the end of the first week onwards. The increase in turbidity is usually being considered as an indication of bacterial growth [18,28]. Overall, due to the low initial color intensity of the real wastewater, it is not possible to evaluate the HA removal by monitoring the color of the media. Therefore, the SEC analysis of the HA content of the wastewater during the fungal treatment becomes crucial for evaluating the HA removal.

The SEC results of the real wastewater shown in Fig 4, confirmed the lower HA concentration in the real wastewater compared to the synthetic wastewater; the UV detector detected a lower intensity (data not shown). Large HA molecules comprised 57%, medium size HA covered 33% and small size molecules made up 10% of the total HA content of the real wastewater. The average MW of the HA molecules in the real wastewater was 1.9 kDa. The SEC analysis of the feeding tank and the control reactor showed less than 15% change in the HA and less than 17% change in the FA content of the wastewater, indicating the stability of the humics.

In the fungal reactor after 5 days of treatment, almost all the large HA molecules were removed from the wastewater, and in total around 80% HA removal was achieved. The adsorption on to the fungal pellets may have had a prominent role in the first days of the treatment. It should be noted that on the same day that 80% HA removal was detected by SEC analysis, only 40% color removal was measured (fig. 3).

After 11 days of treatments, the total concentration (area under the SEC curve) of HA was higher than its initial concentration. This likely can be attributed to the vast increase in the small size HA molecules compared to their initial concentration (from 10% to 50%). The concentration of medium size HA molecules was also increased, but the concentration of large HA molecules was considerably reduced (from 57% to 7%). The average MW of the HA on day 11 was reduced to 0.65 kDa. The decrease in large HA and increase in small and medium size HA, suggest the in-complete degradation of large HA to smaller HA molecules. Corrales Escobosa et al. (2009) also demonstrated the degradation of HA with formation of lower MW soluble compounds by *Fusarium oxysporium* [6].

On day 18, the total concentration of HA was reduced to 27% of its initial value (73% removal). However, when compared to the decolorization results (fig. 3), the color of the wastewater was almost the same as its initial value (100%), indicating no color removal and no HA removal. This clearly demonstrate that the increase in the color of the wastewater is not related to the HA concentration. On day 25, there was an increase in the large HA molecules compared to day 18. This increase in large HA molecules continued more intensely until day 27, suggesting the decay in the fungal activity in the reactor.

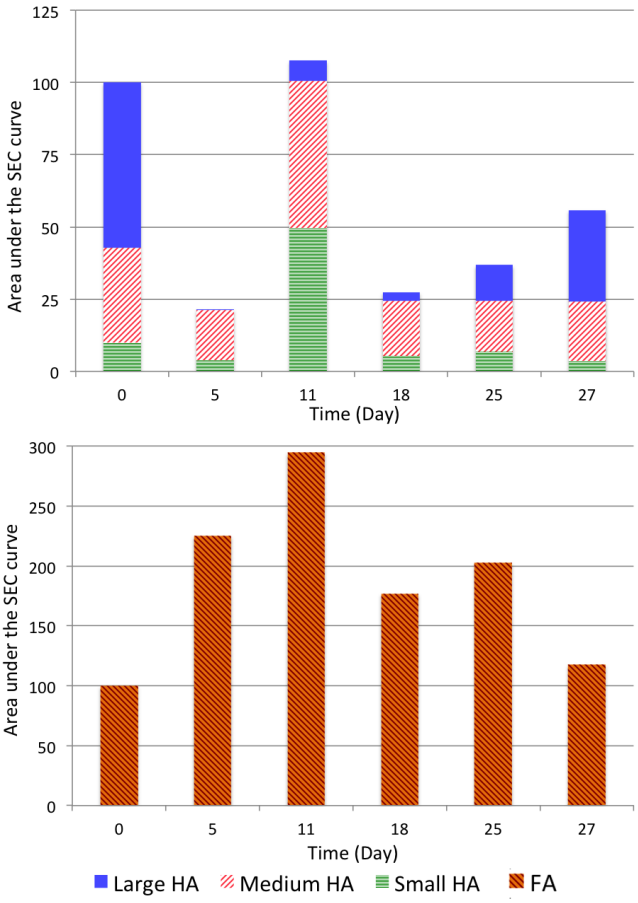

Fig 4. SEC analysis of the real wastewater during the fungal treatment. Top: HA, Bottom: FA. Results are presented as the average of at least duplicate measurements with standard error of less than 2% for HA and less than 4% for FA.

The performance of the reactor along the wastewater treatment showed more than 90% removal of large HA molecules, until day 25. The results are comparable with other authors who also studied the HA removal from real wastewater applying different technologies. Zhai et al. (2016) [29] have reported 66% HA removal efficiency by subcritical water catalytic oxidation technology with a batch reactor. Wang et al. (2016) [30]reported the total removal of HA from concentrated leachates by ozonation. Biosorption of humic acid also has been studied using activated sludge [31] and *Rhizopus arrhizus* [32].

During the fungal treatment of the real wastewater, the FA content of the wastewater was significantly increased. The FA concentration increased to 300% (3 times higher than its initial value) on day 11. The increase in the FA concentration continued with a lower rate (170% and 200% on day 18 and 25, respectively), but the FA concentration decreased to its initial value (100%) on day 27. The increase in the FA concentration suggests the in-complete enzymatic degradation of HA, which resulted in formation of FA molecules. Also, the decrease in the laccase activity correlated with the decrease in the FA concentration after day 11, which suggests the involvement of laccase in conversion of HA to FA molecules. The conversion of HA to FA by laccase has been studied before [15]. Also, Fakoussa and Frost (1999) [33] suggested the correlation between the decreased concentration of humic acids and the increased concentration of fulvic acid. After day 11 when FA concentration decreased and enzyme activity dropped drastically, still significant HA removal was observed. The reason could be due to the biosorption of HA to fungal mycelia, as well as involvement of other fungal enzymes.

The concentration of FA, small and medium HA sometimes increased along the treatment. As indicated before, this increase results from the degradation of large HA into smaller molecules. The average MW of HA was decreased from 1.9 kDa to 0.6 kDa on day 11, and then it gradually increase to 1.8 kDa on day 27. The average MW of FAs in the wastewater was 0.6 kDa. This value started to increase from the second week of the treatment and reached 1.1 kDa on day 27.

The overall measured laccase activity during the treatment of real wastewater was lower compared to synthetic wastewater. The reason could be the interference of some chemicals that are generally present in the real wastewater with the enzyme assay. The interference of tannic acid, a plant polyphenol, and some of its structural-related molecules in laccase assay has been reported before [34]. Also the interference of catalase, a common enzyme produced in aerobic organisms, in the

assay of laccase has been studied before and reported to cause an underestimation of laccase activity [35]. The inhibition of laccase by some chemicals in industrial wastewaters [36] and interference of some metals in the assay of laccase [37] have been also been reported. Although in this study the presence of these molecules was not studied specifically, but the presence of these molecules in the real wastewater is likely, due to the origin of the real wastewater used in this study. The bacterial growth could also have had a negative effect in the laccase activity [16,38]. However, the laccase activity was used as a possible indicator of fungal activity, although some previous works found no clear relationship between the extracellular enzymes and HA degradation [39]. In addition, when working with real wastewater, detection of laccase activity confirms fungal activity, on the contrary the low level of the enzyme is not an indication of fungus inactivity [40].

The lower level of removal obtained working with real wastewater is in accordance with previous studies [41]. This performance deterioration is generally caused by the overgrowth of bacteria, which impose an inhibition on fungal growth and enzyme production [17,42]. Taking into account that the real wastewater was collected from a food processing wastewater treatment plant after going through multiple biological treatment units, it very likely contained more bacterial biomass than the synthetic wastewater.

The observed decrease in efficiency of the reactor treating real wastewater from day 18 might be tackled by changing the process parameters in order to increase the fungal activity after week 3 of the treatment. Shortening the period of biomass renewal or increasing the portion of biomass that is getting renewed could be effective in increasing the fungal activity. Also, using a nutrient source that is more selective for fungi over bacteria could be helpful. Previous studies suggest that fungus-assisted degradation of HA might be controlled using appropriate N- and C-sources required for fungus growth [6].

5.4. Conclusion

It was shown, for the first time, that the fungal reactor could continuously remove HA from synthetic wastewater under non-sterile conditions. Also, it was demonstrated that periodic and partial fungal biomass renewal was crucial in maintaining the fungal activity in the reactor. Overall, the treatment of the real wastewater was less effective than the synthetic wastewater. The HA removal

efficiency started to decrease after 18 days in real wastewater, whereas it stayed almost steady in synthetic wastewater and only decreased when the biomass renewal was not performed. In this study, the degradation of large HA molecules to smaller HA molecules and conversion of HA molecules to FA molecules were observed as results of the fungal treatment.

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Chapter 6

Application of immobilized *T. versicolor* on sorghum in a fluidized bed reactor for continuous treatment of humic-rich industrial wastewater under non-sterile conditions

6.1. Introduction

White rot fungi (WRF) are the most abundant wood degraders in nature and are distinctive among eukaryotes for having a non-specific enzymatic mechanism for the

complete degradation of lignin and lignin-like molecules [1,2]. WRF have shown potential for application in wastewater treatment, due to their ability to degrade recalcitrant organic compounds with molecular structures similar to lignin such as azo dyes [3,4], pharmaceuticals [5,6] and humic acids [7,8]. However, the application of WRF in real wastewater treatment plants has been deferred mainly due to complexities associated with their growth under non-sterile conditions. Since the sterilization of wastewater is obviously not a feasible option, maintaining the fungal growth under non-sterile conditions is crucial. The WRF's growth and enzymatic activity decrease drastically under non-sterile conditions. It has been reported that even in case of a high initial fungal activity and removal efficiency at the beginning of the treatment, it is very hard to maintain the fungal reactor's efficiency for a continuous treatment under non-sterile conditions [9–11]. The low growth rate and enzyme activity of WRF under non-sterile conditions is mainly due to bacterial proliferation. The presence of fast growing bacteria in the wastewater results in an intense competition with WRF for available nutrients. Due to the relatively slow growth rate of WRF, this competition usually leads to bacterial proliferation of the media and ultimately causes inhibition of the fungal growth and its enzymatic activity [12,13]. In Chapter 4, we reported on a successful application of WRF under non-sterile conditions to treat humic-rich industrial wastewater by immobilizing WRF on sorghum as the main nutrient source [14]. Also, in Chapter 5, we reported on the importance of partial renewal of fungal biomass in the maintenance of the fungal activity in a continuous reactor under non-sterile conditions. In this chapter, by combining these two techniques, we report on the application of immobilized fungi on sorghum in a continuous fluidized bed reactor under non-sterile conditions with partial renewal of immobilized fungal biomass. The objective of this chapter was to develop a fungal bioreactor that can continuously remove HA from HA-rich wastewater under non-sterile conditions.

6.2. Material and methods

6.2.1. Fungal strain and chemicals

Trametes versicolor DSMZ 3086 was obtained from DSMZ (Germany) and was pre-cultivated on 3% malt extract agar. All the chemicals, including coal HA, were purchased from Sigma-Aldrich (Germany), unless stated otherwise.

1.2.Immobilization of fungi

The sterilized grain sorghum was provided by Wageningen University (department of Plant breeding, The Netherlands). Immobilization of fungi on sorghum was performed by growing fungi on sorghum (solid state growth), as described before [15]. The fungal granules (fungi immobilized on grain sorghum) were stored in boxes each containing 280-300 granules, and kept at 4°C for maximum 7 days, until further use.

6.2.2. Humic-rich wastewater

6.2.2.1. Synthetic wastewater

The details of the preparation of synthetic wastewater and HA stock solution were explained in Chapter 2. Briefly, synthetic wastewater was prepared by diluting HA stock solution in tap water. HA stock solution was prepared by dissolving 4 g coal HA powder (Sigma-Aldrich) in 200 mL of NaOH (0.1 M), and removing the precipitates by centrifugation and filtration (0.45 μm) [7].

6.2.2.2. Industrial wastewater

Industrial wastewater was collected from the effluent of a wastewater treatment plant of a food-processing company (Eindhoven, The Netherlands). The main characteristics were as follows: pH: 6.9, conductivity: 4.5 $mS \cdot cm^{-1}$, COD: 331 $mgO_2.L^{-1}$, SCOD: 326 mgO_2. L^{-1}, ammonium: 0.46 mg NH_4^+-$N.L^{-1}$, TSS was 5.5 $mg.L^{-1}$ and VSS was 4.5 $mg.L^{-1}$.

6.2.2.3. Bioreactors and operational conditions

The experiments were performed using identical glass reactors. The schematic of the reactor setup is presented in Fig 1.

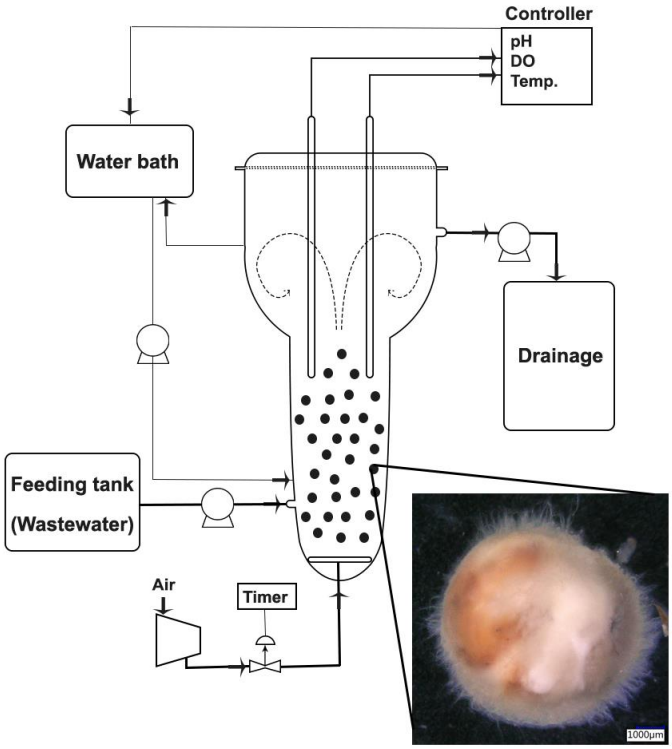

Fig 1. Schematic of the fluidized bed pulse aerated bioreactor setup inoculated with immobilized fungal granules

The reactor was a 3.5 L glass jacketed fluidized bed vessel. The lid was made of PVC and was easily lifted, to facilitate the handling of fungal biomass. Fluidization was provided by aeration through a sparger at the bottom of the reactor. Compressed air (20 $L.min^{-1}$) was supplied through an electric valve connected to a timer, to provide pulse aeration by opening the valve for 3 s and closing it for 6 seconds. The pH, DO and temperature were monitored continuously. Temperature was controlled at 25 °C by pumping water through the reactor's jacket. pH was adjusted between 4.5-5 by addition of NaOH (1M) or HCl (1 M).

Each reactor was inoculated with about 600 immobilized fungal granules (2 boxes). Partial biomass renewal was performed by removing approximately half of the fungal granules (≈300 granules) and replacing them by fresh ones. Hydraulic retention time (HRT) was adjusted at 3 days.

Two identical reactors were operated for each wastewater (real and synthetic). One reactor was inoculated with fungal granules, which will be referred to as the fungal reactor. The other reactor, called control reactor, remained non-inoculated, but the fungus free sorghum grains were added to it at the beginning and at the times corresponding to the biomass renewal in the fungal reactor. The amount of sorghum added to the control reactor was equal to what was added to the fungal reactor as fungal granules.

6.2.3. Analytical methods

6.2.3.1. Size exclusion chromatography

The details of the SEC analysis of humic content of the wastewater were explained before [7]. Briefly, each sample (2 mL) was acidified (pH <2) by adding 20µL HCl (37%), and centrifuged (14000 rpm, 20 min). The acid supernatant was separated as fulvic acid (FA) and the precipitants were re-suspended by adding 2 mL NaOH (0.1 M) and used as HA portion of the sample. Both FA and HA portions of the samples were subjected to SEC analysis. The SEC was conducted using a Phenomenex column (Yarra™ 3 µm SEC-2000, LC Column 300×7.8 mm, Ea) connected to an ultrafast liquid chromatograph (UFLC) (Shimadzu, Prominence) to detect changes in the concentration (area under chromatogram) and MW of HA (and FA) molecules during the incubation with WRF. The areas under the curves, as well as the (weighted) average molecular weights of the eluted substances, were calculated by Labsolution software (Shimadzu). The results of the SEC analysis are presented as area under the SEC curve, indicating the concentration of the detected substances. The SEC results of HA are color coded for large, medium and small size HA molecules, to give a clear view on the changes in the MW distribution of the HA molecules during the treatment. The details of the method used for the presentation of SEC results have been presented in Chapter 2 [7,15].

6.2.3.2. Other analysis

The color of HA was estimated by measuring the light absorbance of the filtered (0.45 µm) sample at 450 nm [7, 15]. The color is presented as percentage of the initial color of the wastewater. The initial color of the wastewater was measured from a sample taken from the feeding tank at the beginning of the reactor operation.

Laccase activity was determined spectrophotometrically in the culture supernatant obtained by filtering through 0.45 µm syringe filters and measured by monitoring the

oxidation of 2,6-dimethoxiphenol (DMP) as described before [16]. The enzyme activity was expressed in enzyme units (U: micromoles.min^{-1}) per liter.

All the measurements were performed in triplicates and the results were presented as the average of three measurements.

6.3. Results and discussions

6.3.1. Synthetic wastewater

The color removal along with the laccase activity during the treatment of the synthetic wastewater are presented in Fig 2. Results are presented as the average of triplicate measurements. Error in color removal measurement was less than 2% and error in laccase activity measurement was less than 9%.

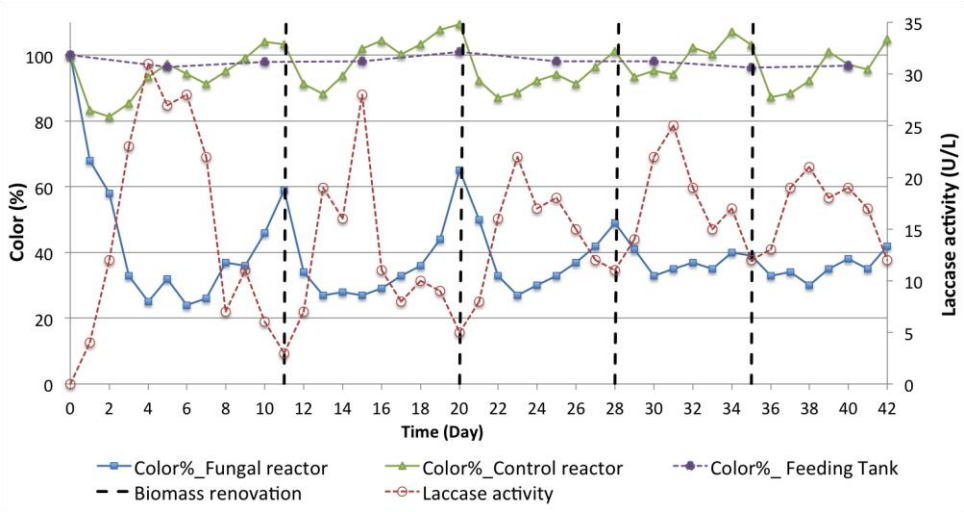

Fig 2. Evolution of the color in the fungal reactor (effluent), control reactor (without fungi) and the feeding tank (wastewater) in the treatment of synthetic wastewater. Laccase activity in the fungal reactor is represented in red with open circles. Vertical dotted lines mark the instances of partial fungal biomass renewal. Time 0, is the inoculation time. The results are presented as the average of triplicate measurements, with standard error of less than 2% for color and less than 9% for laccase activity.

The color of the synthetic wastewater inside the feeding tank was stable (<± 10% deviation) during the treatment, indicating the stability of the HA content of the

wastewater. However, in the control reactor (non-inoculated), almost 20% color removal was observed two days after the inoculation, which probably is due to the absorption of HA to the sorghum. A clear increase in color removal was observed after each renewal of the sorghum, likely providing fresh absorptive capacity. Also, it was observed that occasionally the color of the wastewater inside the reactor was even higher (max 5%) than its initial value, which could be due to the growth of the bacteria, or due to release of some color from the sorghum in wastewater.

In the fungal reactor, after one day of inoculation, more than 30% color removal was detected. Considering the low enzyme activity at this early point of the treatment, the color removal probably was mainly due to biosorption of HA to fungal granules. With increasing enzyme activity in the following days, the color removal efficiency increased to 75% on day 4 and stayed almost stable until day 9, when it started to decrease (increase in color). On day 11, when color reached 60% of its initial value after 3 days of continuous increase, the first biomass renewal was carried out (50% of fungal granules were replaced with fresh immobilized fungal granules). Immediately after the renewal (day 12) a steep decrease in color was observed. Color of the wastewater stayed stable at 25% until day 17, when it started to increase slowly. From day 18 to 20, a relatively fast increase in color was observed, indicating a decay in the fungal activity, which was supported by decrease in the enzyme activity. On day 20 the second biomass renewal was performed (9 days from the first renewal), in order to recover the fungal activity inside the reactor. Following the second biomass renewal, the color decreased to 25% on day 23. However, after day 23 a slow increase in the color was observed. On day 28, 8 days after the last renewal, half of the fungal granules were replaced with fresh granules. The renewal of fungal granules was successful in recovering the fungal activity and reducing the color, as well as maintaining 65% color removal until day 33. The fourth fungal renewal was performed on day 35, 7 days after the last renewal (1 day sooner compared to the last renewal), to avoid a failure of the fungal reactor and maintain a stable color removal. From day 35 to 42 (7 days), a stable color removal of about 60-65% was achieved. Looking at the trend of the color it seems that by reducing the period of the biomass renewal to 7 days, a more stable decolorization was achieved. In order to elucidate the effect of the time intervals between each renewal, the performance of the reactor was calculated based on each period (cycle) in table 1.

Table 1. The performance of the fungal reactor in each cycle with respect to partial biomass renewal (including minimum and maximum WW's color in each cycle).

Cycle	Days	Duration (days)	Color min. (%)	Color max. (%)	Average Color (%)	Average Enzyme activity ($U.L^{-1}$)
1	0-11	11	23	-	46	14.5
2	11_20	9	27	65	36	12.5
3	20-28	8	25	50	38	14.8
4	28-35	7	25	41	37	17.7
5	35-42	7	26	42	36	17.1
5'	35-41	6	26	38	34	18

As it can be seen, by reducing the time between fungal biomass renewals from 9 to 7 days, average enzyme activity increased from 12.5 to 17.7 $U.L^{-1}$, and in the next cycle of 7 days (5^{th} cycle) it was 17 $U.L^{-1}$. If the 5^{th} cycle was stopped after 6 days (Cycle 5' in Table 1) the average enzyme activity would have been 18 $U.L^{-1}$, which was slighlty higher than the 4^{th} cycle (with 7 days retention of fungal biomass). When operating with 9 days interval for biomass renewal, the color of the treated wastewater increased to a maximum of 65%, but with 7 days interval the maximum color was 42%, and with 6 days it was 38%. This shows that shortening the time interval (period) between the biomass renewals from 9 to 7 (or 6) days improves the stability of the reactor's performance with regard to both enzyme activity and color removal. Thus far, the exact reason for this decrease in enzyme activity is not (yet) clear.

The changes in the humic molecules were measured using SEC (Fig 3).

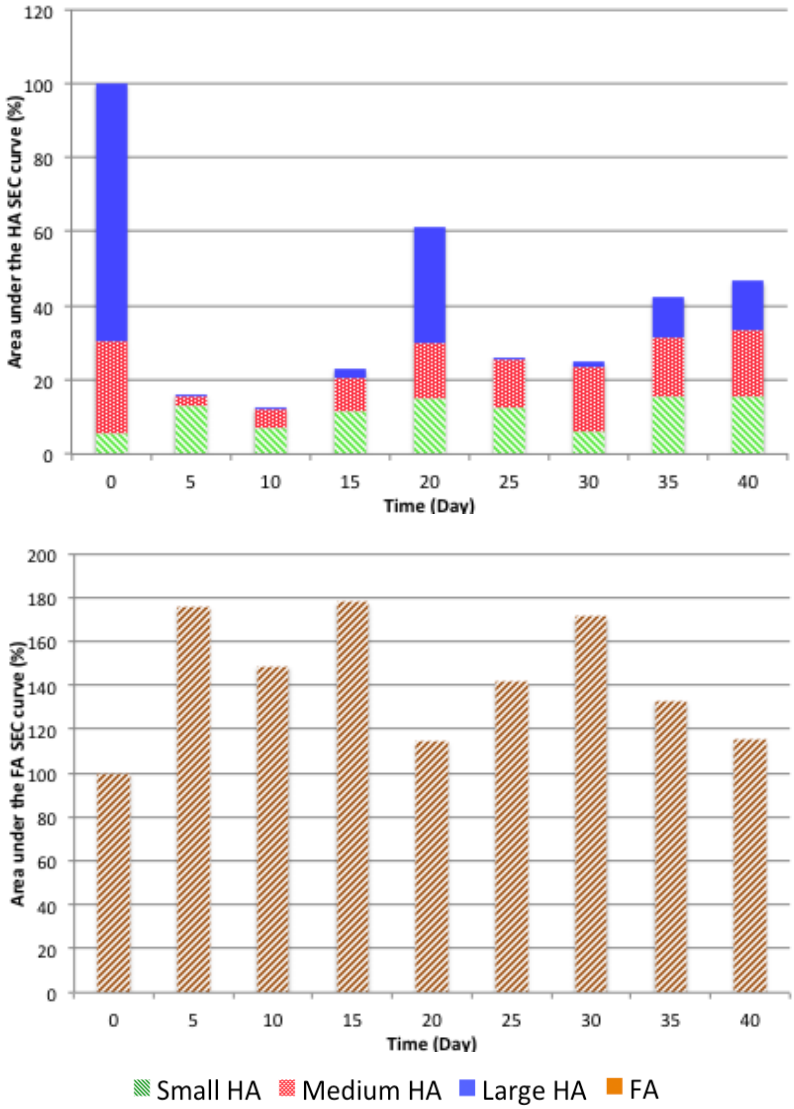

Fig 3. SEC analysis of the synthetic wastewater during the fungal treatment. Top: HA, Bottom: FA. Results are presented as the average of at least duplicate measurements with standard deviation (for duplicates: range of min-max/2) of less than 1% for HA and less than 2% for FA.

Synthetic wastewater was comprised of 70% large HA, 25% medium size HA and 5% small HA molecules. The average molecular weight of the HA content of the wastewater was 2.03 kDa. The average MW of the FA content of the wastewater was 0.5 kDa.

In agreement with the color removal results, SEC analysis shows significant removal of HA, especially the large HA molecules, during the fungal treatment until day 20. On day 20, there was an increase in the HA concentration that largely agreed with the increase in the concentration of large HA. This increase corresponded with the increase in color of the wastewater and drop in the enzyme activity on day 20, when the second fungal biomass renewal was performed (Fig 2). However, 5 days after the renewal, on day 25, large HA were completely removed and total HA concentration was significantly reduced to 25% of its initial value (day 0). Also, almost all the large HA molecules were removed. This observation emphasizes the effect of the fungal biomass renewal on the recovery of the fungal reactor's performance and HA removal activity. , the increase in the small HA molecules during the fungal treatment suggests the degradation of large and medium HA to small HA molecules. Looking at the SEC results of the FA content of the wastewater, it can be generally concluded that the FA concentration during the fungal treatment was increased. The maximum concentration was almost 80% higher than the initial FA concentration. This increase in the FA concentration, concurring with a decrease in HA concentration, suggests conversion of HA molecules to smaller FA molecules.

6.3.2. Real wastewater

The fungal reactor treating the real wastewater was operated identical to the reactor treating synthetic wastewater, except that partial renewal of fungal biomass was performed routinely every 6 days. Results of color removal and enzyme activity are presented in Fig 4. Results are presented as the average of triplicate measurements.

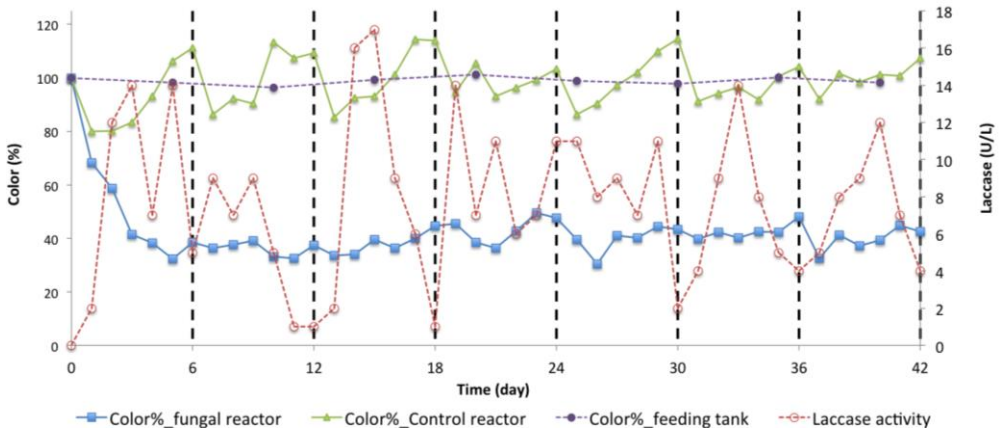

Fig 4. Evolution of the color in the fungal reactor (effluent), control reactor (without fungi) and the feeding tank (wastewater) in the treatment of real wastewater. Laccase activity in the fungal reactor is represented with open red circles. Vertical dotted lines mark instances of partial fungal biomass renewal. Time 0, is the inoculation time. The results are presented as the average of triplicate measurements, with standard error of less than 2% for color and less than 11% for laccase activity.

The color of the real wastewater in the feeding tank was stable during the time of the treatment (<± 5% deviation). The trend of the color of the real wastewater in the control reactor was similar to what was observed with the synthetic wastewater. Overall, the color of the treated real wastewater of the control reactor (fungal-free) was relatively stable with less than ±30% deviation.

In the fungal reactor, the color reached 34% of its initial value on day 5 (65% color removal). A slight increase was observed on day 6, just before the first partial fungal renewal was performed. During the second cycle (day 6-12), the color remained at 35-40% (60-65% color removal). The second fungal biomass renewal was performed on day 12. Following the renewal, color remained at around 40% until day 15, when a slight increase in color was observed, and gradually continued until day 18. During the fourth and fifth cycles (after the third and fourth biomass renewal) color remained between 30-45%. During the last two cycles (6th and 7th), the color showed to be even more stable in the same range. Looking at the laccase activity, results show clearly that the partial renewal of the fungal granules was effective in recovering the enzyme activity in each cycle. Overall, a stable color removal of around 60% (± 10%) was achieved continuously for 37 days of fungal treatment of the real wastewater.

A summary of the reactor's performance for each cycle (partial fungal biomass renewal) is presented in Table 2.

Table 2. The performance of the fungal reactor treating real wastewater in each cycle with respect to partial biomass renewals periods (including minimum and maximum WW's color in each cycle)

Cycle	Days	Duration (days)	Color min. (%)	Color max. (%)	Average Color (%)	Average Enzyme activity ($U.L^{-1}$)
1	0-6	6	32	-	54	7.7
2	6_12	6	33	39	36	5.3
3	12_18	6	34	45	38	8.5

4	18-24	6	36	50	43	9.3
5	24-30	6	30	45	40	8
6	30-36	6	40	48	42	7.3
7	36-42	6	33	45	40	7.5

The difference between the minimum and maximum color intensity observed during the treatment was maximally 15% (cycle 5), which shows the stability of the fungal reactor with respect to color removal from the real wastewater. Compared to the reactor treating synthetic wastewater, the measured enzyme activity was significantly lower in the reactor treating real wastewater. This is in agreement with our previous study (chapter 5) and the possible reasons for this difference have been discussed there. Briefly, it could be due to certain chemical and biological compounds that are present in the real wastewater and can interfere with the laccase activity assay [17]. The interference of tannic acid (plant polyphenol) and its derived molecules [18], catalase [19], and some metals [20] in the measurement of laccase have been reported before. Also, the real wastewater likely contains a higher initial concentration of bacteria compared to the synthetic wastewater. This high initial bacterial concentration could contribute to the inhibition of laccase activity [14,21].

The SEC results of the humic content in the reactor during the fungal treatment are presented in Fig 5 and provide a more accurate overview on how the HA and FA molecules change during the fungal treatment.

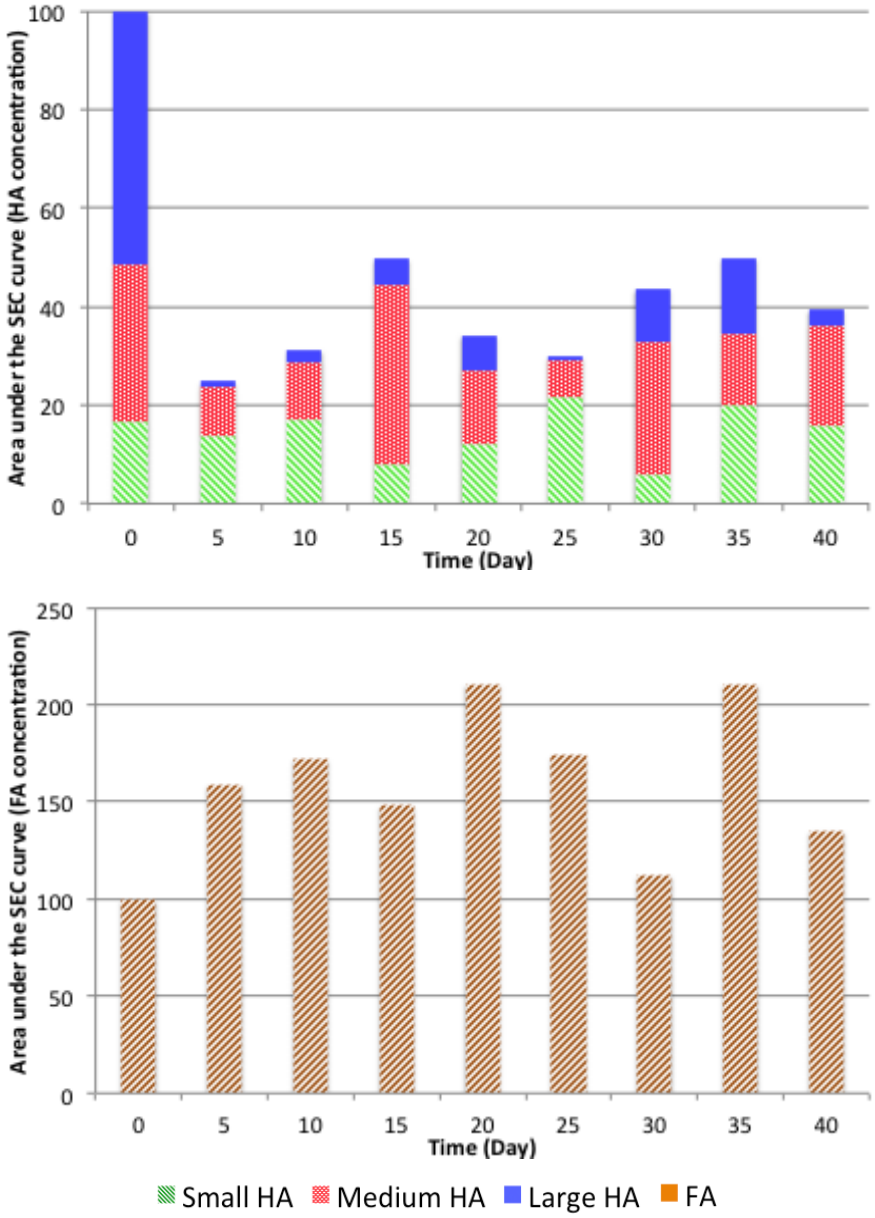

Fig 5. SEC analysis of the real wastewater during the fungal treatment. Top: HA, Bottom: FA. Results are presented as the average of at least duplicate measurements with standard deviation (for duplicates: range of min-max/2) of less than 2% for HA and less than 3% for FA.

The HA content of the wastewater was comprised of 51% large HA, 32% medium and 17% small size HA molecules (Day 0, before the inoculation). The average MW of the HA content of the real wastewater was 1.53 kDa, and for FA it was 0.68 kDa. It is noteworthy that based on the raw results of the SEC analysis, the HA concentration of the real wastewater was lower than it was in the synthetic wastewater (36% lower) and FA concentration was higher (55%) than what it was in the synthetic wastewater (raw data not shown).

After the inoculation of the fungal reactor, the first SEC analysis was done on day 5, and showed more than 75% reduction in HA concentration (area under the SEC curve). Notably, almost 100% removal of the large HA molecules was achieved, 70% of medium HA were removed and the concentration of small HA molecules remained almost unchanged. Biosorption of HA by fungal granules/mycelia, at the beginning of the operation with fresh fungal biomass, may contribute significantly (40-60%) to the removal of HA [7,14]. Following the biosorption of HA to fungal mycelia, degradation of HA by cell-bound fungal enzymes could be hypothesized [7]. However, our results cannot support that. On day 10, a slight increase in the concentrations of large and small HA molecules was detected (compared to day 5). However, on day 15, a significant increase in the HA concentration was observed, which was due to an increase in medium size HA molecules. The concentration of medium size HA molecules, was increased to 14% higher than its initial value (day 0). This simultaneous decrease in the concentration of large HA and increase in medium size HA molecules, suggests an in-complete degradation of large HA to medium size HA molecules. The HA concentration was again reduced on days 20 and 25. From day 25 onwards, the HA concentration slightly increased, however, the concentration of large HA molecules remained below 30% its initial value (>70% removal). SEC analysis of the FA content of the wastewater, revealed that the FA concentration increased during the course of the fungal treatment, similar to what was observed with synthetic wastewater. This observation, in agreement with our previous results (chapter 2,3,4,5), suggests an enzymatic conversion of HA to FA molecules. However, the increase in the FA molecules was higher with the real wastewater (max 210%), compared to the synthetic wastewater (max 177%).

6.4. Conclusion

The periodic partial renewal of the fungal biomass was shown to be effective in maintaining the fungal reactors' activity with respect to HA removal. In the treatment

of the synthetic wastewater in the used set-up, it was shown that shortening the time between the intervals of biomass renewal (from 10 days to 6-7 days) increased the stability of the HA removal in the fungal reactor. During the treatment of the real wastewater, half of the fungal biomass was renewed every 6 days, resulting in a stable color removal (average 60%) from the real HA-rich wastewater for over 40 days. The mechanism of HA removal was shown to involve the degradation of HA to smaller molecules and also conversion of HA to FA molecules. However, the biosorption of HA to fungal mycelia could not be excluded. Overall, the application of immobilized WRF on sorghum was shown to be efficient in maintaining stable HA removal, even with the real industrial wastewater and without the presence of any additional nutrient sources. To our knowledge this is the first time that a fungal reactor is successfully operated under non-sterile conditions without additional supply of carbon or nitrogen sources other than the immobilization carrier (fixed matrix), in this case sorghum.

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Chapter 7

Concluding remarks

7.1. Summary

In order to address the ambiguities and knowledge gaps in the HA removal by WRF, mechanisms of removal were explored in chapter 2. It was shown that the mechanisms of HA removal by WRF involves the degradation of HA to smaller molecules, conversion to FA and also biosorption of HA by fungal mycelia. Furthermore, the ability of laccase to effectively degrade HA was demonstrated and it was shown that the presence of suitable mediators, such as ABTS, have a crucial effect on the degradation process. Also, the contribution of the membrane-bond CYP 450 enzymes to the rate of the HA degradation by fungi was demonstrated. In addition, addressing the ambiguities in the measurement techniques mentioned in chapter 1, it was shown that decolorization was not necessarily representing the degradation of HA, but removal of HA in the general context. This removal could be due to biosorption, in-complete or complete degradation of HA to smaller molecules. Also, it was shown that the degradation or depolymerization of the HA is not necessarily reflected by a change in the average MW of the HA in the wastewater. The reason is that the depolymerization and/or degradation of HA could basically result in three products, smaller HA molecules, smaller non-aromatic molecules, and FA. Except for the first group, the other products from the degradation of HA could not be detected by the SEC analysis (at 254 nm) and were therefore not considered in the calculation of the average MW. Also, it was shown (chapter 2 and 3) that the biosorption of HA to fungal mycelia was not uniform among large, medium and small size HA. The large HA molecules were shown to be more susceptible to biosorption. Therefore, sole biosorption of HA to fungal mycelia could result in a change in MW distribution of the HA, even when no degradation or polymerization have occurred.

In order to address the knowledge gap in the application of WRF to treat real HA-rich wastewaters, and regarding some previous reports on the importance of the origin of the wastewater and its HA content on the HA degradation by WRF, a real HA-rich treated industrial wastewater was collected from a food processing company and was subjected to the fungal treatment (chapter 3). Results showed that WRF could remove HA from the real industrial wastewater, originated from animal and agricultural waste. Also, it was confirmed that biosorption of FA, degradation and conversion of HA to FA were involved in the removal process. In agreement with

the results of chapter 2, it was concluded that although Laccase and MnP were effective in the degradation of HA, there were possibly also other enzymes involved in this process, because some of the degradation patterns could not be explained by Laccase and MnP activities.

Following the successful results of the HA removal by WRF under sterile conditions, *T. versicolor* was applied for the bioremediation of HA-rich wastewater under non-sterile conditions (chapter 4). However, results showed that fungal growth and enzyme activity was severely inhibited under the non-sterile conditions, and consequently almost no HA removal was achieved. This observation was in line with what was explained in the introduction (chapter 1) as the major challenge in industrial application of WRF i.e. application under non-sterile conditions. Our results in chapter 4 indicated bacterial proliferation of the culture, as it has been reported before (summarized in chapter 1). Therefore, a new approach was developed to facilitate the application of WRF under non-sterile conditions. It was hypothesized in this study that the immobilization of WRF on a nutrient source could facilitate the growth of WRF, and thus its competitive strength, under non-sterile conditions. The results showed that sorghum could act as the sole nutrient source for growth and laccase production of *T. versicolor*. Under non-sterile conditions, immobilized fungi could degrade HA in real industrial wastewater when fungal free cells could not grow. Decolorization of HA-rich wastewater was up to 75% using immobilized fungi under non-sterile conditions, and SEC results revealed up to 40% degradation of HA molecules. Nonetheless, the laccase activity was much lower (almost 50%) than that observed in the sterile experiments using real industrial wastewater. Furthermore, a sequential batch experiment was performed to evaluate the feasibility of this method for future application in continuous (or semi-continuous) reactors. Results showed that immobilized fungal granules could significantly remove HA from wastewater in the sequential batch operation under non-sterile conditions, without renewing the fungal inoculum. However, the HA removal efficiency deteriorated in each batch.

In chapters 5 and 6, we tested the application of WRF in continuously fed bioreactors under non-sterile condition to treat HA-rich synthetic and real wastewaters. In chapter 5, we adopted a method that has been reported before in the literature for application of WRF under non-sterile conditions. This method was initially developed for decolorization of azo dyes and later on for removal of some aromatic pharmaceuticals from synthetic and real wastewaters. The core idea of this

method was to keep the fungal culture young and fresh. To this end, fungi were pre-grown under sterile conditions (as pellets), and then used to inoculate the reactor under non-sterile conditions. During the treatment, periodic partial renewals of fungal biomass were performed to keep the fungal culture young and active. It was shown that the fungal reactor could continuously remove HA from synthetic wastewater under non-sterile conditions. Also, it was demonstrated that periodic and partial fungal biomass renewal had a crucial effect on maintaining the fungal activity in the reactor. Comparison of real wastewater with synthetic wastewater showed that the treatment of the real wastewater was less effective than the treatment of synthetic wastewater. The HA removal efficiency started to decrease after 18 days in the reactor treating real wastewater, whereas it stayed almost steady for 30 days in the reactor treating synthetic wastewater.

In chapter 6, we combined the two methods that we were developed for the application of WRF under non-sterile conditions i.e. immobilization of WRF on sorghum as the main nutrient source (chapter 4) and partial renewal of fungal biomass (chapter 5). A pulse aerated fluidized bed bioreactor was applied for the treatment of real industrial wastewater, with renewal of half of the fungal biomass (granules) every 6 days. Finally, a stable decolorization (average 60%) of the wastewater for over 40 days was achieved. Overall, the application of immobilized WRF on sorghum was shown to be efficient in maintaining a stable HA removal, even with the real industrial wastewater. To our knowledge this is the first time that a fungal reactor was successfully operated under non-sterile conditions without additional supply of carbon or nitrogen sources other than the immobilization carrier (in this case: Sorghum).

7.2. Limitations and uncertainties

The humic-rich wastewaters applied in this research were low in COD and BOD. When using wastewaters with higher COD and BOD, different results might be achieved. The reason is that bacterial growth, which can interfere with fungal activity, will be different (likely higher) in the wastewater with a high biodegradable carbon sources.

The presence of humics in the enzyme activity assay might result in an underestimation of enzyme activity. Although other researchers have also reported

this observation, still the research in this area suffers from the uncertainties about the actual enzyme activity in the media.

The degradation of large HA molecules could result in not only smaller HA and FA, but also in non-aromatic molecules, which are not detected in SEC analysis. Although in this study the mentioned degradation pathways were shown qualitatively, the contribution of each pathway was not quantified due to limitation of the analysis protocols and devices.

7.3. Suggestions for future studies

To further improve the fungal reactor and to also facilitate the generation of realistic data for cost analysis the following activities are suggested:

- Optimization of the HRT, inoculum volume and the portion of fungal biomass for renewal (in this study it was 50%).
- Examination of the potential applications of the produced fungal biomass
- Examination of the potential of other carriers (besides sorghum) as the support and nutrient source for immobilization of fungi
- Scaling up of the fungal reactor (+20L)
- Carrying out a cost analysis based on the results of the scale up (+20L) study

The fungal reactor that has been developed in chapter 6, could be used to treat other wastewaters and degrade other contaminants. Azo dyes and pharmaceuticals (like carbomazepin) have been shown to be degraded by the same species used in this thesis. Therefore, application of the reactor with immobilized fungi (chapter 6) could be effective in treating these compounds under non-sterile conditions.

7.4. Final remarks

The application of immobilized fungi on sorghum along with performing partial renewal of fungal biomass, showed to be effective in establishing a stable fungal reactor under non-sterile conditions. The applied approach as described in chapter 6 has the potential to be used in upscaling of fungal bioreactors to further develop a pilot plant and possibly an industrial scale bioreactor.

The successful results of the fungal reactor developed in this thesis (chapter 6), was achieved using a real wastewater. However, it should be noted that this wastewater was tertiary treated effluent from a wastewater treatment plant. Therefore, the in this thesis proposed reactor setup is being suggested as a post treatment. Although the application of this reactor as a main or pre-treatment might be possible, the results of this thesis cannot necessarily support it.

When it comes to the application of WRF in wastewater treatment, it should be noted that the targeted compounds are non/hardly biodegradable. So far, application of WRF is the main biological method to treat these compounds. However, non-biological techniques such as ozonation and UV treatment have been shown to be effective in degradation of these compounds. Comparative analyses supported by life cycle assessment and environmental impact studies should reveal which technique, bio-treatment with WRF or the mentioned physicochemical techniques, will be more cost-effective and sustainable on the long term.

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Zahmatkesh, M., Spanjers, H., van Lier, J.B. Continuous fungal treatment of humic-rich wastewaters under non-sterile conditions: application of a fluidized bed bioreactor with partial renewal of fungal biomass. Submitted.

Zahmatkesh, M., Spanjers, H., Toran, M.J., Blánquez, P., van Lier, J.B.J.B., 2016. Bioremoval of humic acid from water by white rot fungi: exploring the removal mechanisms. AMB Express 6, 118.

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Curriculum vitae

Mostafa Zahmatkesh was born on 11th September 1982 in Tehran, Iran. He received his Bachelor degree in Chemical engineering- Process design and his Master degree in Chemical engineering-Biotechnology. After his Master he worked as a Bioprocess engineer in ACECR and also in Royan institute. In 2012 he joined the Sanitary engineering group of TUDelft, as a PhD candidate. His areas of research are bioprocess engineering, bioreactor design, wastewater bio-treatment, Mycoremediation and enzyme production. Recently, he is more involved in bioprocess development for industrial production of antibacterial phages as food safety products.