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

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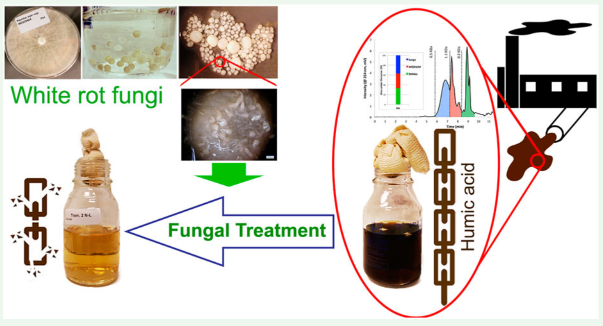
ABSTRACT

This paper presents the results of fungal treatment of a real industrial wastewater (WW), 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, Trametes versicolor, Pleurotus ostreatus and Pleurotus pulmonarius* were tested to remove humic acids (HA) from a real humic-rich industrial treated WW 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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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

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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 WW have been conducted using synthetic WW, 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 affect its biodegradability by WRF [16]. Therefore, the results that have been achieved by application of WRF in synthetic WW could be challenged when real WW 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 relation 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 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 WW from a food-processing company. Therefore, the HA used in this study was naturally originated from animal and agricultural waste. Decolorization of the WW during the fungal treatment was compared with the results of size-exclusion chromatography (SEC) analysis to clarify the ambiguities about the interpretation of decolorization of HA, regarding its relation to the degradation of HA. The reliability of the average MW calculation 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 explained by distinguishing biodegradation, bioconversion and biosorption of HA.

2. Material and methods

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 the 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.

2.2. Media

2.2.1. Industrial wastewater

Industrial WW was collected from the effluent of a WW treatment plant of a food-processing company (Eindhoven, The Netherlands). The main characteristics of this WW were as follows: soluble COD: 260 (±6) mg/L, total COD: 262 (±8) mg/L and biochemical oxygen demand (BOD_5): < 8 mg/L. The ammonium concentration ($N\text{-}NH_4^+$) and total suspended solid (TSS) were not quantifiable (negligible values). The SEC chromatograms of the

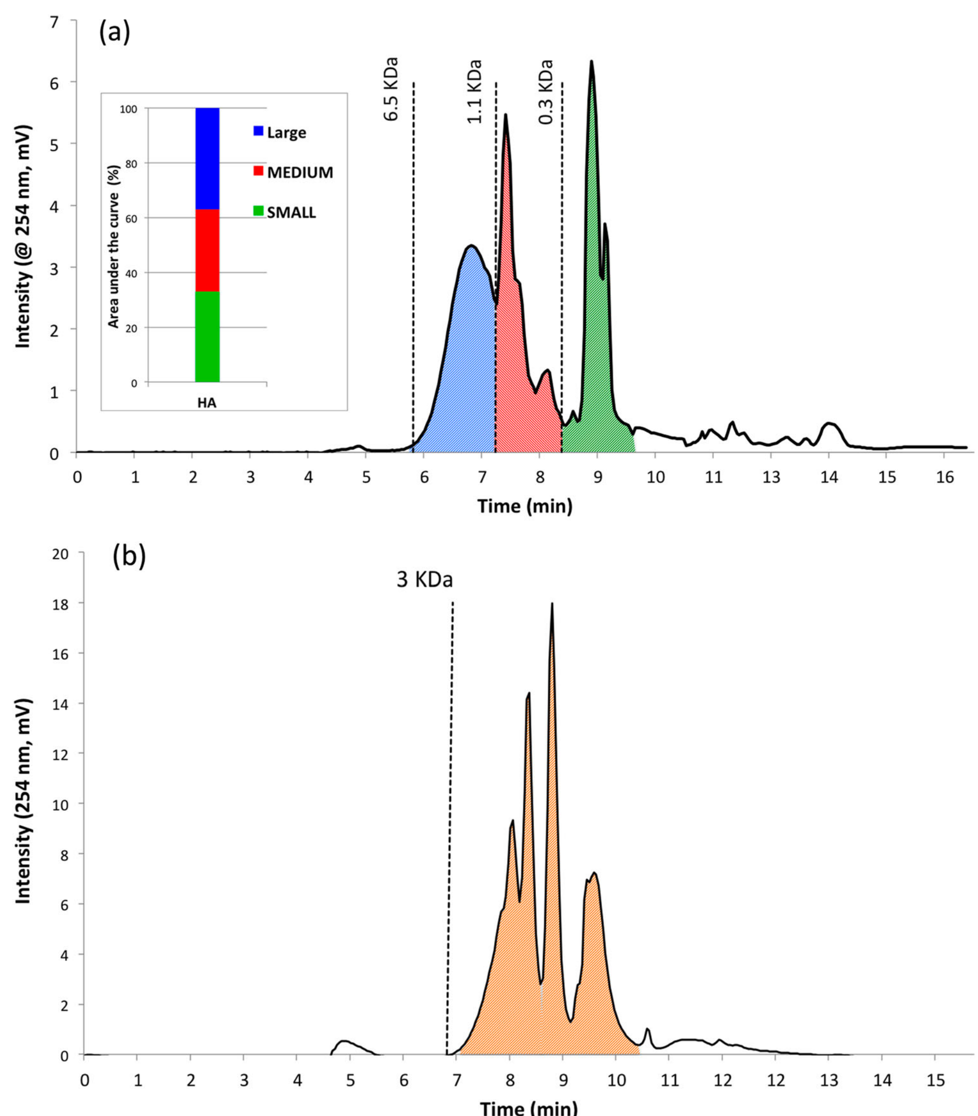

Figure 1. SEC chromatogram of HA (a) and FA (b) content of the wastewater.

HS content (HA and FA) of the WW are shown in Figure 1. The WW was kept at 4°C for a week after the collection and autoclaved (120°C, 15 min) before starting the experiments.

2.2.2. Defined media

Defined media were 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; $MgSO_4 7H_2O$, 0.05 g/L; $CaCl_2$, 0.01 g/L and trace element solution, 1 mL/L [18]. Glucose was used as the main carbon source in a final concentration of 10 g/L and ammonium tartrate was used as the main nitrogen source. For nitrogen-limited media (NL), the final concentration of ammonium tartrate was 20 mM, 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.

2.3. Experimental procedures

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 × 0.5 cm^2) of pre-cultivated fungal agar (see Section 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 Section 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 MW distribution of HA and FA substances. All controls were made in duplicate and incubated identical to mycoremediation flasks.

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 h. At the end, samples were withdrawn and filtered through 0.45 µm filters [24].

2.4. Analytical methods

2.4.1. Size-exclusion chromatography

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 20 µL HCl (37%) and centrifuged (14,000, 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 contents of the samples were analyzed by SEC. 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 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 ultrapure water supplemented with 10 mM sodium phosphate buffer (pH 7). The flow rate of the mobile phase was 1 mL/min 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.

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 the 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).

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 aluminum cups (100°C, 48 h). 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. Results

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 Figure 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 Figure 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)

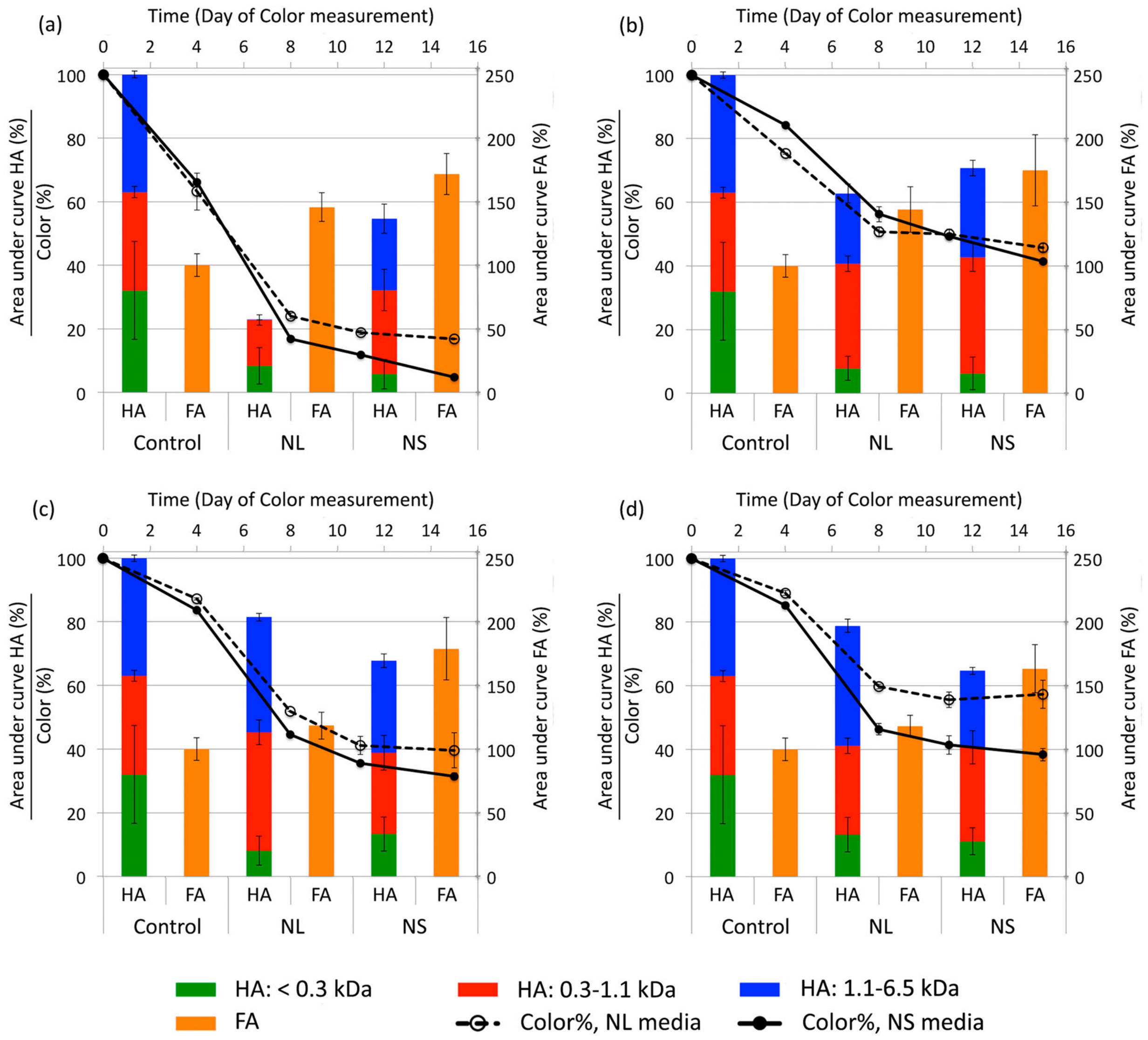

Figure 2. HA color removal during the fungal treatment and SEC results of the HA and FA content of the wastewater (area under the curve) before (uninoculated control) and after the fungal treatment (after recovery of sorped HA) in NL and NS media. (a) *T. versicolor*, (b) *P. chrysosporium*, (c) *P.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

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 average 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 Figure 2. SEC results of the 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).

The results of the fungal treatment of the humic-rich WW are shown in Figure 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 Figure 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 the 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 Figure 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 (Figure 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 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 Figure 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 the 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.

According to the results of color removal in Figure 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 average 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 average 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 average MW of the total HA complex. Therefore, monitoring the average 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 (Figure 2).

In the case of *P. chrysosporium*, the color removal in NL and NS media was 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 Figure 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 by 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

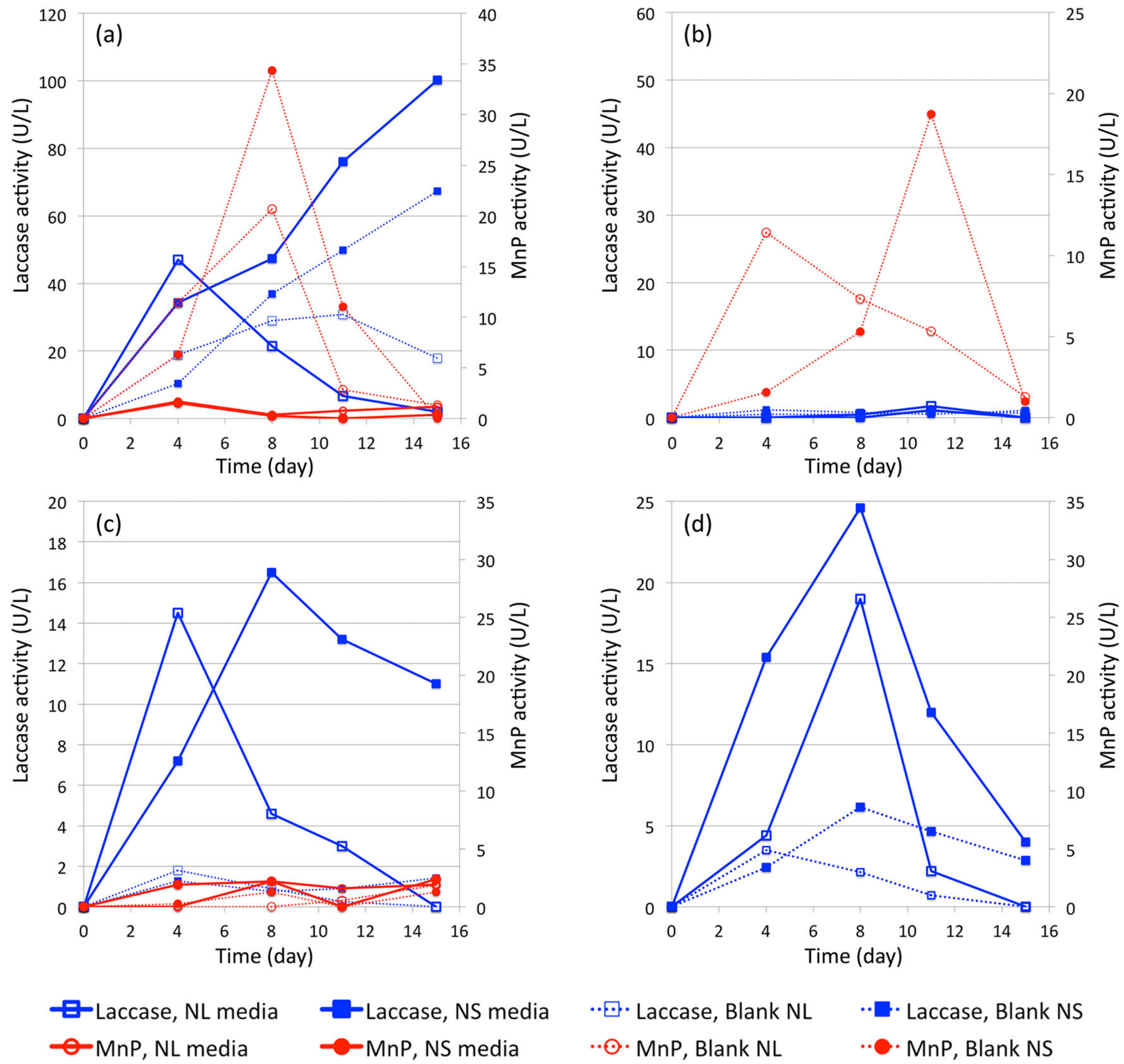

Figure 3. Laccase and MnP activity during the incubation period with (solid lines) and without (blank, dot lines) the presence of HS. (a) *T. versicolor*, (b) *P. chrysosporium*, (c) *P. pulmonarius* and (d) *P. ostreatus*. Note: differences in *Y*-axis scales.

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.

3.2. 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 Figure 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.

As it can be seen from Figure 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 MnP activity after 8 days (in HS-free controls) and maximum laccase activity of 100 U/L 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 extracellular enzymes as a secondary metabolite under limited nitrogen concentrations, 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 Figure 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 MnP activity after 12 days, but in NL media, the maximum enzyme activity reached a lower and sooner maximum of 12 U/L 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 Figure 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.

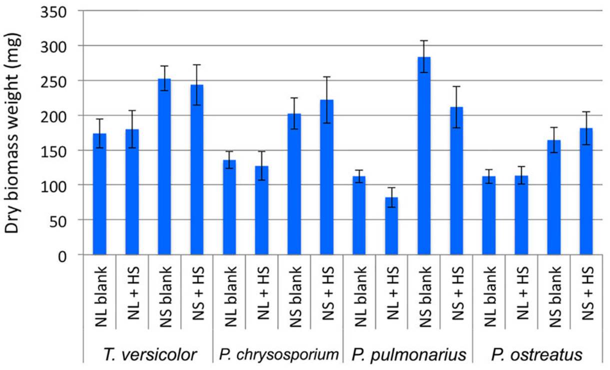

Figure 4. Growth of WRF after 15 days of incubation in NL and NS media, with (+HS) and without (blank) the presence of HS.

Overall, by comparing the results of *T. versicolor* and the other tested fungal strains in Figures 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 was detected.

For all four tested WRF strains, the laccase activity was higher in the media containing HS, which indicates 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. 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 Figure 4.

From the results shown in Figure 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 a 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 Section 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].

4. Discussion

From the results of HA removal by *T. versicolor* and *P. chrysosporium*, that the decolorization of HA could not necessarily represent the degradation of HA. *T. versicolor* could degrade more HA in NL media compared to NS media, since there were complete degradation of large HA in the NL media and incomplete degradation of large HA in the NS media. However, when looking at 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 Figure 4, *T. versicolor* produced significantly more biomass in the NS media than in the NL media (due to nitrogen limitation in the NL media). Consequently, more biosorption of HA was achieved during the incubation in the NS media. A similar observation could be made in the 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 the 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 was 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 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, which was not detectable via the SEC analysis. The complete mineralization of HA molecules, that is, 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 proven 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 activities. 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].

5. Conclusions

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

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