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TREBALL FINAL DE GRAU

Bio-remediation of humic acids from industrial wastewater using White-Rot Fungi

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1-Introduction

Humic acid substances (HA) can be frequently found in waste water coming from industries, specially those related to food processing. HA are highly soluble compounds characterised by a long lifetime as well. Their high solubility and their slow degradation rate make them recalcitrant compounds difficult to deal with (Asgher, Bhatti et al. 2008).

Due to their nature as recalcitrant pollutants, there is growing concern about their disposal. However, this has proven to be a difficult problem to solve and research is being carried out with regard to different techniques that could be applied to deal with them. Particularly, this project aims to develop HA removal by the use of White-Rot Fungi (WRF).

The main effects that they produce in the water that they pollute are organoleptic: they give it a dark-orangish colour and a coffee-ish smell. Their successful removal from effluent waste water is interesting from a social approach because it would therefore lead to a cleaner environment.

On another topic, HA appear to inhibit the growth of bacteria in the digesters and they can also cause fouling of membranes. Thus, their removal could significantly increase the efficiency of water treatment units (TUDelft).

WRF are a physiological group of fungi capable of biodegrading lignin that owe their name to the white layer from rotting that is produced by the degrading of cellulose over the wood they remain on (Pointing 2001). Cellulose and HA molecules have some shared particularities that, along with the non specific nature of the enzymes, made it possible to think that WRF might be able to degrade the latter as well (Asgher, Bhatti et al. 2008). However, the understanding of the mechanisms of the enzymatic processes involved is beyond the scope of this research.

Since the mechanisms involved on the removal of HA by WRF from wastewater are broader than the (bio-)degradation in which the enzymes they produce act as catalyzers, the concept of bio-remediation is included as well. (Bio-)remediation will refer in this paper to the actual removal of HA from wastewater, disregarding wether the mechanism involved is (bio-)degradation, adsorption or any other third mechanism that has not been considered.

1.1-Aim of the research

This study pretends to focus on wether WRF could be a possible solution to treat effluent with HA substances.

In order to achieve this, 3 different strains will be tested:

- Trametes Versicolor
- Phanærochate Chrysosporium
- Plearotus Sajor Caju

The first question this research aims to answer is whether any of these strains is able to remediate HA from wastewater.

Mainly, 2 different mechanisms are expected to occur:

- Adsorption of the HA by the mycelia grown by WRF, so that it can then be easily removed from the water by a filtration process.
- Bio-degradation of the HA that would be feasible thanks to the extra-cellular non-specific enzymes that the WRF produce.

Provided that any of the given strains shows clear evidence that they are indeed producing a removal of HA, it is as well the intention of this study to give an insight of the processes that take place. This is, to understand to which extent the remediation of HA is due to adsorption by the mycelia or to bio-degradation enhanced by the said extracellular enzymes.

After all, the ultimate goal is to provide a method that can be successfully applied on industrial conditions to remove HA from wastewater. The feasibility of a continuous reactor will be tested to face this final research question.

With regard to this report, its intention is to provide exhaustive information regarding the materials and methods that were used during the experiments whose description is subsequently given, a significative summary of the results that were obtained and the conclusions that from them can be deduced.

My research project is framed within the latest phase of Mr. Mostafa Zahmatkesh' PhD thesis. The previous work embraced, among other issues, research and experiments related to the media conditions that optimize fungal growth for our particular strains and a prescreening of 10 different strains of WRF on HA medias from which 3 where selected. It is also remarkable that the investigation as a whole has been done in cooperation with the Department of Environmental Chemistry of the Autonomous University of Barcelona, where similar procedures with some variances were applied.

1.2-Structure of the research at the laboratory

Research has been executed at the microbiology laboratory and it embraces the following:

During a period of time comprised between the months of September and December of 2015, 5 different badges have been carried out. This is:

- Prepared media sterile: where the media was consistent of demi water to which HA and compounds needed for the development of the fungi were added.

- Effluent sterile: where the media was consistent of the wastewater from which it is the main goal of the project to remove the HA and to which compounds needed for the development of the fungi were added.

- Immobilisation sterile: where the same wastewater was used, but this time carriers with the fungi were added to the media instead.

Up to this point, all badges were carried out on sterile conditions. The subsequent ones were not.

- Immobilisation non sterile: exactly as the previous one, but on non sterile conditions. This badge was done twice.

The explanation of the specific details of all badges will be further developed.

Taking into consideration the observations and results of the badges executed so far, this technique has been adapted to be used on continuous reactors from January to March 2016. 3 different reactors have been carried out:

- Continuous reactor with synthetic effluent

- Continuous reactor with industrial media

- Continuous reactor with synthetic effluent (modified)

Information concerning the specific details of these experiments will be further discussed.

Finally, the last badge carried out concerns the appreciation of the HA that is adsorbed by the fungi rather than bio-degraded. Fungi mycelia was killed in the autoclave and then washed out of any extra-cellular enzymes with water and put in media containing HA. This experiment was supposed to be realized in 4 days, but instead 2 contiguous weeks were required because of feasibility issues and so half of the badge was carried out each week. Details will be given.

Colour measurements are performed as an indicator of the amount of humic acid dissolved in the wastewater. Lignin, laccase and manganese peroxidase enzyme activity are also checked because they are the extracellular enzymes produced by WRF related to the degradation of HA (Grinhut, Hadar et al. 2007). Eventually, the HA concentration is precisely recorded with a High-Performance Liquid Chromatography (HPLC) device to evaluate the correlation between the colour of the samples and its concentration on HA and to obtain highly concise results. However, the further analyses of the information provided by the HPLC assays will not be extensively developed in this thesis.

2-Materials and methods

All work has been done under normal lab conditions with regard to light and temperature unless the opposite is stated.

2.1-Fungi stock cultures

These stock cultures are essential for the preparation of the badges, since they provide the fungi that are the main object of study.

3 different strains are prepared:

- Trametes Versicolor
- Phanærochate Chrysosporium
- Plearotus Sajor Caju

All work is done under a laminar hood, previous cleaning of the working surface with ethanol, and with a flame source nearby. Hand gloves are also required. These measures apply for each time that sterile conditions are required.

-Preparation of the media:

800 mL of demi water to which it is added:

- 24 g of Malt Extract (3%)
- 8 g of Agar (1%)

After proper mixing, this media is autoclaved so that it becomes sterile.

Any time that autoclaving is required, it applies a temperature of 121 ºC during 15 minutes.

With the media at 30ºC —before it solidifies—, it is poured into sterile Petri plates that are intentionally prepared for the growth of aerobic organisms. Petri plates are filled for half its capacity, which approximately accounts for 40 mL. Then, waiting until it cools down and solidifies.

With the only help of a loop that every now and then should be sterilised with the flame, the previous cultures are cut into a mesh and a couple of pieces are placed into the new Petri plates. It is important that they remain open the least time possible. Once finished, they should be kept under the laminar hood without much light going into them (photosynthesis must be avoided) until they are grown. Then, they are sealed with para film and stored in the fridge.

Cultures should be renewed every 5 weeks from previous cultures. Every 3 to 4 months cultures should be renewed from the original strain, which is kept in the freezer.

Since this procedure is rather sensible with regard to contamination of the plates, a generous amount of them is produced each time and afterwards, when the fungi are already grown, a sight valuation is carried out to dispose any wrong plates. This analysis is not difficult, since the white colour of the studied fungi contrasts with the black or grey dots meaning contamination.

2.2-Preparation of the different media used for the badges:

2.2.1-Prepared media sterile

To 4 bottles containing L 1,5 demi water, the following compounds are added:

Compound	Concentration (g/L)
KH2PO4	0,2
MgSO4*7H2O	0,05
CaCl2	0,01
Glucose	10
Trace elements	1 mL

TABLE 1-COMPOSITION OF THE PREPARED MEDIA STERILE

Each of the bottles are either N-L (add 0.35 g/L Ammonium tartrate) or N-S (add 4.8 g/L), so that there are 2 of both types.

-Preparation of the trace element solution used above:

The following are added to L 1 of demi water:

Trace element	Concentration (g/L)
MgSO4	3
MnSO4	0,5
NaCl	1
FeSO4*7H2O	0.1
COCl2	0,1
ZnSO4*7H2O	0.1
CuSO4	0,1
AlK(SO4)2*12H2O	0,01
H3BO3	0,01
Na2MoO4*2H2O	0,01

TABLE 2-COMPOSITION OF THE TRACE ELEMENTS

18 empty jars of 300 mL capacity —each of them sealed with a cotton stripe that prevents any microorganism, but not oxygen, from going inside— are required for the cultures.

The 4 bottles and the 18 empty jars are sterilised on the autoclave.

Also, each bottle is either with HA stock solution or not.
So that we have:

•N-L: Nitrogen-limited media with humic acid stock solution

•N-L blank: Nitrogen-limited media without humic acid stock solution

•N-S: Nitrogen-sufficient media with humic acid stock solution

•N-S blank: Nitrogen-sufficient media without humic acid stock solution

-Preparation of the humic acid stock solution:

•200 ml of demi water with 0,1 M NaOH and 4 g of humic acid that are mixed for 20 minutes

•Centrifuge of the solution at 12000 rpm for another 20 minutes

•Preparation of 100 mL of 0,5 M phthalate buffer (pH 4,00), to which the liquid phase of the previous solution is added after being centrifuged

•PH adjustment from 5,05 to 4,65 with 37% HCl

The following work is done under sterile conditions.

To the medias that require it, 10 mL of humic acid stock solution have been added. This has been done with sterile 0,45 μm filters (so that humic acid solution becomes also sterile)

The medias are distributed within the 18 jars so that each one has 150 mL of the respective media. This way, we obtain the next jars: 6 N-L, 6 N-S, 3 N-L blank, 3 N-S blank.

After the medias have been distributed, inoculation of the fungi so that:

3 strains x 2 types of media x 2 specimens each, plus blank x 3 types of strain x 1 specimen each

N-L		N-S	
with HA	blank	with HA	blank
2 specimens	1 specimen	2 specimens	1 specimen
x 3 strains	x 3 strains	x 3 strains	x 3 strains

TABLE 3-OVERVIEW OF THE SPECIMENS

Inoculation is done from the most recent ready culture plate following the same steps as for the preparation of the fungi stock cultures. This time 3 pieces of the mesh are added.

Jars must not leave the hood without a cotton stripe sealing them (they keep them sterile but at the same team allow oxygen in for the fungi to grow.

Once finished, the jars are placed in the incubator: room temperature (25 ºC), 170 rpm, humidifier required, without light.[1]

[1] Incubation period varies from 7 to more than 20 days and depends on the badge itself. Further information is provided with the results.

It is remarkable that the incubation is done in shaking mode (170 rpm) so that shear stress is applied to the fungi. This way, fungi grow in nice spherical colonies that are better spread inside the media rather than an amorphous mass at the bottom. It is acknowledged that the given unit is completely dependant on the device that is used; therefore, a better indication would be that the shaking should be enough so that well-rounded fungi colonies are produced but not too much to prevent them from being destroyed.

2.2.2-Effluent sterile

The same procedure as above, but this time effluent is used instead of demi water. This means that humic acid must not be added to the media (it already has it) and that there will be no blank specimens for this badge.

After all the procedure is done, the following are obtained: 2 N-L specimens and 2 N-S specimens, for each of the 3 strains.

2.2.3-Immobilisation sterile

For the immobilisation badges it is important to have previously prepared the carrier.

-Preparation of the carrier:

Working on sterile conditions: a couple of pieces of fungi mesh are inoculated to a package of carrier so that 1 package is prepared for each strain (3 in total). Packages must be prepared for aerobic breathing. They are closed and should remain in a dark environment at room temperature. They need to be shaken once every 3 or 4 days, ensuring that fungi grow upon all carriers and that mycelia does not make carriers stick to each other. Eventually, they should be kept in the fridge to prevent overgrowing. Sterilised sorghum seeds are used as a carrier.

This time effluent, demi water, and demi water with humic acid are used. No compounds are added except for the demi water with humic acid: 75 mL of humic acid stock solution to L 1,4 of demi water, following the previous methodology.

All bottles are autoclaved.

Working on sterile conditions: the medias are distributed into jars so that there are: 2 specimens with effluent (150 mL), another 2 specimens with demi water with humic acid (150 mL), and 1 specimen with demi water (300 mL), per each strain. A spoonful of carrier is put into each jar according to the pertinent strain that should develop.

2.2.4-Immobilisation non sterile (old)

Same procedure as for the immobilisation sterile but this time only effluent and demi water are used.

No sterile conditions should be implemented and medias are not autoclaved.

2.2.5-Immobilisation non sterile (new)

Same procedure as the old one, but this time effluent was autoclaved because fungi did not develop as expected the first time. This might have been because the microbial concentration of the media was too elevated.

2.3-Taking samples for the assays

Once jars are ready to start the incubation period, and during it until the recovery procedure begins, sample supernatant has to be extracted from them in order to execute assays that keep trace of the fungal activity.

Every few days samples are required to check colour reduction and enzyme activity. In this case, a 5 ml sample per jar is extracted.

Once or twice per badge, samples are collected for the HPLC. In this case, 8 mL are collected instead.

For the badges that were started under sterile conditions, the extraction must be done under sterile conditions but, once this is done, sample supernatant does not require them.

Samples are taken with pipettes and put inside falcons. Also syringes and 0,45 μm filters are used in order to filter them.

Pipette tips (with their tip cut at about 1 cm from its end, to prevent mycelia getting stuck) are previously autoclaved.

Once the assays are finished, the remaining supernatant is stored in the freezer.

2.4-Assays on the spectrophotometer

2.4.1-Colour assay

Checking the absorbance of 1 mL sample supernatant at wavelength 450 nm. For the last badge, colour is also checked at wavelength 600 nm.

Disposable 1.5 mL plastic cuvettes are used.

First blank measurement is done with demi water.

For all the assays performed at the spectrophotometer it has to be checked that there are no air bubbles when starting the measurement, It can alter the results. When different phases are put inside the cuvette, the same pipette is used to mix them.

The unit of any of the measurements that relate to absorbance in this project is the absorbance unit [AU].

2.4.2-Laccase activity assay

Checking the absorbance difference ratio per minute at wavelength 468 nm, during 2 minutes

Disposable 1.5 mL plastic cuvettes are used.

First blank measurement is done with demi water.

Compound	Volume in sample	Stock solution	Final concentration
Sodium malonate pH 4.5	0,2 mL	250 mM	50 mM
2,6-DMP	0,2 mL	5mM	1mM
Supernatant	0,6 mL	-	-
Total	1 mL		

TABLE 4-COMPOSITION OF A SAMPLE FOR THE LACCASE ACTIVITY ASSAY

It is important that in all the stock solutions for this assay (and for all assays regarding enzyme activity) ultra pure water is used.

Then they are added in this order and in the given amounts: sodium malonate, DMP, sample supernatant.

The reaction happens more or less fast depending on the enzyme activity. In order to grant that the change in the absorbance will be recorded, the experiment should be started immediately after sample supernatant is added.

As stock solutions are used regularly, they are kept in the fridge and used over time.

2.4.3-Manganese peroxidase activity assay

Checking the absorbance difference ratio per minute at wavelength 238 nm, during 2 minutes

Since it is on UV range, a quartz cuvette is used. After each experiment, the cuvette is washed with a load of demi water.

The procedure goes as follows:

-It is added in this order and in the given amount: sodium tartrate, sample supernatant and MnSO4

-Check blank

-Quickly added H2O2 and the measurement is started

Compound	Volume in sample	Stock solution	Final concentration
Sodium tartrate pH 5	0,2 mL	0,5 M	0,1 M
Supernatant	0,6 mL	—	—
MnSO4	0,1 mL	1mM	0,1 mM
H2O2	0,1 mL	1mM	0,1 mM
Total	1 mL		

TABLE 5-COMPOSITION OF A SAMPLE FOR THE MANGANESE PEROXIDASE ACTIVITY ASSAY

H2O2 stock solution is always prepared the same day that has to be used.

2.4.4-Lignin peroxidase activity assay

Checking the absorbance difference ratio per minute at wavelength 310 nm, during 2 minutes

A quartz cuvette is used again for the same reasons and in the same way.

The procedure goes as follows:

-We add in this order and in the given amount: sodium tartrate, sample supernatant and veratryl alcohol

-Check blank

-Quickly add H2O2 and start measurement

Compound	Volume in sample	Stock solution	Final concentration
Sodium tartrate pH 3	0,2 mL	0,5 M	0,1 M
Veratryl alcohol	0,1 mL	4 mM	0,4 mM
Supernatant	0,6 mL	—	—
H2O2	0,1 mL	5,2 mM	0,52 mM
Total	1 mL		

TABLE 6-COMPOSITION OF A SAMPLE FOR THE LIGNIN PEROXIDASE ACTIVITY ASSAY

This assay was solely performed on 6/10/15 for the first badge in the 12th day of incubation.

2.5-Preparation of the samples for the HPLC

1,8 mL of sample supernatant is added to a 2 mL plastic disposable vial.

20 μL of 37% HCl are added to it. This is done so that humic acid becomes insoluble and can be isolated. Let the vials be for a day in the incubator, so that they shake.

Next day, vials are centrifuged for 20 minutes at 15000 rpm.

Then, fulvic acid (liquid phase) can be extracted and put inside another vial.

1.8 mL of 0,1 M NaOH are added to the remaining solid phase (humic acid). This is to surely solubilize all of the humic acid.

It is also advisable to check the pH of randomly selected vials with indicator strips. PH should respectively be extremely low and extremely high, for the fulvic vials and for the humic vials.

Samples are kept in the lab at normal conditions until the day the assays on the HPLC are done. It is better if all the samples relative to the same badge are assayed altogether to ensure equal assay conditions because of the sensitivity of the device.

2.6-The recovery day

The recovery day is the day when the badge is finished. It is usually after 2 weeks of the starting of the badge, but this number varies widely depending on the badge and the situation.

Steps to be followed:

-Extracting sample supernatant from the jars (8 mL samples). At this point, it is not required to work under sterile conditions since only a few hours left cannot substantially change the cultures.

-Regular assays on the spectrophotometer

-Preparation of the vials for the HPLC

-Dry Mass Weight (DMW)/Recovery sample supernatant: since they are both destructive assays, either one or the other is performed with each jar.

2.6.1-DMW

Weighting cups (with their respective filter paper) are pre-weighted and numbered.

The biomass of the jar is split from the liquid phase with the help of a vacuum filter and the filter paper.

The filter paper, the weighting cup and all the collected biomass are dried out. Then, the next day, they are all weighted together again so that the difference with the first result stands for the biomass that was in the jar.

2.6.2-Recovery sample supernatant

The purpose of this procedure is to obtain vials for the HPLC that take into consideration the part of the fulvic and the humic acid that might have been absorbed by the fungi.

The whole jar is steered with a magnet in order to destroy the mycelia. At the same time 0,4 g NaOH is added to it. They should be kept mixing for 2 hours.

After that, they are sonicated for 5 minutes each.

10 mL are extracted and put inside a falcon. Then, 50 μL of 37% HCl is added and they are left shaking in the incubator for the night.

Next morning, they are centrifuged for 15 minutes at 145000 rpm.

1,8 mL are extracted from the top and put inside a vial. This will tell the fulvic concentration at the HPLC.

The rest of the liquid phase is thrown away and the remaining solid (humic acid at the bottom of the falcon) is mixed with 10 mL of 0,1 M NaOH.

1,8 mL are extracted from it and put inside a vial. This will tell the humic concentration at the HPLC.

2.7-Other assays performed

Assays regarding other different topics have been carried out without following a specific pattern:

-Prepared media sterile: humic acid of the media was measured at the beginning of the badge with the same protocol as for DMW, the 12th day lignin peroxidase activity was also measured (the only time this was done), and DMW on the recovery day.

-Effluent sterile: DMW was performed on the recovery day.

-Immobilisation sterile: humic acid was measured at the beginning as for the first badge; it was also intended to estimate the amount of carrier that a spoonful accounts for, but using different balances at different steps made the results profitless.

-Immobilisation non sterile (new): the pH of the culture jars was measured on the 4th day of incubation. A descriptive diary of the visual characteristics of the jars was also kept.

The intention of this section is to enumerate all the relevant assays that were performed apart from the usual ones. Detailed information about their figures can be found at the data file concerning the badge to which they belong.

2.7.1-Immobilisation non sterile (new), descriptive diary

The mass of the carrier and of other microbial life present in this badge would make it impossible to achieve accurate figures for the fungal biomass with the DMW procedure. Instead, notes have been taken all along the incubation period regarding the visual characteristics of the inside of the jars.

The following is a faithful transcription of what they exactly say:

•Day 3 (3/12/15)

Growth: Blank: Trametes and PSC are growing. Both are covering the beads (sic). Some free very small granules.

Some observation for the jars with Effluent
Color: Trametes obviously reduced color. Phanerochate has not changed that much. PSC is between Phan & Tram.

All of them are turbid except Blank Tram. Phan blank was the most turbidity among the blanks, but with effluent, it has the lowest turbidity.

•Day 4 (4/12/15)

PSC has grown a lot of mycelia since last afternoon. It is also removing color, but not as much as trametes, which has grown less. Thus is not related to absorption,
Jar BI Effluent without cotton stripe has developed a lot of growth, filaments are visible. It has removed some colour.

•Day 7 (7/12/15)

Trametes much less colour.
PSC and Phan more or less the same.
Tram 1 no apparent growth of mycelia. Tram 2 considerably more, plus some granules (very small, outside the carriers)
PSC and Phan 1 similar growth (not much) around the carriers
PSC 2 growth outside the carriers.
Not many black stains visibles as last badge.
For the blanks: phan no apparent growth. Tram growth outside the carriers. PSC growth around the carriers (and more developed than PSC eff)

•Day 9 (9/12/15)

Some bacteria granules are growing in Tram 2 and Tram blank. Tram 1 does not grow much but it seems to be doing almost as ok as tram 2.

2.8-Continuous reactors

After all the badges concerning jars were carried out, a new phase of the research project is developed, which involves the use of continuous reactors. This is the ultimate attempt to create a method that can be used for industrial purposes. Thus, conditions are much less regulated: no sterile conditions apply and the producing of the media is less strict as well with regard to concentrations.

At the laboratory, 2 identical continuous reactors could be used for this purpose, and so the different badges can be overlapped in time. The 3 badges that were carried out are as follows:

•Synthetic media reactor: from 22/01/16 to 17/02/16, synthetic media is used.

•Industrial media reactor: from 04/02/16 to 17/02/16, industrial media is used. Sterile conditions are not applied; however, the bacterial concentration on the industrial media that had been stored since September in the fridge proved to be excessive for the good developing of the fungi, and therefore the media was autoclaved before used.

•Modified module with column: from 24/02/16 to 16/03/16, synthetic media is used again. The intention was to use industrial media but the concentration of HA on the newly acquired industrial media turned out to be insufficient because there had been a fire in the industry that provides it the previous month and it was not working perfectly, and so it was decided to use synthetic media instead. The motivation for the modification of the module lays on the observations from the previous badges: there were areas of the reactor that remained stagnant and grew significant sludge.

Immobilised fungi were always used.

2.8.1-Reactor design

As it has already been stated, both reactors were at the beginning identical as described.

They consist of a single L 6 cylindrical tank. The tank is made of glass so that the inside is fully visible and has openings at different heights. An opening at approximately L 5 from the bottom is used as the outlet of the continuous reactor.

As part of the structure of the reactor, there is also a water jack around the tank where water is always being circulated from an isothermal water tank at approximately 26 ºC.

At the top lid of the tank there are incisions where probes can be placed; in this case, 2 probes are used: to measure the oxygen concentration and to measure the pH. Hanging as well from the top lid there is a threaded bar on which 2 discs are placed. The discs are made of plastic and full of small holes so that air and media can get through but fungi cannot. The intention was to create a volume inside the tank (in between the 2 discs) were the fungi are concentrated so that losses of biomass due to the outlet are reduced.

Because of mycelia and carriers frequently escaping the volume limited by the discs, the outlet resulted often clogged and sometimes it even overflowed from the top lid. To prevent these events, a pump is used to help the media go outside of the tank.

There are also 2 pumps more pumping respectively inside the tank from the top lid media and antifoam. The retention hydraulic time is approximately set at 3 days.

At the bottom of the reactor there was a sealed outlet in case that it needs to be emptied. A ventilation system is also installed there: it consists of air forced into stone filters so that small bubbles are produced and agitate the media to ensure it is constantly mixed and the desired shear stress for the fungi. This system should be placed in a way that the bubble flow is similar everywhere in the tank to prevent secondary currents that lead the carriers to stagnant points. This system also stabilises the oxygen concentration at more or less 6 mg/L, so that there is enough of it for the fungi to grow.

-Modified module with column: one of the reactors was modified after the initial badges were finished. The object of this intervention was to eliminate the stagnant areas at the bottom that were caused by an imperfect ventilation system. The cylindrical shape of the tank was joined to a cone shape that steadily reduced its diameter to reach the bottom where a circular stone filter was placed.

Figure 1 shows the sketch of the initial continuous reactor as it has been described.

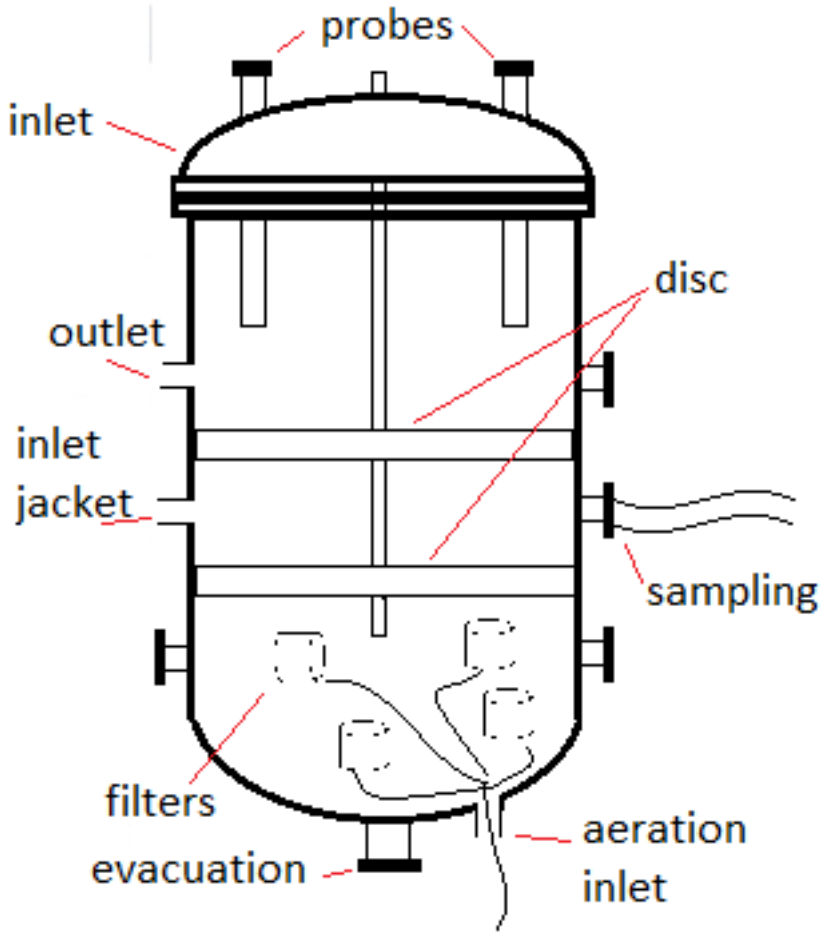

FIGURE 1-SKETCH OF THE CONTINUOUS REACTOR

2.8.2-Preparation of the synthetic media

The preparation of the synthetic media is similar to the one used for the previous badges. However, in this case the amount of media required is substantially higher: at around L 20 every 10 days for just one reactor. Thus, the procedure was adjusted so that it was the least troublesome possible to produce such an amount[2].

800 mL of demineralised water with 0.1 NaOH and 16 g of HA are mixed during approximately during 20 minutes with 400 mL of demineralised water with 0.5 M phthalate buffer. Hence, we obtain L 1,2 of HA stock solution.

The stock solution is then centrifuged at 15000 rpm during another 20 minutes and after that, poured from the falcons to another vase trying to leave the sediment at the bottom. Then, the stock solution is filtered with a 0.45 micras pressurised filter.

To a tank containing L 20 of demineralised water, 500 mL of HA stock solution are added. The rest of it is stored as stock solution for when it is required.

To check that the concentration of HA was similar for all the times this procedure was executed, the colour after mixing was measured in the same way it has already been given for the previous badges analyses protocols. The correct value from the spectrophotometer was set at 0.155 with an acceptance margin of 15%.

Sometimes, the resulting media did not adjust to these values and some more stock solution or demineralised water was added. If water had to be added, it had the same concentration of buffer than the container. This was done with a trial and error approach.

2.8.3-Sampling from the reactor

Before taking the sample from the reactor the air flow is stopped. Then, one of the outlets is opened and sample supernatant is taken from there. Samples from the tank containing media are taken as well, via the pump that introduces it in the reactor. As a rule of thumb, samples from the reactor are taken daily whereas samples from the tank containing only media are taken every 3 days.

After that, samples stand still for some minutes to let the mycelia sediment at the bottom and then they can be filtered with 0.45 micra filters.

With these samplings, the same assays as for the previous badges are performed. This is: measuring of the colour and the enzyme activities (only laccase and manganese peroxidase).

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[2] The main difficulty consisted on filtering the HA stock solution, since HA is partially soluble and at the concentrations that were being used filters were easily clogged.

At the starting and the ending of the badge, as well as once every week samples are prepared also for the HPLC measurements according to the protocols previously described.

2.8.4-Data recorded

All the files containing the data recorded for the reactors, the previous badges and any other experiments are annexed to this document. However, to give a first impression of which are the unprocessed outputs of the experiments with the continuous reactors, this first overview is provided:

•From the taken samples, results are obtained for colour and enzyme activity measurements daily. In the case of the tank with media, colour measurements are obtained every 3 days and it is unnecessary to check enzyme activity.

•From the probes, oxygen concentration and pH are recorded once a day in the reactor. Sometimes the probe for oxygen concentration got wrapped in small particles of mycelia. If the oxygen concentration was unusually lower without any reason, the probe was pulled out and carefully cleaned. The measurement was read again once it became steady.

•HPLC samples are collected and stored to be analysed as a pack after the badge is finished. Data resulting from these analyses will not be considered as part of this thesis.

•Any relevant observations are also written down on a daily diary. This observations range from incidents such as overtopping of the lid by the media in the reactor because of clogging to the dates at which new carriers with fresh fungi are provided to the reactor.

2.9-Adsorption experiment

Along with the last continuous reactor experiment, a badge concerning the adsorption of the mycelia and the carriers was executed.

The idea underneath is to use deactivated specimens that do not produce enzymes anymore and introduce them in media with HA to see the adsorption that they produce. This badge is structured as follows:

•Immobilisers: only the carriers

•Immobilisers + “Strain”: the carriers after a particular fungi strain has been previously inoculated and grown on them

•Free “Strain”: mycelia from a particular fungi strain that was previously grown on liquid media under sterile conditions

The 3 different strain of fungi that were analysed on previous badge have been used.

For each of the resulting categories, 3 different proportions were used: 2, 4 and 6 spoonfuls, being this quantity remarkably dependent on the category that was being tested.

Each one of the resulting specimens is duplicated, so that finally the badge consists on 42 specimens.

This experiment was supposed to be realized in 4 days, but, because of feasibility issues, half of the badge (Immobilisers alone and free fungi) were tested on a week and the other half (immobilisers with fungi) were tested the next one.

2.9.1-Weighting

In order to assess the quantities that were being used, the amounts of 2, 4 and 6 spoonfuls were poured into individuals caps that had been previously weighted and their weights were measured right after and after letting them rest overnight in the oven to dry them out.

In the subsequent table, the weight of each cup has been subtracted to the weighting after drying of that cap to obtain an estimate of the amount of matter that was being used each time:

Dry weight of the specimens (g)			
Type	**2 Spoonfuls**	**4 Spoonfuls**	**6 Spoonfuls**
Immobilisers	1,494	2,9359	3,93715
Free Tram	0,0219	0,03275	0,06285
Free PSC	0,02525	0,0711	0,08085
Free Phan	0,0081	0,01845	0,02955
Imm+Tram	0,56715	1,06795	1,67065
Imm+PSC	0,5628	1,1589	1,95745
Imm+Phan	0,16785	0,28085	0,54965

TABLE 7-WEIGHTS OF QUANTITIES USED FOR THE ADSORPTION EXPERIMENT

As it can be observed, the amounts vary on a more or less linear way for each type. Naturally, the highest weights are found in the carriers alone, because they have a greater density; and then carriers with fungi and free fungi respectively. The most significant differences appear with the free fungi, since their variety on shapes and sizes makes it difficult to work with spoonfuls of the same proportions.

2.9.2-Preparation of the media

A HA stock solution was prepared in the same way that the previous times with the stock solution for the media concerning the reactors.

Approximately L 7 of demineralised water in a glass bottle and 42 jars of 300 mL capacity were sterilised on the autoclave.

It should be noted that this experiment was not executed under sterile conditions because it was thought that its duration of 4 days was not long enough for bacterial concentration to have a relevant influence on the outputs. However, materials were prior sterilised to ensure that this is the case.

HA stock solution was added to the water and mixed properly. A sample was collected, filtered and analysed; being its final resulting colour 0,124

All jars where then added 150 mL of the media. Note that because in reality the second half of the badge had to wait for a week before being started, the media for it was stored in the lab in normal conditions and was autoclaved a second time immediately before its preparation.

2.9.3-Preparation of the fungi and the carriers

- Immobilisers

Carriers alone did not require any further preparation. The boxes containing them under sterile conditions were stored in the fridge until they were required to start the experiment.

- Immobilisers with fungi

First, carriers where inoculated fresh fungi from petri plates under sterile conditions with the same procedure that has been previously described and kept at room temperature and no light for its further development, occasional shaking as well.

After a week, these carriers in which the development of the fungi was obvious but not as extended as previous times (the incubation period had been longer) were inoculated to L 2 glass bottles containing L 1,5 of demineralised water that were previously autoclaved.

It was important for the inside of the jars to remain sterile but at the same time, oxygen was required for the development of the fungi; thus, cotton stripes were used to seal them. It was important as well that there is a considerable amount of air space left in the inside of the bottles because they were to be shaken thoroughly during the incubation period.

Each jar contained 2 boxes of carriers inoculated with the same strain.

The incubation period inside the jars lasted for 1 week.

- Free fungi

The same autoclaved equipment and methods as before were used. This time, the content of a petri plate culture is poured inside a jar (so 1 jar per strain, again) in small pieces.

They should develop as nicely rounded granules. The size of these granules depends on the strain and the shear stress applied. At around 10 days after inoculation, the media becomes dense enough in granules for our experiment and they are ready for the next phase.

The carriers did not require any further preparation before they were added into the media. As for the free fungi and the immobilisers with fungi: it is desired that there is no interaction between the media and the enzymes the fungi produce because only the adsorption produced by the mycelia and the carrier are the object of study in this experiment. Hence, after the incubation period the jars were autoclaved again.

Up to this point, the fungi were not capable of producing any more enzymes but yet there were still some that had been produced previous to the autoclaving. The jars were then washed thoroughly with demineralised water several times and keeping the granules and/or carriers inside. As much water as possible was then removed from the jars.

There remained a liquid phase that was not feasible to remove: it consisted partially on remaining water and partially on an oil that is produced by the fungi themselves. This oil made the fungi particularly slimmer and it was difficult to produce acceptably similar spoonfuls for the experiment.

2.9.4-Scheme of the adsorption experiment

The main indicator for this experiment is colour of the samples as analysed by the spectrophotometer. Samples were taken and filtered with the following scheme:

•Right after the inoculation of the spoonfuls. This was too early for the adsorption to begin, but it was aimed to see the inferences in the colour that the adding of some liquid phase along with the fungi could have.

•1 hour after inoculation

•1 day after inoculation

•2 days after inoculation

•Recovery day (3 days after inoculation). After adding of NaOH

•Recovery day (3 days after inoculation). After steering

The first 4 samplings consisted on taking the samples, filtering them, measuring the colour at the spectrophotometer and preparing samples for the HPLC.

The last 2 samplings required manipulation of the specimens and are further discussed.

•Recovery day, 1st step:

In the evening of the 2nd day after inoculation 0,1M NaOH was added to each of the specimens and they remained in the incubator overnight.

Next morning samples were taken as before.

•Recovery day, 2nd step:

Specimens were then steered with a magnet until the mycelia was destroyed. This process took 2 hours. After that, samples were also sonicated for 5 minutes.

Samples where then taken as usual.

2.10-Used devices

In table 8, there is a list of the specific lab equipment that was used for the experiments:

Device	Model
Centrifuge	Sorvall ST 16 Centrifuge 75004380
Laminar flux hood	SC-BHC-IIA2
Incubator	New Brunswick Innova 40/40R
Autoclave	Fedegari FVA/A1
Spectrophotometer	Genesys 10S UV-Vis
HPLC	Shimadzu Nexera Quaternary System

TABLE 8- DEVICES IDENTIFICATION

3-Review of the results

3.1-Inventory of the results

It follows the inventory of the annexes that contain the detailed data obtained from all the experiments previously described. The data is organised in excel files whose name indicates the badge that they belong to and it is ordered in the chronological order in which the experiments were realised. A brief explanation of what they are will be as well submitted. Further analysis, conclusions and any relevant graphs and tables will be based on them.

- Prepared media sterile.xls

It consists of 4 sheets. They are: "enzyme protocols", in which the protocols used for the measurement of the enzyme activity at the spectrophotometer are summarised; "colour" contains the data about the colour assay as well as the weighting of the HA stock solution and the grown fungi and the data from the one day that the lignin peroxidase activity was measured; "laccase" contains the data about the laccase activity assay; and "Mn. peroxi." shows the data about manganese peroxidase activity.

- Effluent sterile.xls

It consists of 3 sheets. "Colour", "Laccase" and "Mn. peroxi" showing respectively the data for these assays. "Colour" contains as well the data of the weighting of the fungi after the badge was finished.

- Immobilisation sterile.xls

Again: "Colour", "Laccase" and "Mn. peroxi.". "Colour" contains as well the weighting of the HA that the effluent used as media has and also the weighting of the mass that a tablespoon contains as an average.

- Immobilisation no sterile (old).xls

"Colour", "Laccase" and "Mn. peroxi"

- Immobilisation no sterile (new).xls

"Colour", "Laccase" and "Mn. peroxi". "Colour" also contains pH measurements of the specimens at day 4th of incubation.

The following files are a diary of the reactor experiments. They contain almost daily data on oxygen concentration (DO), pH, colour, laccase and manganese peroxidase activity and any relevant observations:

•Synthetic media reactor.xls (from 22/01/2016 to 17/02/2016)

•Industrial media reactor.xls (from 04/02/2016 to 17/02/2016)

•Modified module with column.xls (from 24/02/2016 to 16/03/2016)

Adsorption color.xls contains 2 sheets: “Color” and “Weight”, regarding these 2 measurements during this experiment

As it has been asked that no more than 5 files should be annexed and that they should be pdf files, the previous have been merged into 1 single pdf file that preserves the said order.

Finally, a scanned version of the original laboratory diary is submitted with the name: notebook.pdf.

3.2-Presentation of the results

It is the intention of this chapter to analyse the main features that can be identified in the submitted files containing all the data available and to provide a clear summary as well as relevant plots and tables.

3.2.1-Colour removal on initial badges

It was the main goal of this research project to find a method that could be used under industrial conditions to separate HA from wastewater effluent. The main attribute that can be assessed as a consequence of water containing HA is a dark orangish colour. Therefore, this is the attribute on which the evolving of the specimens is primarily judged and the procedure to assess it has been already described as the colour assay at the spectrophotometer.

The data is presented as colour removal, but the measurement that the assay provide is light absorbance at certain wavelength by the sample. A higher absorbance implies a higher colour. The colour removal is then presented as a ratio between the current absorbance and the initial one. The colour that is produced by the fungi because of their growth is identified as the colour from blank specimens and subtracted from the previous formula so that:

$$Colour\ removal = \frac{Absorbance^{0} - Absorbance^{i} + Absorbance^{iblan}}{Absorbance^{0}}$$

All badges have as well an error bar. This error bar is the result of taking into consideration that each one of its categories is indeed composed by 2 identical specimens. As a general overview, the

differences are lower than 10% of the original colour, which will be accepted as the margin of error of the experiment

Figure 2 shows the evolution of the colour removal for the first 5 badges that were carried out. This evolution is shown from day 1 (inoculation day) to the last day (recovery day) before the destruction of the samples and is evaluated as a reduction from the colour of the sample before inoculation on day 1. The colour removal is calculated as a percentage of the starting colour (i.e. before inoculation), the colour that is produced by the fungi because of their growth is subtracted.

With regard to the badge “Immobilisation non sterile (old)”, it can be seen that the colour removal is very poor (or even negative) compared to the other badges. At that moment, it seemed that this was due to bacterial proliferation in the stored industrial effluent that was being used for the badges and it was decided that a second badge “Immobilisation non sterile (new)” would be performed in identical conditions but this time the media would be sterilised before using it. As a result, the second badge performed way better leading to the conclusion that, indeed, the bacterial concentration was, at that point, too high for fungi to develop properly. The consequences of this conclusion were that the industrial effluent would be sterilised before use in the following experiments. A considerably higher error is shown for the former of the said badges, but this is merely disregarded considering that the whole badge was repeated because of the given reasons.

Taking into consideration the 4 badges that did not encounter the previously explained incident, it is noticeable that, in general terms, there is a significant color removal for all strains; moreover, it is particularly remarkable for Trametes Versicolor. For this strain, color removal usually reaches the 80% both in nitrogen-limited and nitrogen-sufficient environments.

There is one exception to this behaviour: Trametes Versicolor in nitrogen-sufficient environment for the “Prepared media sterile” badge. The most reasonable explanation to it is that the fungi grew too much and too fast and so exhausted the resources too early; optical evidences supported this explanation, since on the recovery day these specimens presented highly developed mycelia that was already starting to rot. Another reason that could partially stand as well for this case is that fungi produce colour as they keep growing; the evolution of the colour for the blank specimens will be further discussed.

The other strains show a more or less nice behaviour in most of the badges but, comparatively speaking with Trametes Versicolor, their effectiveness with regard to colour removal is much lower. Amongst the 3 studied strains, Trametes Versicolor was consequently selected to be studied on a continuous reactor. Particularly important for this decision was the performance of the 3 strains on the badge “Immobilisation non sterile (new)”, since, as it was already explained before, the conditions on the reactor are non sterile and the inoculated fungi are previously immobilised.

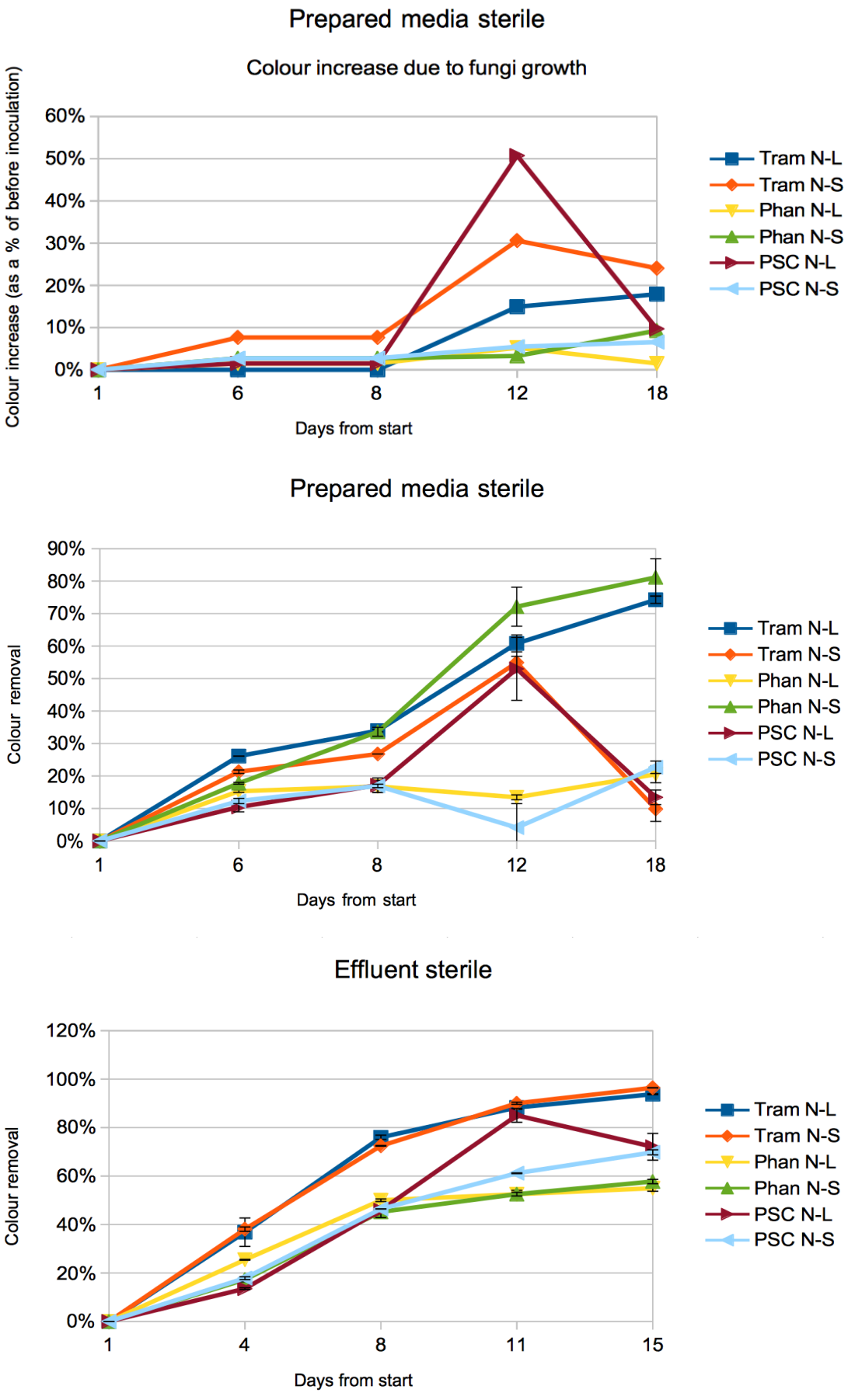


FIGURE 2-COLOUR RESULTS FOR THE INITIAL BADGES: REMOVAL AND GROWTH ON BLANK

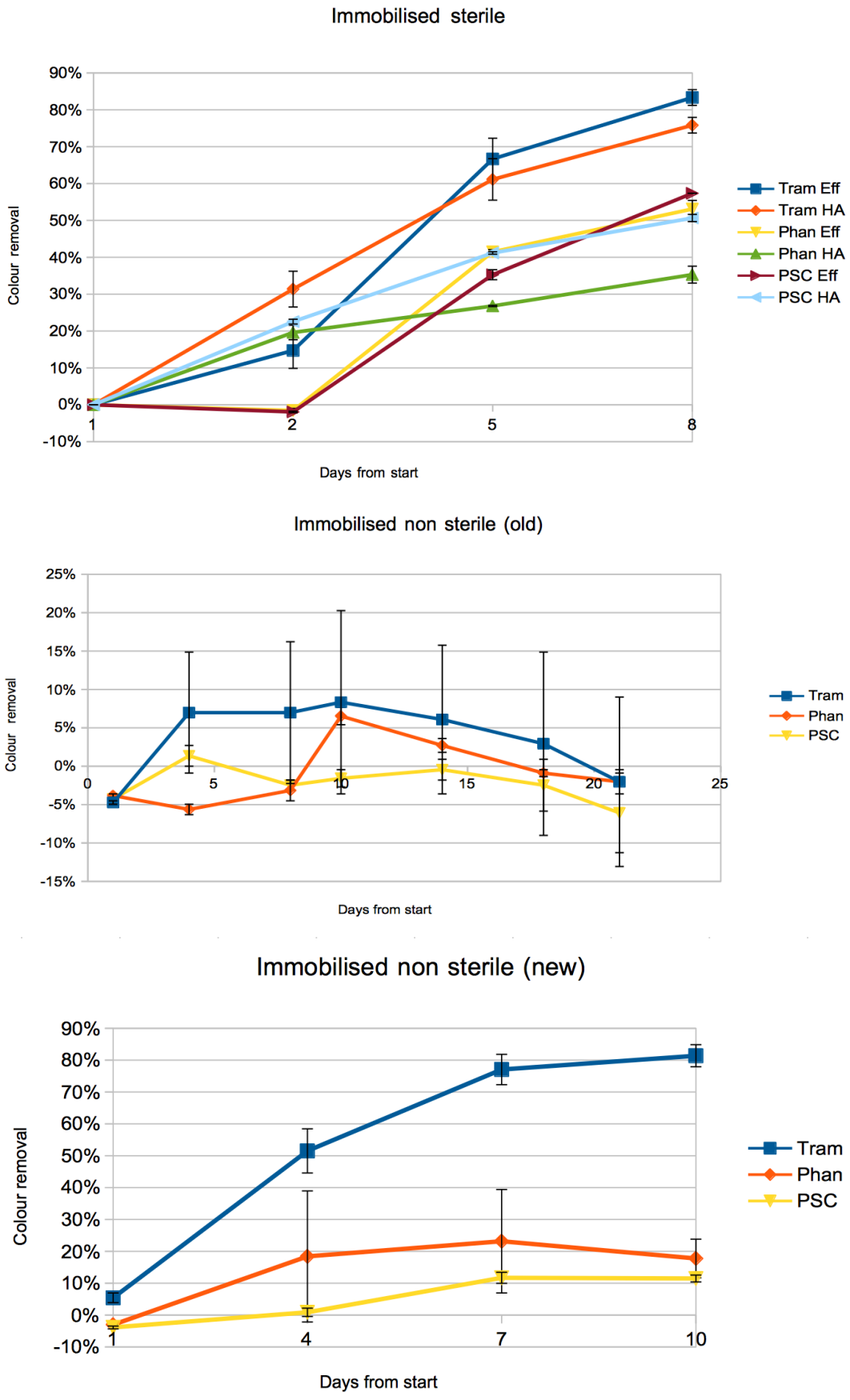


FIGURE 2-COLOUR RESULTS FOR THE INITIAL BADGES: REMOVAL AND GROWTH ON BLANK

3.2.2-Colour removal on reactors

Figure 3 shows the evolution of the colour removal for the reactor badges. This colour removal is again evaluated as a reduction of colour that takes this time the colour measurement for the inlet media as a reference point.

However, there were some differences in the colour of the inlet throughout the experiment and even in some cases these differences were significant enough that correction actions had to be executed: the threshold that was imposed was that more than 15% of colour difference from the first measurement at day 1 was to be corrected. Taking all of this into consideration, the graphs shown in Figure 3 take as reference value for colour the last colour measurement done to the inlet media, and this measurement was generally repeated every 2 or 3 days.

At a first glance, one could not see a general positive trend: diary records of events are completely necessary to understand what is happening at any point. Thus, the main relevant events are hereby summarised for each of the reactors:

- Synthetic media reactor

Immobilised fungi were added on days 1, 12 and 20.

On day 6 the colour of the inlet media was considerably higher, this led to a darkening of the outlet as well. Corrective actions were applied.

On day 19 the tank containing the inlet run out of it, which meant that for some hours there was only anti-foam being pumped into the reactor. This incident led to a considerably lighter effluent because of the white colour of the anti-foam.

- Effluent media reactor

Immobilised fungi were added on days 1 and 7.

- Modified module with column

Immobilised fungi were added on days 1 and 9.

On day 8 a considerable portion of the bacterial mass was removed because there was the concern that it was troubling the proper development of the fungi.

As a general overview, it can be stated that the reactors showed the highest rates of color removal after the inoculation of fresh fungi and kept going on for about a week.

An exception to this overview would be the first week for the “Synthetic media reactor” if we only look at Figure 2, but it has to be understood that there is an uncertainty about the real colour of the inlet media as well.

A better example on this reactor would be the recovery of color removal that took place on days 20 to 24 when fresh fungi were added again; after that, it is conceivable that bacteria concentration was troublesome and so colour removal decreased again.

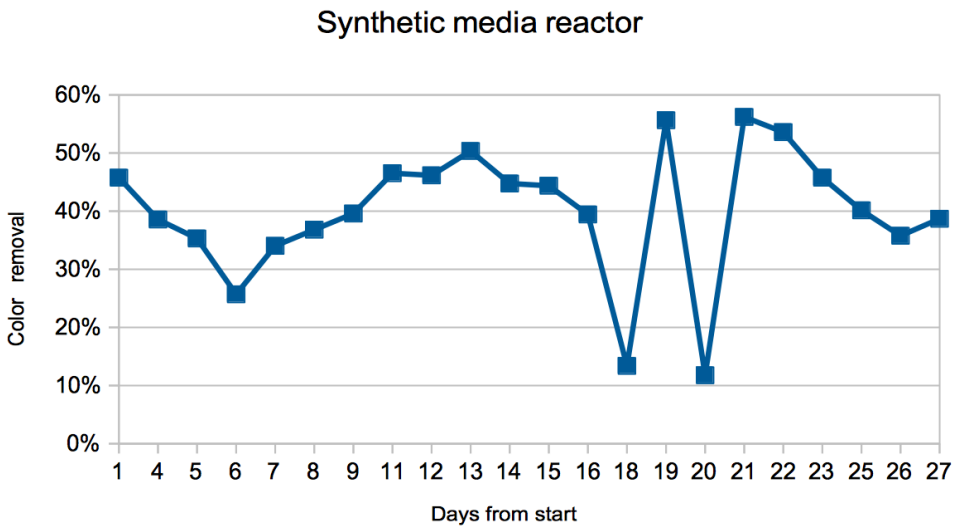
The synthetic effluent was set at a pH of approximately 4.5 with the help of a buffer and the trend was that it would not vary during the experiment. The situation is different for the industrial effluent: buffer was not added and therefore pH was as delivered by the industry, which was at around 6.5. Fungi are known for being able to influence the pH of the media while they grow (Samson 2016) and in this case Trametes Versicolor was not an exception: the initial pH of 6.5 was reduced to around 4.5 without any intervention.

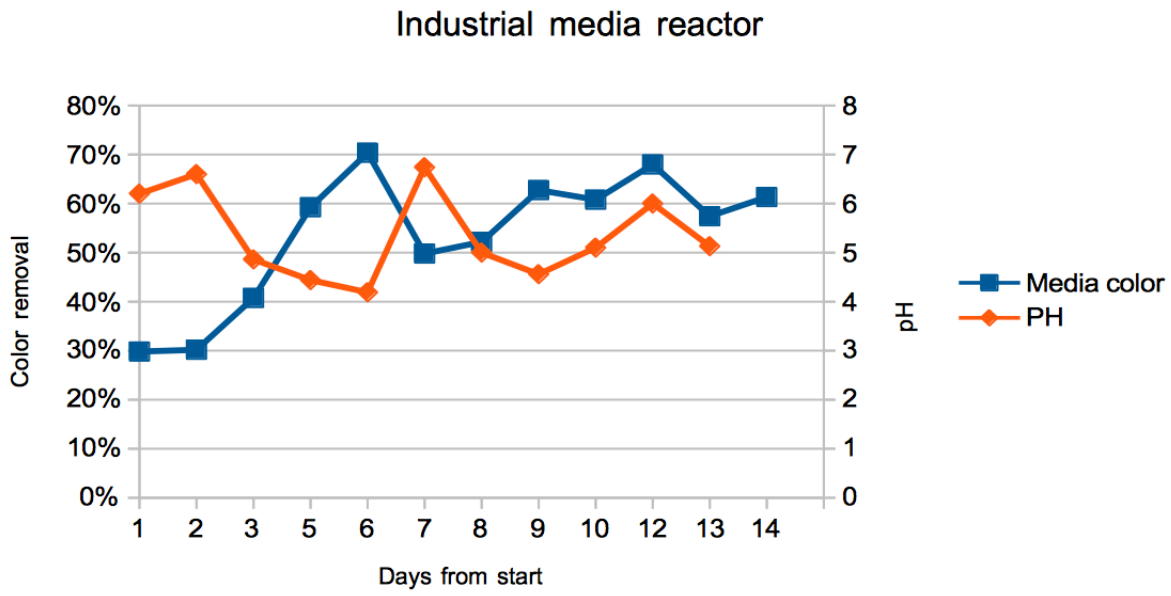
It can be observed that there is a first step of decreasing of the pH to the desired range after which color removal increases substantially. If we take into consideration that laccase enzymes from Trametes Versicolor are reported to have a rather low optimum pH of activity at around 3-4 (Stoilova, Krastanov et al. 2010) it seems an evidence that laccase play a main role on the remediation of HA.

This scenario is repeated after day 7, when fresh fungi are added again.

There was some concern about the bacterial concentration of the media inside the reactors. The aeration system had some flaws that created regions inside the reactor that were close to stagnant. These areas were a particularly good spot for bacteria to develop but also sometimes fungi were accumulated in these areas where they lacked the shear stress and the amount of oxygen that they required for a proper growth. Because of all of this, a new module for the reactor was used for the final badge: its improvement that have already been described meant the eradication of these stagnant areas and, as it can be seen in Figure 2, there was a considerable improvement of the functioning of the reactor.

For this last reactor, it can be perfectly seen that there is a good colour removal following the week after inoculation. Then, there is an abrupt decrease that is sorted out by the addition of fresh fungi. The last days show an increase of the pH and a decrease of the colour removal that can be justified by an increasing bacteria concentration.





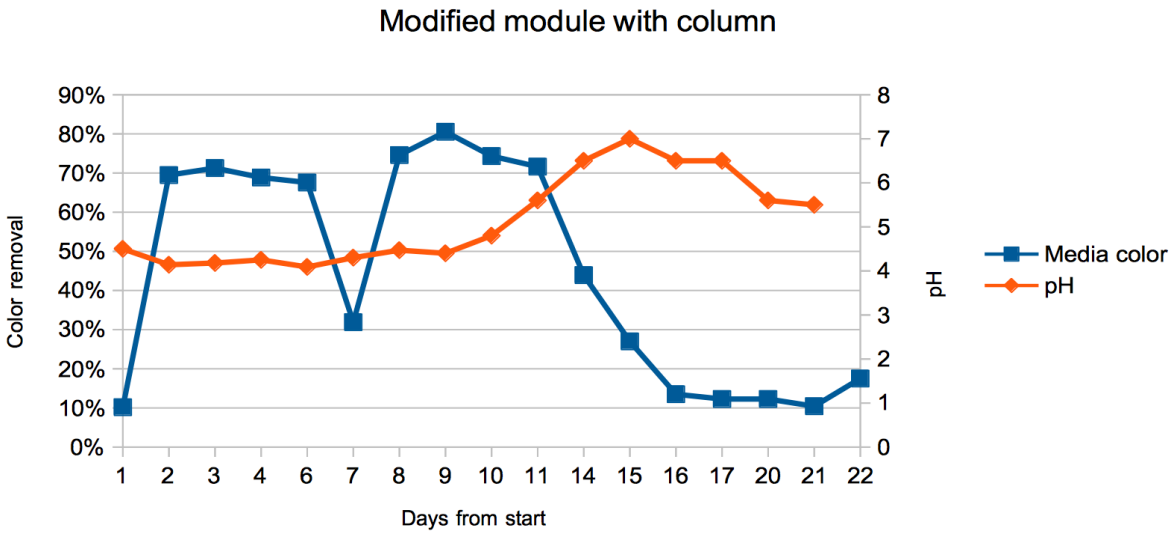


FIGURE 3-COLOUR REMOVAL ON CONTINUOUS REACTOR BADGES

3.2.3-Adsorption experiment

Figure 4 relates to the removal of colour that was caused in 150 mL specimens only due to adsorption of the carriers, free fungi or immobilised fungi respectively.

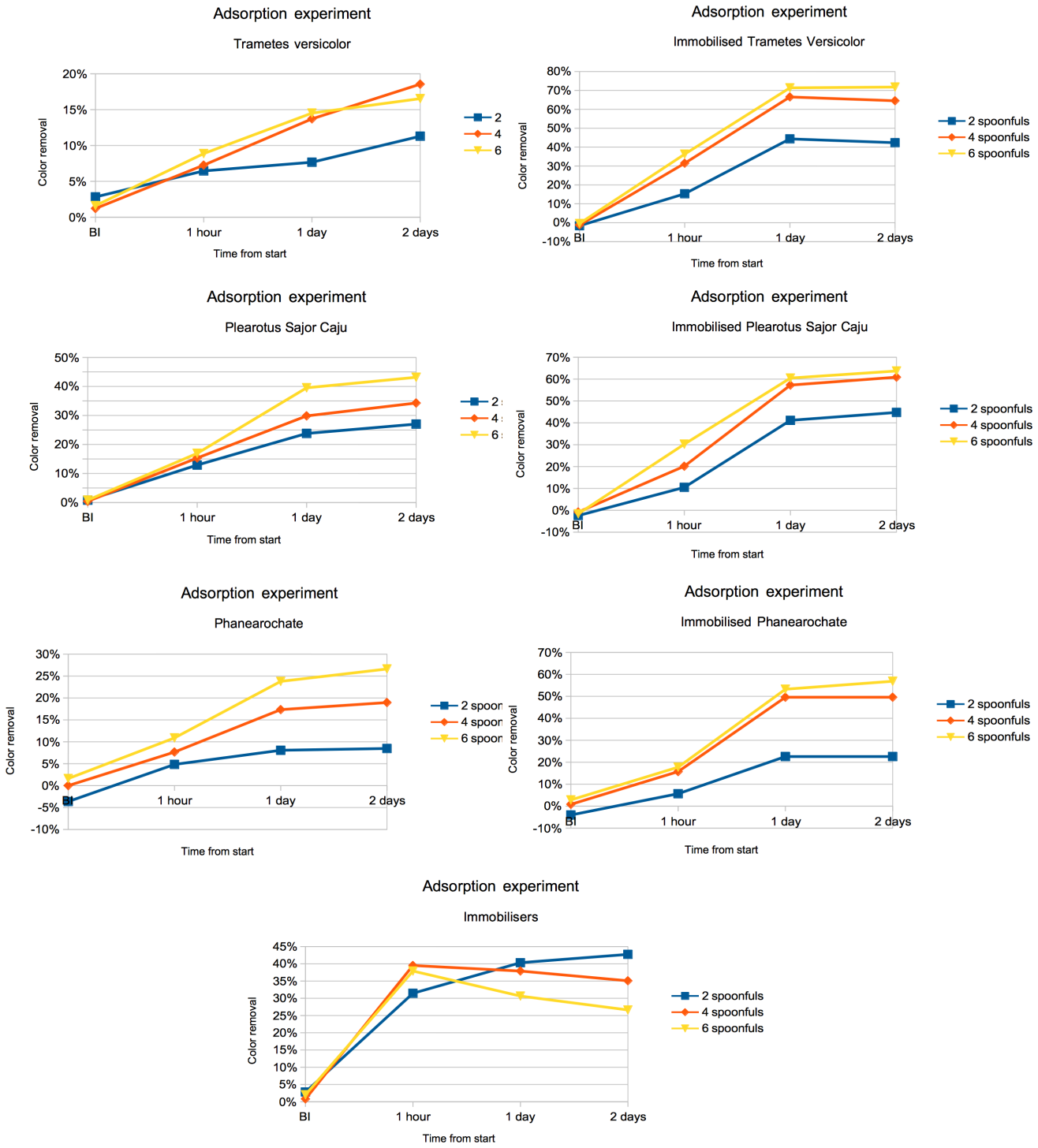


FIGURE 4-COLOUR REMOVAL ON THE ADSORPTION EXPERIMENT

Carriers alone had a very high initial adsorption (during the first hour) but the trend was that they released some of the HA during the following days. For the free fungi, the adsorption was less pronounced but steady and after 1 or 2 days the adsorption would decrease, presumably until it stopped. Immobilised fungi performed the highest colour removal, although it clearly stabilised after the 2nd day.

Adsorption appears to have a great influence in the color removal for the first 1 or 2 days but then it stops. This idea does not have a clear meaning with regard to the initial badges with 150 mL jars because mycelia would develop during the whole experiment and thus some colour removal due to adsorption can be considered to happen throughout the whole period. Actually, to have a better insight of this particular point, HPLC measurements would be necessary: particularly those that relate to the samples taken after destroying the mycelia (and thus the liberation of the HA that might be trapped inside) at the end of the badge. However, when talking about the reactors it can be expected that during a first period after the inoculation of fresh fungi, colour removal is mainly due to adsorption. Later on, enzyme activity results will as well serve as an evidence of this.

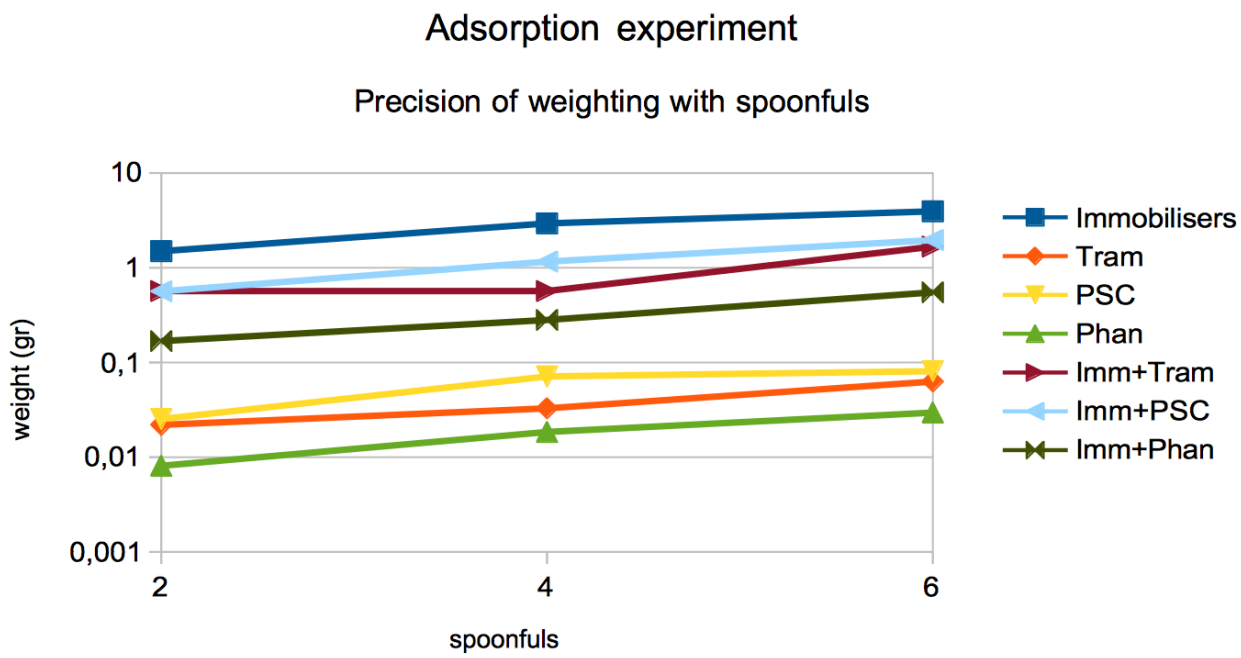


FIGURE 5-RELATION BETWEEN SPOONFULS AND ITS WEIGHT FOR THE DIFFERENT STRAINS AND IMMOBILISERS

Figure 5 illustrates the difficulties involved in making accurate inoculations with the right amounts. The specimens were measured in spoonfuls and this method is, of course, imprecise. However, there was no feasible method that could have improved the precision in this procedure.

As it can be seen, the mass of each spoonful varies widely depending on the nature of the tested subject. One could think that the volume that is taken by each spoonfuls is much more likely to be similar. Volume measurements were not executed; however, it should be also taken into

consideration that different strains showed different size of granules and therefore it was also difficult to achieve a similar volume for different strains.

As a whole, it can be said that proportions were well kept for different amounts of spoonfuls in a strain but that when comparing different strains a same number of spoonfuls does not mean either the same mass or the same volume.

3.2.4-Enzyme activity on initial badges

Whereas the colour assays show easily followable trends and events, such as the inoculation of fresh fungi on a reactor, can be traced down; results concerning the analyzed enzymes are way more vague and their interpretation presents some difficulties.

Enzyme activity was recorded at the spectrophotometer as the change of the absorption of the sample at certain wavelengths (238 nm for Manganese Peroxidase and 468 nm for Laccase). These results are then converted into enzyme activity (μM/min) applying the next formula:

$$EA = \frac{v \cdot Abs}{V \cdot d \cdot c} \cdot 10^6 \left[\frac{\mu M}{min} \right]$$

where

EA: Enzyme activity [μM/min]
v: volume of supernatant [mL]
V: total volume of the sample [mL]
d: distance that the light beam travels in the cuvette [cm]
c: extinction coefficient of the used substrate at the given wavelength [$M^{-1}cm^{-1}$]

The substrates to which the extinction coefficient refers are 2,6-DMP for the Laccase assay and Hydrogen Peroxide for the Manganese Peroxidase assay. Their extinction coefficients at the given wavelengths are, respectively, 27500 (Toribio, García-Martín et al. 2009) and 43,6 $M^{-1}cm^{-1}$. (BioBasic 2011)

The distance that the light beam travels is 1 cm.

Even when both activities have been adjusted to EA units, it has to be remarked that these results are not comparable between each other in terms of absolute numbers because the used substrate differs.

Moreover, it should be noted that the measurement of enzyme activity by fungi is a topic yet to be fully understood and research is being carried out. The chosen methods are imperfect for they present some noise that could not be removed: more precisely, it has been reported by previous investigations that DMP is oxidized in the presence of HA (Rui, Gao et al. 2006) and that peroxidase activity is inhibited by it as well (Pflug 1980). This means that laccase activity is

allegedly thought to be lower than measured because the oxidation of the substrate DMP would occur only by the presence of HA itself, and that MnP activity is lower than it would be with a same amount of enzyme in a media that lacked HA.

A logarithmic scale has been applied to all enzyme activity graphs because the great differences in magnitudes depending on the strains.

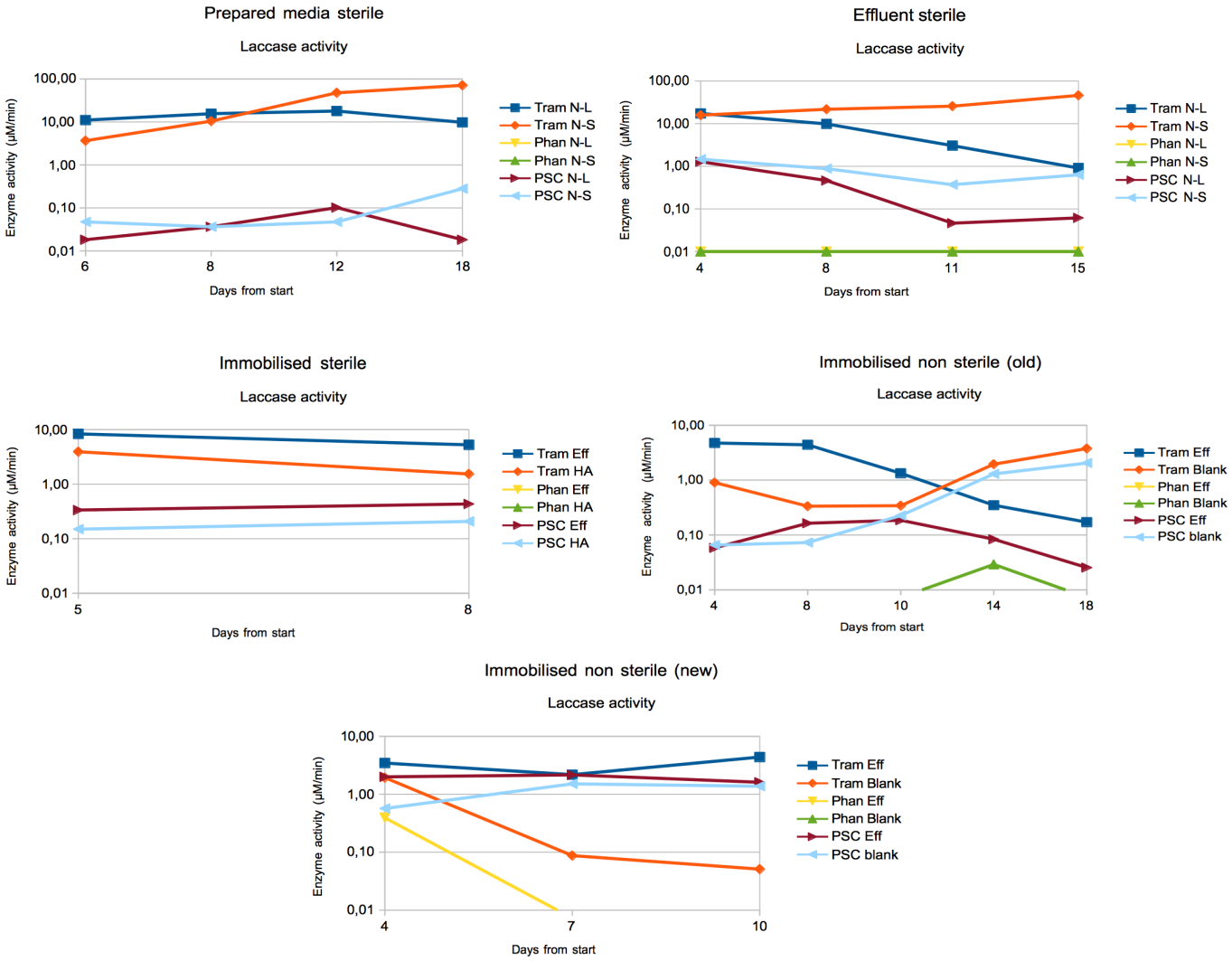


FIGURE 6-LACCASE ACTIVITY RECORDED FOR THE INITIAL BADGES

In spite of the above stated, the recorded data is useful in terms of proving the (un)existence of the said enzymes. From the Laccase activity graphs related to the initial badges, it can be concluded that Trametes Versicolor and Plaerotus Sajor Caju specimens generally do produce this enzyme but Phanaerochate does not (its values are completely residual and likely to be due to spectrophotometer precision),

Graphs recording MnP show less clear results. There is no predominant strain that shows higher results than the others, and there is no particular trend. Also, results at the spectrophotometer were difficult to track down and we prior know that MnP activity appears lower at the assays where in

presence of HA. However, with these results it can be said that the given strains do produce MnP at a certain rate.

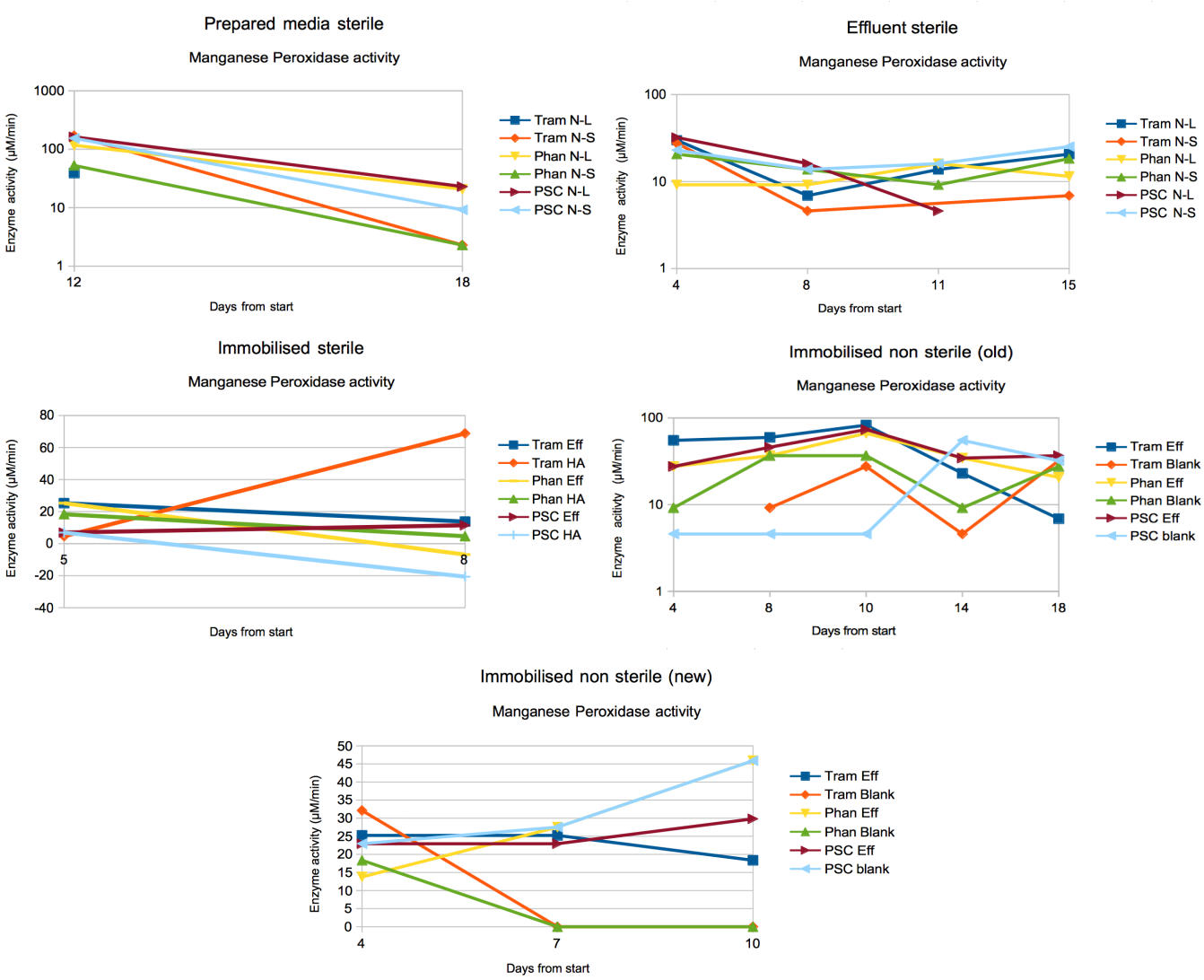


FIGURE 7-MANGANESE PEROXIDASE ACTIVITY RECORDED FOR THE INITIAL BADGES

3.2.5-Enzyme activity on reactors

Once again if we take a look at the graphs concerning the enzyme activity on continuous reactors, Laccase activity seems more obvious than MnP. But again it should not be forgotten that indeed Laccase activity is overestimated because of HA presence.

In spite of this, it was used as a proxy of the state of the fungi to decide when was the optimal time to inoculate fresh fungi: it would decrease steadily from its relatively high values recorded the first days of its inoculation and reach bottom values a day before the colour of the reactor increased substantially. Therefore, at a certain point of low Laccase activity it could be considered that it was the perfect moment to inoculate fresh fungi if the colour of the reactor outlet was to be maintained at a low colour.

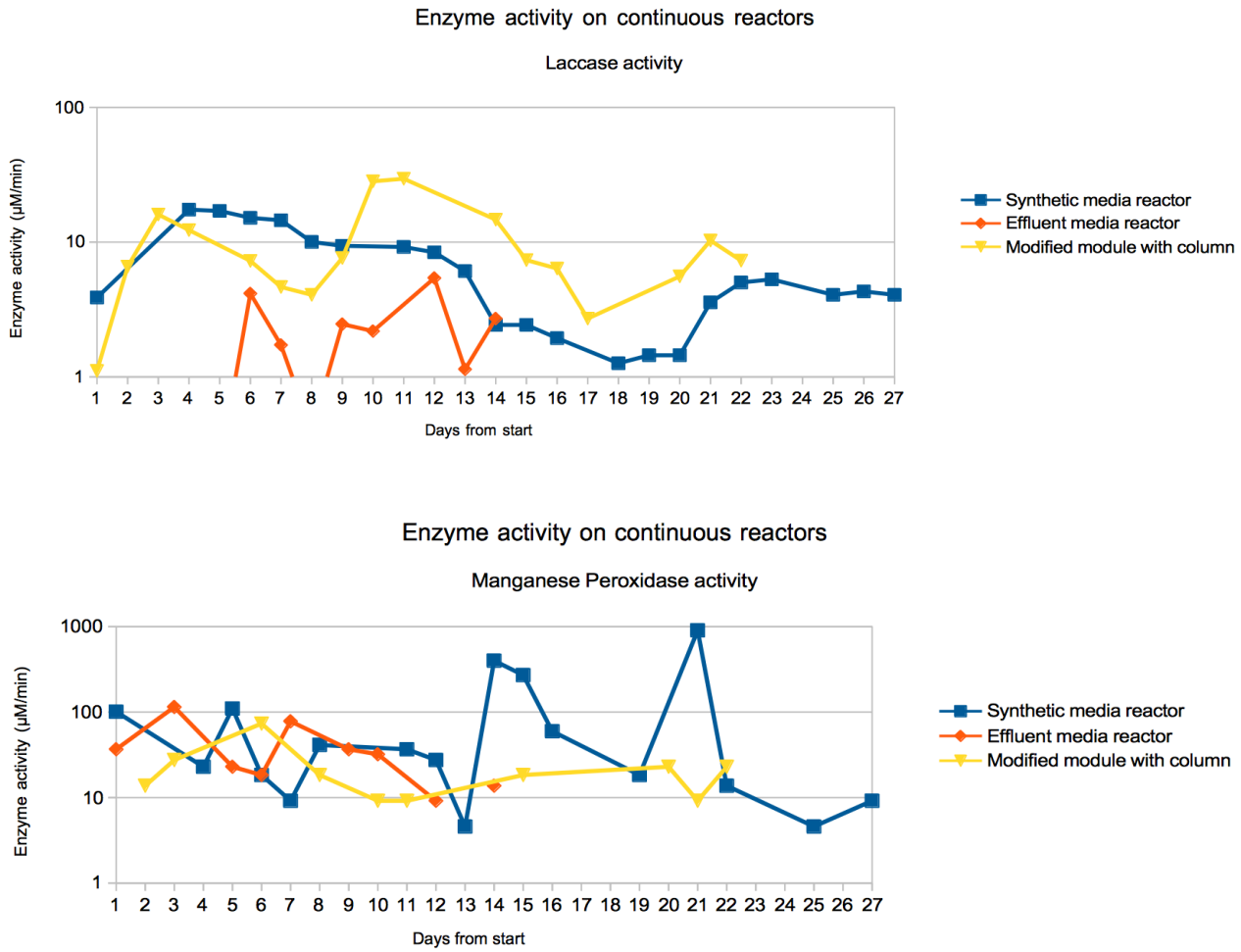


FIGURE 8-ENZYME ACTIVITY RECORDED FOR THE CONTINUOUS REACTOR BADGES

3.2.6-THE HPLC results

As it has been explained before, the HPLC results provide the most reliable evidences and constitute a final checking of the previous observations that have been done with the spectrophotometer results. However, its processing and comprehending are of extreme difficulty and require of very advanced knowledge on the matter and therefore are regarded as out of the scope of this thesis.

In spite of the above stated, an example of the results is provided to give an insight of what is pursued with this experiment.

Figure 9 corresponds to the HPLC results for the HA fraction of a N-L media containing HA before the inoculation of fungi from the badge “Prepared media sterile”.

Through the principle of liquid chromatography, the HA molecules present in the sample will pass by the detector of absorbance at a certain time depending on their weight. HA is a fraction of the Humic substances that are characterised by some specific chemical properties, however they still

constitute a group of varied molecules for which, considering its range of molecular mass (Grinhut, Hadar et al. 2007) it is expected an approximate time of 7 minutes at the HPLC to get to the detector. The rate of arrival follows a normal distribution of probability. Also, because of the range of weights, different peaks are observed corresponding each one to a different HA type of molecule.

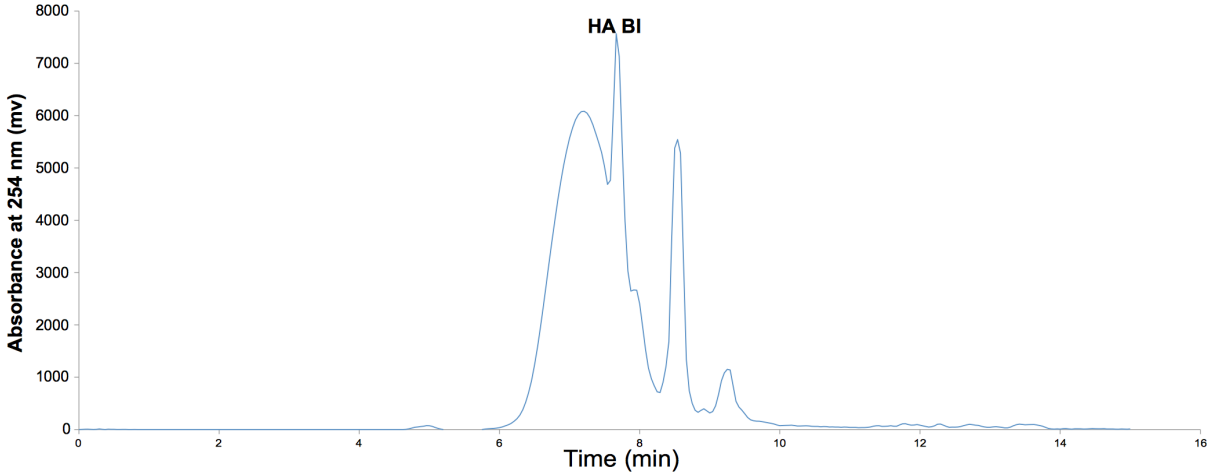


FIGURE 9-HA RECORDING AT THE HPLC

After the inoculation of the fungi, both adsorption and biodegradation of HA are expected to occur. Therefore, a succeeding sample of the same specimen would present a graph on which the area under the line is less because of the adsorption but also some other peaks would be expected to appear before in time because biodegrade molecules have a lower weight.

The separation of HA and FA as it was explained in the methodology is of vital importance since FA have a similar weight and it would have been not possible to tell the difference in the HPLC. However, the procedure for doing so was quite depending on human accuracy and usually some material (both HA and FA) got lost from the samples. This was an important added factor to the interpretation of the results since sometimes samples were not showing reliable results but even that was hard to tell.

4-Overview

With regard to the performance of the different strains on the remediation of HA:

•There is clear evidence that there is a colour reduction and this is correlated to a reduction of the concentration of dissolved HA. This HA might have been degraded or adsorbed.

•Trametes Versicolor is the strain that most consistently showed this remediation and its effectiveness reached 70-90% of colour removal.

•The 3 strains showed significant colour removal. Production of laccase enzyme has been tracked for both Trametes Versicolor and Plaerotus Sajor Caju. As for Manganese Peroxidase, there are weaker trails of its production for all of the strains. They all showed significant absorption ratios.

Focusing on the strain that played the main roll in this investigation, i.e. Trametes Versicolor:

•Removal of colour by this strain is generally good, up to 90%. However, it depends on the specific conditions that are applied and, for continuous reactors that could be used as a industrial process, 75% was achieved.

•There are clear evidences that extracellular enzymes Laccase and MnP are produced by this strain, and therefore, they arguably play a roll in color removal.

•Adsorption by the mycelia (and the carriers, when used) is another mechanism by which HA are removed from water. Its importance is particularly high during the first 24 hours after inoculation.

•The growing of these fungi produces as well some colour in the media. This means that the removal of HA is slightly higher than the apparent by just taking into consideration the colour of the sample.

Concerning the role of Laccase and MnP enzymes on the remediation of HA by WRF:

•It has been reported in previous studies that Laccase and MnP enzymes act as catalyzers in the bio-degradation of HA.

•As said before, there is enough evidence to ensure that there is Laccase production in 2 of the selected strains and weaker trails of MnP for all of the strains as well. However, given that HA media significantly reduces the apparent activity shown by the spectrophotometer for MnP, it is accepted that there is evidence enough of the production of this enzyme as well.

•Taking into consideration the 2 previous statements, it follows that these extracellular enzymes can be taken into consideration when analysing the processes that lead to the reduction of dissolved HA in water by the given strains. However, no clear correlation has been found and this might be because of these processes being far more complex than prior expected.

A lot has been learnt about the mechanics involved in the remediation of HA in continuous reactors:

•The importance of a proper aeration system that keeps the growing of the fungi

•Keeping the bacterial concentration at low rates is also determinant and this can be achieved by removal of biomass but also by optimizing the shape of the reactor in a way that the stagnant areas are prevented.

•Adding of fresh fungi has to be done periodically. Although its frequency is presumably depending on each individual reactor and should be tested case by case.

4.1-Conclusion

Going back to the main aim of this research, which was to achieve an effective method to remediate HA from industrial wastewater, the results are very positive: the selected strain shows high ratios of removal when applied to a continuous reactor. Furthermore, there has been a process of optimization and refinement of this method that has shown a clear improvement from the first reactor assayed to the last one.

The only missing step on this direction that is not feasible for obvious reasons would be a final scaling up of the method to industrial dimensions.

As for the mechanisms involved in the removal of HA by fungi: they are presumably way more complex than prior expected. Extensive further research is required to learn the specifics about them. Furthermore, other mechanisms than those initially proposed are presumably to be taken into consideration, such as the degradation of HA by in-cellular enzymes after they have been adsorbed by the mycelia, or by other extra-cellular enzymes than those considered in this research.

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