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

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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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White rot fungi; wastewater treatment; non-sterile; humics; sorghum

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]. HS can cause serious technical problems in water treatment plants, such as membrane fouling [18,19] and the deterioration of adsorbents [20]. The presence of HS 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, HS 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 colour, 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

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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%), fibre (~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 [39–41].

In our present study, sorghum was used as a 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.

2. Material and methods

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.

2.2. 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.

2.3. 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 conditions (see sections 2.5.1 and 3.5), to compare the results of immobilized fungi on sorghum as nutrient source with the results of free fungal pellets with defined media as the nutrient source.

2.4. HS-rich wastewater

2.4.1. 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.

2.4.2. 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, total COD: 283 (±8) mg/L and biochemical oxygen demand (BOD5): <10 mg/L. The ammonium concentration (N-NH4+) and total suspended solid were not quantifiable (negligible values). The treated wastewater was kept at 4°C for a week after the collection and before starting the experiments.

2.5. Experimental procedure

2.5.1. 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 into two sets. The flasks in the first set were subjected to sampling during the incubation period for colour measurement, enzyme activity and size exclusion chromatography (SEC) analysis. The other set of flasks was kept intact and only used at the end of the incubation for the recovery procedure (see section 2.5.2). 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 section 2.3) and inoculated with five pieces of fungal agar (∼ 1 cm^2). The flask containing free fungal pellets was 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.

2.5.2. 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 h. At the end, samples were withdrawn and filtered through 0.45 µm filters [10,43].

2.5.3. 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 2 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 three times with sterilized water and were added to flasks containing synthetic wastewater. The flasks were incubated for 48 h and monitored for changes in colour. After 48 h, the recovery procedure (see section 2.5.2) was performed and samples were analysed via SEC.

2.5.4. 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 section 2.5.1). 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.

2.6. 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 (14,000, 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 analysed 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 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).

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

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 [44]. The enzyme activity was expressed in enzyme units (U: micromoles/min).

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

3. Results

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 Figure 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.

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 Figure 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].

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 analysed by SEC. The synthetic wastewater showed a relatively narrow FA peak starting from 0.5 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 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.

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

The HA colour removal along with the results of the SEC analysis and the enzyme activities are shown in Figure 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 colour and less than 10% in the area under SEC curve for HA and FA, indicating the high stability of HA molecules (data not shown).

During the fungal treatment, the humic concentration was significantly reduced, as it is shown by the colour reduction in Figure 3. The results of the colour removal are already corrected for the changes in the colour of the HS-free control flasks. Most of the colour was

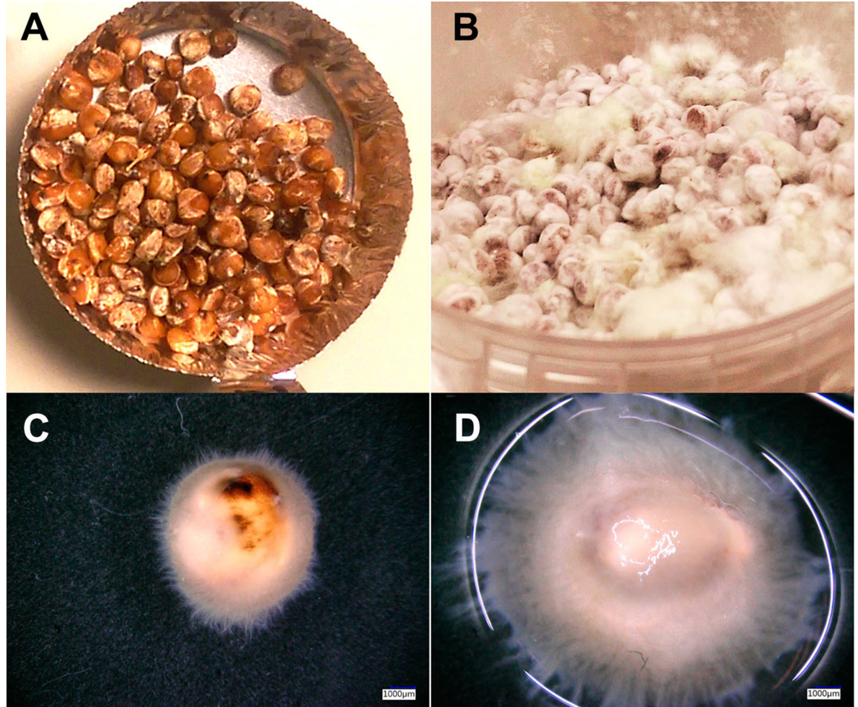

Figure 1. Immobilized fungi. (A) Sorghum (as carrier), (B) Pre-grown fungi on Sorghum, i.e. immobilized fungi (after 3 weeks of incubation in solid phase), (C) Immobilized fungal granule after 2 days of incubation in liquid phase (water), (D) Immobilized fungal granule after 14 days of incubation in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

removed after 8 days of incubation when 70% colour 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

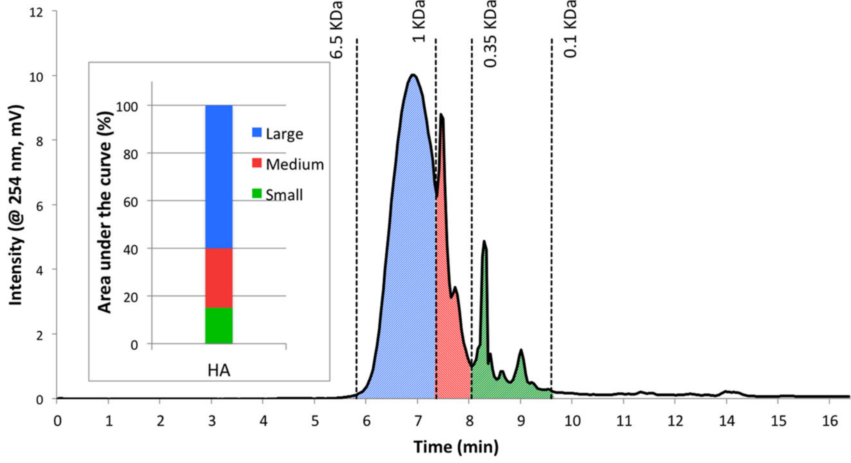

Figure 2. SEC chromatogram of HA complex (synthetic wastewater). The chromatogram is divided into three zones based on the main peaks: large HA molecules (left), medium size HA molecules (middle) and small HA molecules (right). The area under the curve was normalized and presented in the stacked column graph.

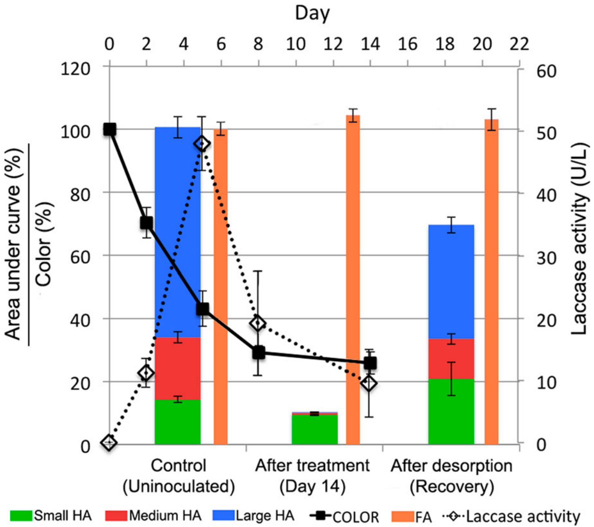

Figure 3. Fungal treatment of the synthetic wastewater under sterile conditions. SEC results of HA and FA are presented as area under the curve (left Y-axis and lower X-axis). Colour removal (%) and enzyme activity (laccase) were monitored during the incubation period (upper X-axis). Results are presented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

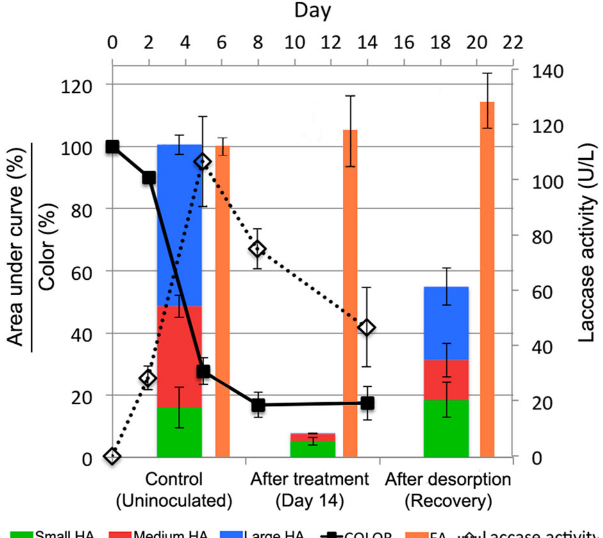

Figure 4. Fungal treatment of the real wastewater under sterile conditions. SEC results of HA and FA are presented as area under the curve (left Y-axis and lower X-axis). Colour removal (%) and enzyme activity (laccase) were monitored during the incubation period (upper X-axis). Results are presented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

these ratios with the initial composition of the HA complex (66% large, 20% medium and 14% small), it is apparent that there is a shift towards the lower size molecules in the HA complex. The average MW of the HA after the recovery procedure was about 1.14 kDa. After the recovery of sorped HA, the SEC results (Figure 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 after 5 days and then decreased to 10 U/L after 14 days. The increase in the laccase activity during the first 5 days correlated with the high colour 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].

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 Figure 4. The molecular size distribution of HA was different from the HA in the synthetic wastewater (Figure 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 colour and less than 7% in the area under SEC curve for HA and around 14% for FA (data not shown).

Colour of the industrial wastewater reached its minimum of 18% (82% colour 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 composed 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 colour, and when the enzyme activity started to decrease after 5 days, the rate of colour removal was also reduced. This correlation between laccase activity

and colour removal suggests the involvement of laccase in the degradation or conversion of HA, although concomitant absorption of HA cannot be excluded.

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 colour, around 10% change in area under the SEC curve of HA and less than 15% for FA in the uninoculated controls (data not shown).

The results of the HA removal by immobilized fungi as well as free fungal cells (non-immobilized) are presented in Figure 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) and maximum 10% colour 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 were 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% colour 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 after 5 days, and then decreased to 27 U/L, although it was recovered to 38 U/L on day 14.

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

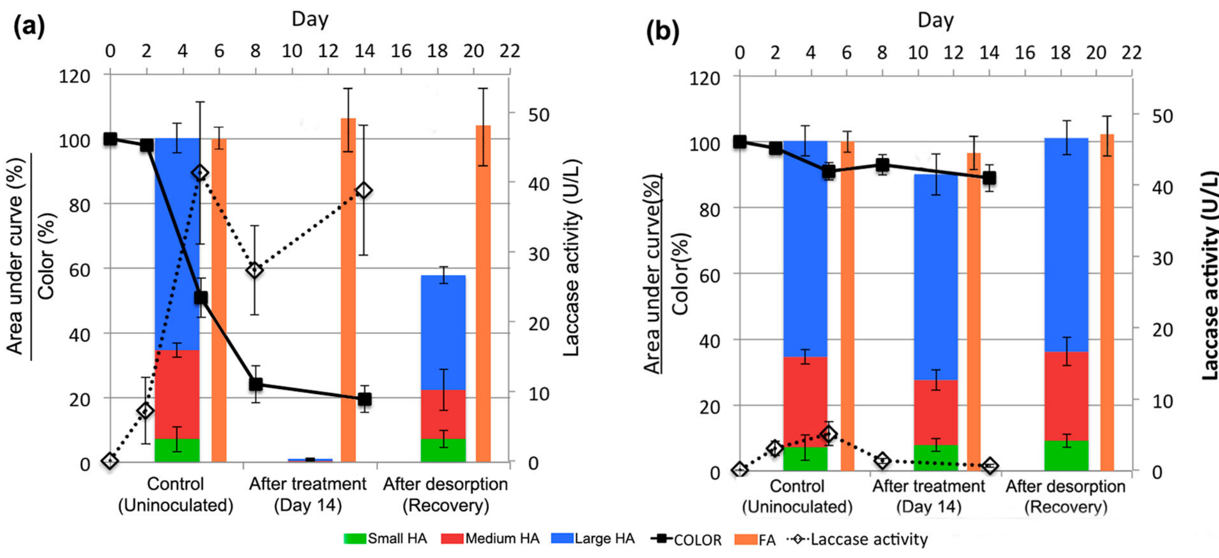

Figure 5. Fungal treatment of real wastewater under non-sterile conditions with immobilized fungi (a) and with free (non-immobilized) fungal cells (b). SEC results of HA and FA portions of the media are presented as area under the curve (left Y-axis and lower X-axis). Colour removal (%) and enzyme activity (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.

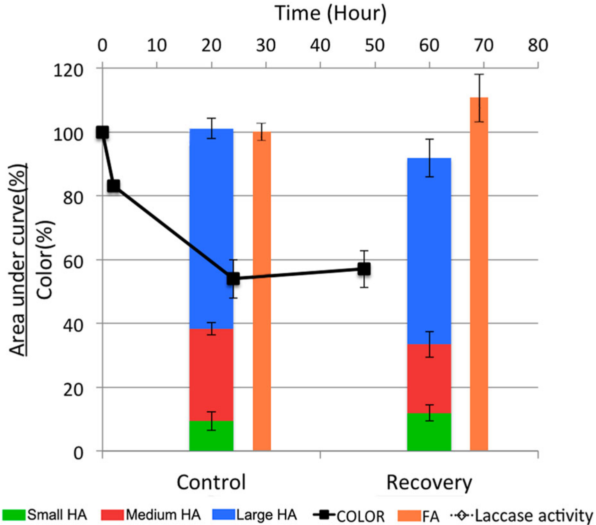

Figure 6. HA removal by deactivated immobilized fungi. SEC results of HA and FA are presented as area under the curve (lower X-axis). Colour removal (%) was monitored during the incubation period (upper X-axis). Results are presented as the average of triplicates ± SD.

fungal mycelia was measured by colour removal and presented in Figure 6.

Deactivated fungal mycelia could remove 55% of the colour after 24 h, although some of the colour was recovered later on, resulting in about 40% colour removal after 48 h. The increase in colour 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 was 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 h of incubation. Overall, the total recovery efficiency was more than 95%.

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 Figure 7.

The first batch lasted 7 days, and when about 78% decolourization 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% decolourization was detected, also a recovery of laccase activity in the media to 33 U/L 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 after 4 days in the second batch feeding, when about 70% decolourization was measured. When the reduction in the laccase activity and decrease in the rate (slope) of decolourization 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

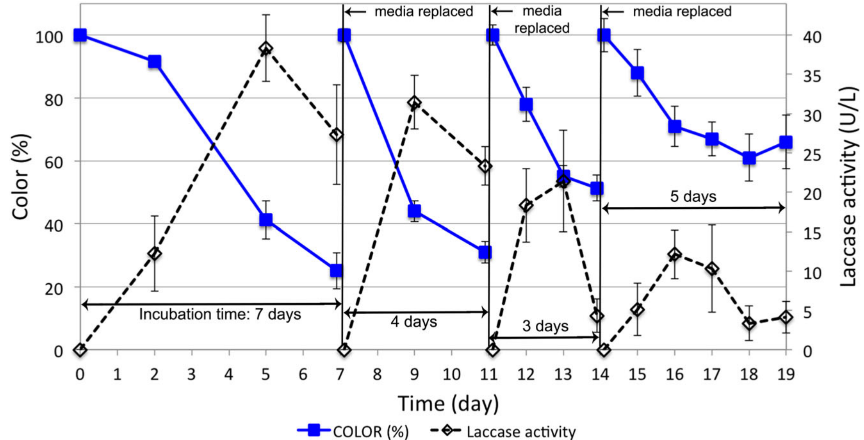

Figure 7. Colour removal and laccase activity during the sequential batch experiment using immobilized fungi under non-sterile conditions. Results are presented as the average of duplicates and error bars indicate the min and max values.

20 (U/L) to less than 5 (U/L). 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% decolourization was achieved after 3 days of incubation in the third batch. In the fourth batch, 40% decolourization was achieved after 4 days, and after that colour slightly increased. The increase in colour 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) and then started to decrease to less than 5 (U/L) in day 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%) than 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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