# Micropollutant degradation by *Schizophyllum commune*

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# Preface

About two years ago, I decided to study my master's in "Bio-Inspired Innovation" at Utrecht University. This decision did not only mean to move from my hometown to lovely Utrecht but also changing my focus from medical biology to the kingdom fungi. I clearly remember being excited but also nervous about starting the longest experimental research project in my study life. During these months, which have been challenging with the pandemic, I did not only learn a lot about my model organism *Schizophyllum commune* but also about myself and ambitions for my future career.

I want to thank Prof. Han Wösten for allowing me to work in his great team and always encouraging his students with new project ideas. Also, I want to thank my supervisor during the whole project, Brigit van Brenk, for her support and open door for my questions. Lastly, I am grateful to the microbiology stuff for always being helpful with problems in the laboratory and creating a welcoming working environment. It has been a pleasure being part of the fungal microbiology team.

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# I. Layman's Summary

Accumulation of micropollutants such as pharmaceuticals, herbicides, and dyes in surface water can cause long-term effects on humans' health and ecosystems. This underlines the urgency to remove these pollutants. However, the current water purification systems are not designed to remove micropollutants, calling for a new methodology to remove these molecules from wastewater. This research aimed to generate a fungal strain of here *Schizophyllum commune* that is capable of efficiently degrading organic micropollutants. This fungus is known to degrade lignin, which shows structural similarities to MPs, and therefore a good candidate fungus for degradation of micropollutants in wastewater. Sexual and asexual S. commune strains were used to assess degradation. The tested strains indicated great differences in their degradation capacities, which indicates diverse degrading enzyme activities within the strains. Next, I tried to set up a method to improve a strain's degradation capacity. This set-up, called induced evolution, stresses the fungi with a MP-like dye, whose concentration increases with every fungi's surviving generation. I performed the induced evolution with spores, homogenate and protoplasts finishing three, two and one generation, respectively. First results indicate that the evolution improved the lines' toxicity resistance but not their degradation ability compared to the control. However, the homogenate line suggests improved a trend toward degradation ability. In summary, the results emphasise the genetic variation of *S. commune* and its potential improvement for MP degradation.

# II. Abstract

The occurrence of micropollutants (MP) in surface-, ground- and drinking water has become a rising problem within the last decades. A promising water purification method, called reverse osmosis, purifies 80% of the water but also releases effluent wastewater, with high MP content. The chemical structure of organic micropollutants resembles the structure of lignin, a compound known to be degraded by some microorganisms. The model organism of this research, *S. commune*, is known to produces laccases, a family of multicopper oxidases that is part of the lignin modifying enzymes. Taking *S. commune's* high genetic variation into account, the first research aimed to assess the heterogeneity of MP degradation by 16 of its isolates. Furthermore, it was tested how the crossing of strong monokaryotic degraders influenced their degradation ability. Both lines were carried out by a new quantitative method of assessing the hue and saturation levels over three weeks. Four out of 16 strains showed a successful degradation of at least one dye. The results did not indicate a correlation between fruiting body formation and degradation and therefore suggests a degrading activity during the first stages of dikaryotic growth before the formation of fruiting bodies. The combination of the strong monokaryotic degraders did not improve its degradation capacity. The second research aimed to set up a laboratory induced evolution methodology to improve the degradation ability of Crystal Violet by monokaryotic WT strain *4.8a.* Spores, protoplasts, and homogenate were used

for the evolution experiments. They showed an increasing resistance against the toxic with further generation but did not significantly improve their degradation ability compared to the control. The spores showed a significantly decreased in its CV degradation ability within three generations. The protoplasts generated one generation, which is suggested to be mutated. The homogenate line suggests a trend toward improvement. Results showed a significant improvement in the degradation ability of its second generation compared to its first generation. Adaptations of the methodology are suggested to improve this promising set up.

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# 1. Introduction

Over the last decades, our society is facing the increasing problem of polluted water by micropollutants (MPs), which appear in the surface-, ground- and drinking water. Water can be contaminated with various organic and inorganic compounds summed up as MPs generated by industry, municipalities, agriculture, and accidental spills (Schwarzenbach *et al.*, 2006). These MPs, such as pharmaceutically active compounds (PACs), personal care products (PPCPs), endocrine disruptors, pesticides, and industrial chemicals, accumulate in the water. Due to their complex structure, which is in some toxic dyes resembled by the presence of the characteristic benzene-ring, MPs are hard to degrade. Accumulations of these contaminants can cause unknown long-term effects on human health and aquatic life systems (Sharma *et al.*, 2016).
One commonly used approach to purify and desalinate industrial wastewater is the reverse osmosis method. This method achieves up to 80% recycled water and 20% effluent wastewater. The latter has five-fold higher concentration of MPs and is transferred to the wastewater treatment plant (WWTP). However, WWTP is designed only to remove macro pollutants such as organic matter, nutrients and suspended solids (Evgenidou *et al*. 2015; Kaiser *et al*. 2014), so MPs are not removed efficiently. It can be concluded that there is an urgent need for new, useful technology.

Species belonging to the white-rot fungi (WRF) show a high bioremediation potential due to their broad specificity to attack substrates by intracellular or extracellular enzymes such as extracellular ligninolytic enzymes (LMEs) that include laccase (EC 1.10. 3.2), manganese peroxidase (MnPs, EC 1.11.1.13), lignin peroxidases (LiPs, EC:1.11.1), and versatile peroxidase (VPs, EC 1.11.1.16) (Janusz *et al*., 2013; Rivera-Hoyos *et al*., 2015). Fungal laccases catalyse the oxidation of various aromatic substrates (Giardina *et al*., 2010) while LiPs and MnPs oxidise, with high oxidation-reduction potentials, non-phenolic aromatic compounds (Glenn *et al*., 1983). VPs combine the substrate specificity characteristics of both described enzymes (Pérez-Boada *et al*., 2005). With the help of these LMEs, WRF fungi can degrade not only lignin but some MPs as well (Tuomela & Hatakka, 2011). That insight allows a potential biotechnological application to use WRF fungi for MP degradation actively.

One known representative of WRF basidiomycetes is the worldwide distributed *Schizophyllum commune*. It is a fast-growing fungus with a sequenced genome and often used as the model organism for mushroom forming fungi (Ohm *et al*., 2010). Research revealed that *S. commune* wild-type *4.8* encode laccase genes, activated during mushroom formation, but not the other LMEs (Ohm *et al*., 2010). However, genetic investigations revealed an extensive genetic variation within *S. commune* isolates (James *et al*., 1999). This leads to the hypothesis that these isolates may vary in LME compositions, thereby having different abilities to degrade MPs.

This research aimed to assess heterogeneity of MP degradation in *S. commune* isolates and to set up a method to generate strains with improved MP degradation abilities.
MP degradation ability of 14 pre-screened dikaryotic *S. commune* isolates and their mono- and dikaryotic WT were investigated with a new fast quantitative method by observing hue and

saturation differentiation of dyes homologous to MP to assess their degradation ability. Isolate *209, 223, 228, 4.8a* and *4.8ab* successfully degraded at least one dye and did not show a clear correlation between fruiting body formation and degradation ability. Strong monokaryotic degraders, which were isolated from spores of *228*, were crossed to observe the degradation behaviour of the resulting dikaryons. Eventually, the combinations of the effective monokaryons did not lead a better dikaryotic degrader. This might be due to the potential interfering of each one's degradation pathways caused by the genetic variation in between *S. commune* strains.

Further, the monokaryotic *S. commune* wild type *4.8a* was selected to investigate the degradation of Crystal Violet (CV). This research aimed to develop an adaptive induced evolution method, which improves the speed of its degradation capacity for CV. The strain was explicitly exposed to CV by increasing the concentration of the toxin with each modified generation of the fungus. Tests were performed with spores, homogenate and protoplasts achieving three, two and one generation, respectively. The CV degradation ability was measured via hue and saturation assessment, and two kind of CV assays. The modified lines did show an improved resistance against the dye by surviving higher concentrations of the toxin with further generations, but did not evolve a significantly better degradation ability than the control.

# 2. Methods and Materials

## 2.1 Strains and Cultivation

For the decolourisation and desaturation assessment , monokaryotic and dikaryotic *S. commune* isolates (Table 1) were grown for 21 days on 10 mL minimal medium (MM) with 1.5% agar (Dons *et al*., 1979) at 25°C in the dark. MM contained either or not 5 µg $mL^{-1}$ Crystal Violet (CV), 5 µg $mL^{-1}$ Malachite Green (MG), 100 µg $mL^{-1}$ Remazol Brilliant Blue R (RBBR), 10 µg $mL^{-1}$ Rose Bengal (RB) or 30 µg $mL^{-1}$ Orange G (OG). Strain *4.8a* used as the monokaryotic control and *4.8ab*, used as the dikaryotic control, were like the other isolates separately grown on all dyes in technical triplicate.

*Table 1: S. commune strains, pre-screened by Nina and Toon were used in the first research line to assess their degradation ability. Marked isolate (228) was used for further experiment in its monokaryotic form.*

| Dikaryotic Isolates | Controls |
|---|---|
| 184 | 4.8a |
| 187 | (monokaryotic) |
| 193 | |
| 209 | 4.8ab |
| 211 | (dikaryotic) |
| 221 | |
| 223 | |
| 228 * | |
| 234 | |
| 238 | |
| 264 | |
| 277 | |
| 278 | |
| 298 | |

Monokaryons were obtained by growing fruiting bodies (three days with parafilm on MM at 30°C in the dark, followed by four days at 25°C in light) and harvesting their spores in 1 mL MiliQ (MQ) water. Spore solution was counted with the Bürker-Türk (Brand GmbH+CoKG) and 30,000 spores $mL^{-1}$ were inoculated on solid MM with 0.5% activated charcoal. Single hyphae were selected using a binocular and inoculated on solid MM in 96-well plates to grow them as separate monokaryons on MM for three days at 30°C in the dark.

For the laboratory-induced evolution experiment, spores and

protoplasts of *4.8a* were incubated individually incubated in regeneration medium (1M $MgSO_4.7\ H_2O$, 2x YT, 1x phosphate buffer) for three days at 30°C in the dark and transferred on solid MM containing defined concentration of CV (Table 2) to be incubated at 30°C in the dark.
Stocks were routinely renewed and stored at 4°C.

### 2.2 Decolourisation and desaturation assessment

Hue and saturation of *S. commune* isolates were scanned weekly on the 7$^{th}$ incubation day for three weeks (Epson Perfection V370 Photo, 24-bit colour, 1200 dpi). The scanning was processed by the ImageJ-win64 software and the Excel 365 program to study the growth and degradation ability by applying the data to the Hue Saturation Brightness (HSB) model (Levkowitz & Herman, 1993; Brenk B. 2020, unpublished work). In the HSB model, all colours are defined by a degree between 0 and 360°. One assumption is that a clear visually detectable colour changes with 30°. Saturation level range between 0 % and 100%, counting from desaturation (loss of the intensity of the colour by turning into white/greyish) to saturation (intensifying the colour). To visualise the data decolourisation and desaturation are represented by their development from the first until the last day of measurement. A successful decolourisation is defined by a difference of at least 30° between the two measurements. A successful desaturation is defined by a decrease of at least -10% between the two points of measurements. Degradation of a dye is assumed if the data of one strain shows decolourisation and desaturation for one dye. Therefore, a successful degradation is defined by a decolourisation of at least 30° and desaturation of at least -10%.

### 2.3 Protoplast isolation of *S. commune* WT 4.8a

An adjusted method (van Peer *et al.* 2009) was used to collect protoplast. Monokaryotic strain *4.8a* was grown in 25 mL MM in 50-mL Greiner tube as a standing culture for three days at 30°C in the dark. The culture was homogenised for 30 s in a blender (Waring), diluted twofold in MM, and was shaken at 200 rpm in 30°C overnight. This was repeated the next day. Mycelium was pelleted at 4700 rpm for 15 min and 40 mL $MgSO_4.7\ H_2O$ was added. Mycelium was again pelleted at 4700 rpm for 15 min, and 15 mL lysing enzyme mixture (7.5 mL 2x yeast tryptone – extract (YT), 300 mg powdered lysing enzymes (Visser P., Lugones LG. 2020, unpublished work), 0.9 mL 0.5 M Malic acid, 2.5 mL MQ-water and 3.96 g 1 M $MgSO_4 \cdot 7H_2O$) was added per 100 mL culture. The culture was gently shaken for overnight at 30°C (Clark *et al*. 2004). Protoplast amount was checked with the Bürker-Türk for a minimum of $8x10^7$ protoplast per mL. 15 mL MQ- water was added and shaken at 2000 g for 10 min to prevent formation of two separate phases. After filtering the sample, using a sterilized Miracloth, 15 mL of 1 M sorbitol was added, and the solution was gently shaken for 10 min. Protoplasts were repeatedly pelleted by centrifuging at 2000 g for 10 min, resuspended in 1 mL 1M sorbitol and filled up to 40 mL with 1M sorbitol. Protoplast amount was checked with the Bürker-Türk for at least $10^{12}$ protoplast per mL (Gehrman *et al.*, 2010), pelleted by centrifuging at 2000 g for 10 min and finally resuspended 1 M sorbitol for further use.

## 2. 4 Set up for laboratory adapted induced evolution experiment

Colonies of *4.8a* parent generation (p) were selected to generate protoplasts and homogenate (homogenized for 45 seconds). Spores derived from *4.8a* combination with *4.8b*. All types of sample were grown in liquid regeneration medium ($MgSO_4 \cdot 7H_2O$, YT and phosphate buffer) containing different strong concentration of CV (Table 2) at 30°C in the dark for at least 4 days. Considering the mutation rate of 1923 x $10^{-8}$ per haploid genome / base /generation and 300 SNPs per colony (Gehrman *et al.* 2017), it is recommended to work with at least $10^{12}$ cells to possibly mutate 5 stop codons. Due to limited research time, cultures with $10^7$ protoplast and $10^9$ spores per mL were used to perform the experiment. Homogenate cultures were concentrated as 100 mg homogenate per mL liquid MM. Colonies, which survived the highest concentration of CV (f1), were pelleted (20 min, 2000 g) and transferred on solid MM to grow as stock to prepare harvesting for the next generation. This cycle of growing and harvesting colonies from MM containing CV was repeated, except that each further generation was exposed to a higher CV concentration. Each generation was carried out in technical triplicate. *4.8a*, not exposed to any CV shock, was used as the control. Three measurements were conducted, to compare the degradation ability of CV within the different types of samples and generations. Colonies from every generation were transferred on solid MM containing 5 µg $mL^{-1}$ CV to assess the decolourisation and desaturation level (same methodology as 2.2). Additionally, two types of CV assay were conducted.

*Table 2: CV concentrations in regeneration medium per generation [µg $mL^{-1}$]*

| **CV concentrations *p* was exposed in** | **CV concentrations *f1* was exposed in** |
|---|---|
| 0 | 15 |
| 5 | 20 |
| 10 | 25 |
| 15 | 30 |
| 20 | 35 |
| 25 | 40 |
| 30 | 45 |
| 35 | 50 |

## 2.5 Crystal Violet assays

Two types of CV assays were set up and eventually compared. For the 30 minutes real times kinetics, 1 mL a 4-days old *4.8a* culture, grown at 30°C in dark in liquid MM, was centrifuged (2 min, 20000 g). 200 µl of the supernatant was transferred to a 1 mL cuvette and been filled up with 800 µl distilled water containing a CV concentration of 5 µg $mL^{-1}$. Immediately after adding the solution to the culture was the CV concentration spectrophotometrically (Du 800 spectrometers, Beckman Coulter) measured over exposure time of 30 min (592nm). For the second approach, the CV concentration was measured over three weeks. After growing the *4.8a* culture for one week at 30°C in the dark in liquid MM, CV was added to adjust the concentration of 5µg $mL^{-1}$ CV in the medium. 2 mL of the culture was centrifuged (2 min, 20000 g) and pipetted in a 1 mL cuvette. CV absorbance (592 nm) was measured once weekly from the first day on over three weeks. Data was evaluated via Excel using the calibration line of $y = 0.0907x+0.0049$ to define the CV concentration. Both experiments were carried out in diplo.

### 2. 6 Statistical analysis

Data was analysed for their significance by multiple comparison via One-way ANOVA and Tukey's honestly significant difference (HSD) post hoc test in IBM SPSS statistics (Version 26). In all cases, p- value of 0.05 was used as the "cut-off".

# 3. Results

## 3.1 Investigation in the genetic variation of *S. commune*

The high genetic variation of *S. commune* may result different degradation capacities of various dyes by its isolates. These differences were investigated by assessing their decolourisation and desaturation of five dyes over three weeks. Additionally, it is studied whether there might be a correlation between fruiting body formation and degradation. Further, studies were carried out with the strong RBBR degrader, strain *228,* to compare the degradation ability of its mono-, dikaryon and inter-strain crossings on RBBR.

### 3. 1. 1. Degradation ability and developmental stages of *S. commune* isolates on dyed plates

The decolourisation and saturation assessment delivered results about the degradation ability of 14 dikaryotic *S. commune* isolates, and the mono- (*4.8a*) and dikaryotic *(4.8ab*) model (Table 3). Decolourisation and degradation level for each dye specifically can be found in the appendix (Figures 1 -10).

CV was successfully degraded by monokaryon *223* and *4.8ab*, and MG was degraded by *209, 223* and *4.8ab*. RBBR was successfully degraded by strains *223, 228* and *4.8a*, whilst RB was only degraded by 223. In summary, five out of 16 tested strains were able to degrade at least one of five dyes. The most successful degrader is strain *223* by degrading CV, MG, RB and RBBR. OG was not degraded by any of the strains.

The isolates showed different developmental stages after three weeks, which are distinguished in mycelial growth, primordia, and fruiting body stages (Table 3). *223*, and *4.8a* did only develop early primordia stages on CV and MG. Likewise showed *209* and *228* a development of primordia on MG and RBBR, respectively. In summary, six out of nine degraders showed early primordial stages but none of them grew fruiting bodies. Therefore, it can be assumed that in this case there is no clear correlation between fruiting body formation and degradation. An additional observation is the occurrence of exudates for three out of 14 isolates (strains: *187, 264* and *283*) for different dyes (Table 3, Figure 11 in the Appendix). None of these strains degraded the dye on which they secreted the substrate and all showed different stages of fruiting body formation. Isolates *187* and *264* developed primordial stages, whereas strain *283* developed fruiting bodies. The exudates took different colours for the dyes; the secrete seemed yellow on OG and RB, clear on RBBR and more brownish on CV and MG.

*Table 3: Degradation, decolourisation, and fruiting body formation ability of 14 dikaryotic S. commune isolates, 4.8a and 4.8ab. Categorisation in successful degradation (+), poor degradation (-), and primordial (marked in blue) and fruiting body formation stages (marked in orange). Strains showed exudates marked with * (n = 3).*

| Isolate | CV | MG | OG | RB | RBBR |
|---|---|---|---|---|---|
| *184* | - | - | - | - | - |
| *187* | - * | - | - | - | - * |
| *193* | - | - | - | - | - |
| *209* | - | + | - | - | - |
| *211* | - | - | - | - | - |
| *221* | - | - | - | - | - |
| *223* | + | + | - | + | + |
| *228* | - | - | - | - | + |
| *234* | - | - | - | - | - |
| *264* | - | - | - * | - * | - * |
| *277* | - | - | - | - | - |
| *278* | - | - | - | - | - |
| *283* | - * | - * | - * | - * | - * |
| *298* | - | - | - | - | - |
| *4.8a* | - | - | - | - | + |
| *4.8ab* | + | + | - | - | - |

### 3. 1. 2 Comparison of strong mono- and dikaryotic RBBR degrader

Based on the result, that dikaryon 228 is a strong degrader of RBBR, this follow-up experiment was aimed to find successful monokaryotic degraders of its siblings, and to perform crossings for an even stronger dikaryotic RBBR degrader eventually.

Firstly, 15 monokaryons were isolates from spores and grown over three weeks on RBBR containing MM. The colour difference within three weeks presents the decolourisation (Figure 1). The three strongest decolourising strains are monokaryons 9, 13 and 8 by changing the colour of about 111°, 10° and 5°, respectively. The saturation levels are distinguished in desaturation and saturation (Figure 2). The former refers to a decrease of saturation over time so that the colour blue turns greyish/whitish, translated to negative values. The latter is an increase in the colour's intensity, referring to the positive values. Monokaryons 13, 8 and 7 showed the most substantial desaturation level (-25%, -23% and -20% respectively), whereas only monokaryon 9 demonstrated an additional saturation of 21% over time.

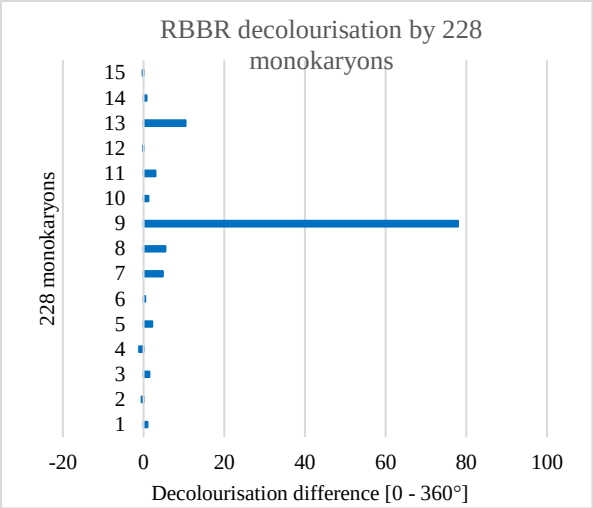


*Figure 1: Decolourisation of RBBR over three weeks by 228 monokaryons. Data presents average of 15 monokaryons (n=3).*

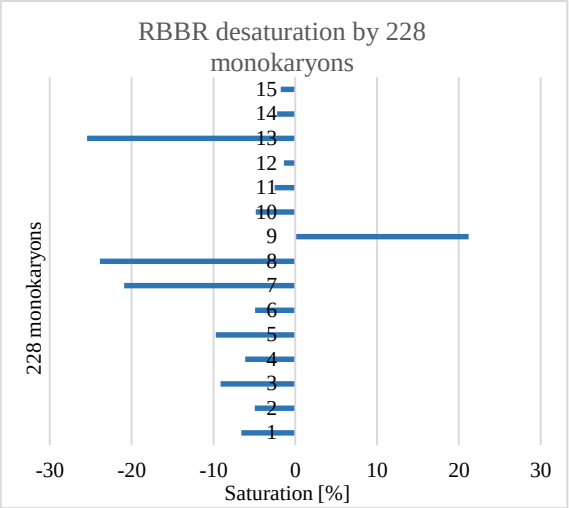


*Figure 2: Desaturation of RBBR over three weeks by 228 monokaryons. Data presents average of 15 monokaryons (n=3).*

Remarkably, monokaryon 9 shows a different development compared to the other monokaryons by colorising the blue dye into brown instead of grey like the other monokaryons (Figure 12 in the Appendix) and has, therefore, a significantly different (p = 0.05) decolourisation behaviour compared to the other strains during the weeks. In conclusion, monokaryon 8 and 13 turned out to be the strongest degrader and therefore, these two monokaryons were planned to be crossed. As their matching types were not compatible, crossings with strong monokaryotic RBBR degrader *4.8a* (see Table 3) were performed and compatible combinations of *8x4.8a* and *13x4.8a* achieved. The degradation ability of RBBR by these combinations was put in comparison to the dikaryotic *228* parent and the three monokaryons separately (Figure 3 and 4). Parent dikaryon *228* degraded RBBR most successfully by decolourizing it about 190° and desaturating it about -40%. Combination *8x4.8a* degraded the poorest (decolourisation: 1°, desaturation: - 4%), closely followed by the combination *13x4.8a* (decolourisation: 3°, desaturation: - 9%). Monokaryon 8, 13 and *4.8a* separately decolourized in the range between 5 to 50° and desaturated in the range between 12 to 25%. It can be concluded that the degradation ability of the monokaryons decreased in the combination with each other. The combinations did not improve the monokaryon's degradation capacity.

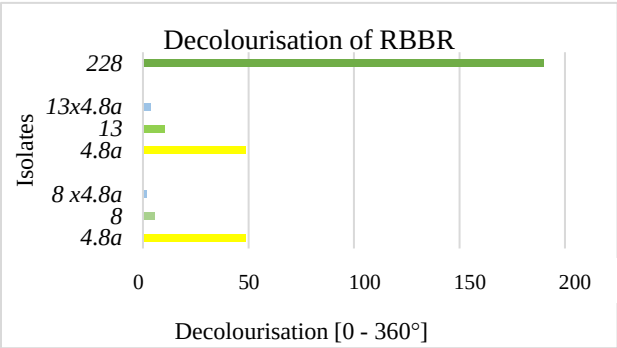


*Figure 3: Decolourisation of RBBR over three weeks by 228 (green), 8x4.8a (blue), 13x4.8a (blue), 8 (green), 13(green) and 4.8a (yellow). Data presents average of each strain (n=3).*

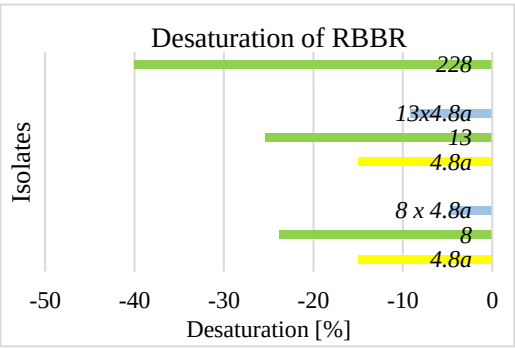


*Figure 4: Desaturation of RBBR over three weeks by 228 (green), 8x4.8a (blue), 13x4.8a (blue), 8 (green), 13(green) and 4.8a (yellow). Data presents average of each strain (n=3).*

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## 3. 2. Laboratory adaptive induced evolution set-up for *S. commune*

The second research aims to set up a method for an induced evolution experiment for S. commune, exemplary using strain *4.8a*.

### 3.2.1 Set-up and samples

*4.8a* protoplasts, homogenate, and spores (*4.8ab*) were shocked in high CV concentrations, which were increased with each generation (see Table 2). In first approach, the samples were shocked in SCMM and regeneration medium containing CV. Cultures, incubated in SCMM resulted in a higher mortality than the ones in regeneration medium. Therefore, regeneration medium was used as the basis medium for shocking cultures with different CV concentrations. The culture, which survived the highest CV concentration, was used for the further experiments to analyse their CV degradation capacity. With each generation the cultures could survive a higher concentration of CV (Table 4). Spores generated three, homogenate two and protoplast one generation. Remarkably, the second generation of homogenate tolerated nearly twice of the CV concentration as its first generation.

*Table 4: Max CV concentration protoplasts, homogenate and spores survived as shock (n=3).*

| Max CV concentration | Protoplast | Homogenate | Spores |
|---|---|---|---|
| First shock (f1) | 15 µg $mL^{-1}$ | 25 µg $mL^{-1}$ | 10 µg $mL^{-1}$ |
| Second shock (f2) | | 45 µg $mL^{-1}$ | 20 µg $mL^{-1}$ |
| Third shock (f3) | | | 25 µg $mL^{-1}$ |

### 3.2.2 CV assays

Two kind of CV assays, a 30-minute real time kinetics and a weekly assessment over 21 days of each sample, were conducted to spectrophotometrically to measure the CV absorbance over different time frames. The decrease of CV absorbance gives information about the degradation of the toxin in the culture. The first methodology demonstrates that the speed of the CV degradation measured in mol $L^{-1}$ $min^{-1}$ is close to zero. Due to the fact that there is no correlation between CV adsorption and the amount of exposure time to CV (Table 1 in the Appendix), it can be concluded that there is no visible degradation during 30 min. The CV assay over three weeks (Figure 5) indicates degradation within this time frame. Over three weeks, three spores' generations, two homogenate generations, the first protoplast generation and the negative control decreased the CV concentration from around 5 µg $mL^{-1}$ to less than 1 µg $mL^{-1}$. All samples degraded with around 3 µg $mL^{-1}$ most amount of CV during the first week. A blank control without any fungi, shows a decrease of CV in between the first, second and third week of 0.68 µg $mL^{-1}$, 0.4 µg $mL^{-1}$ and 0.7 µg $mL^{-1}$, respectively. This natural decay is possibly caused by photodegradation (Caine *et al.*, 2002). Triarylmethanes like CV have been reported to have a low photostability and show a fading of intensity over time when they are exposed to light. Although all samples have been incubated in the dark at 30°C, they automatically have been exposed to light during the preparation for the measurements. The natural decay is not included in the diagram.

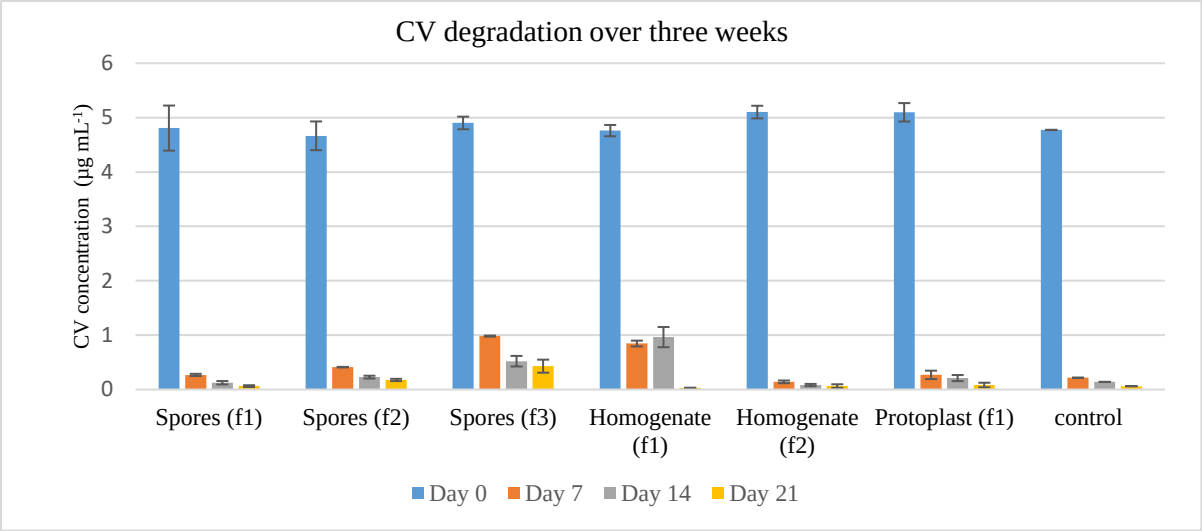


*Figure 5: Average development of CV degradation over three weeks by spores (f1, f2, f3), homogenate (f1, f2), protoplasts (f1) and control (n=2). Calibration line: y = 0.0907x+0.0049. R² = 0.9895.*

During the first week (Figure 5), the control and homogenate f2 show the lowest CV concentration with 0.21 µg $mL^{-1}$ and 0.14 µg $mL^{-1}$, respectively, and are together significantly different from the other samples (p = 0.05). In week 2, homogenate f1 (0.96 µg $mL^{-1}$) and spores f3 (0.52 µg $mL^{-1}$) decreased the poorest amount of CV and are significantly different from the control (0.13 µg $mL^{-1}$). For the last week, spores f3 contained a significantly higher CV concentration (0.09 µg $mL^{-1}$) than the control (0.06 µg $mL^{-1}$). In the homogenate line, the f1 and f2 generation indicate a significantly different CV degradation during the first two weeks. The second generation lowers the CV degradation significantly faster than the first generation. Within the spore line shows the third generation a significantly lower CV degradation over the first and last week than its previous generations.

### 3.2.3 Decolourisation and desaturation assessment

Decolourisation and desaturation assessment were carried out for all the samples (Table 4) over three weeks and provide comparable data for the CV assay. Due to an infection, spores f3 and homogenate f2 deliver data until the second week.

Figure 6 indicates the decolourisation development per week. After the first week, spores f1(109°) colorized CV significantly less (p=0.05) than the control (260°) and the other sample. In the following week following week, all samples range in a similar colorization of CV. In the last week, homogenate f1 (69°), protoplast (50°) and spores f2 (109°) colourized the dye significantly different than the control (29°). The spores showed significantly different decolourisation levels within their generations. During the first week shows the first generation of the spores a significantly different colour than its second and third generation. The second generation of the homogenate shows a significantly different colour than its first generation after one week. The development of the saturation levels (Figure7) presents for the first week a significantly different (p=0.05) saturation by the first generation of homogenate (16°). Within the last two weeks significantly increased the first generation of spores its saturation level in comparison to the control. This additional saturation is significantly different from control and the second generation of its own line, which both desaturated CV. Remarkably, the protoplasts

did also increase its saturation level and showed an unusual mycelial growth (Figure 13 in Appendix).

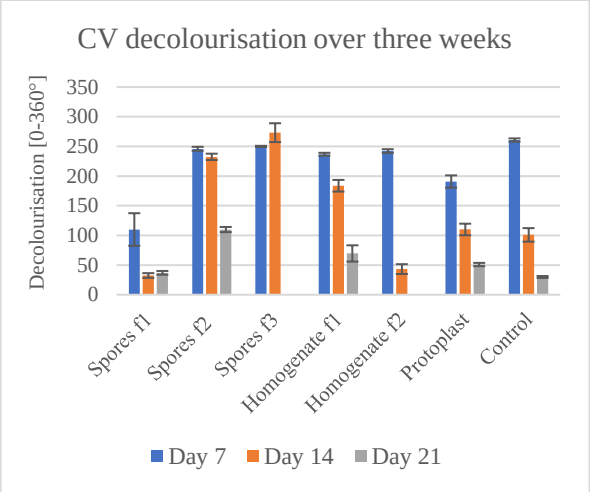


*Figure 6: CV decolourisation over three weeks by spores (f1 – f3), homogenate (f1-f2), protoplast (f1) and control (n=2).*

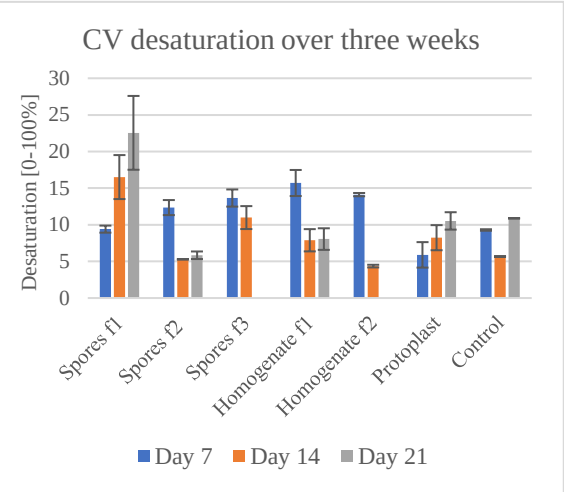


*Figure 8: CV desaturation over three weeks by spores (f1 – f3), homogenate (f1-f2), protoplast (f1) and control (n=2).*

# 4. Discussion

## 4. 1 Investigation in the genetic variation of *S. commune*

The quantitative analysis of 16 *S. commune* strains revealed their different degradation capacity and fungal development on toxic dyes. Out of this batch, the dikaryon *223* degraded the most dyes. The degradation ability of *S. commune* has been related with the ability to produce members of the multicopper oxidase (MCO) family, which are able to oxidise a broad range of substrates like polyphenols and include lignin modifying enzymes (Komori and Higuchi, 2015; Solomon *et al*., 1996). Two kind of laccases (*lcc1* (ID: 2509814) and *lcc2*(ID:1194451)), and four laccases like MCOs (*mco1* (ID: 2621035), *mco2* (ID: 2634619), *mco3* (ID: 2516955) and *mco4* (ID: 2483752)), have been reported to be expressed in *S. commune* (Ohm *et al.*, 2010). A comparison of the enzyme production during their developmental stage revealed that the activity of the previously mentioned enzymes is higher in dikaryons than in the monokaryons of *S. commune* (Madhavan *et al.*, 2014). Madhavan *et al.*, measured a strong regulation of *lcc2* during the sexual development and dikaryotic growth, which declined with the development of primordial stages. The research group measured no detectable enzyme activity in the extracts of dikaryotic primordia, which leads to the presumption that the most activity happens at the beginning of dikaryotic growth. This observation aligns with the high degradation ability of the tested dikaryotic strains, from which six out of eight the dikaryotic successful degradations showed early primordial stages but no fruiting bodies.

Furthermore, it was remarkable that there was the strong degradation of RBBR by half of the successful degrading isolates. Two other studies have described successful decolourisation of

this specific dye by other *S. commune* isolates like the Korean strain (IUM1114 and IUM1800) and the Brazilian isolate (CCB307) (Choi *et al.*, 2020; Machado *et al.*, 2005; Okino *et al.*,2000) Investigations in its degradational process have demonstrated that among others, laccase and peroxidase activity is needed to oxidase RBBR (De Jonget *et al*.1992; Freitag & Morrel 1992). The study of Okino *et al.* analysed 116 different Brazilian fungi of which one *S. commune* strain was able to degrade RBBR with extracellular laccase and peroxidase activity (Okino *et al,* 2000). Since *S. commune* is showing a tremendous genetic variation, it can be hypothesised that the three isolates can oxidise RBBR with the help of active peroxidase although the sequenced WT *4.8* did not show members of LO2 family.

Continuing with the analysis of RBBR degradation, it is mentionable that monokaryon 9, derived from dikaryon *228*, shows a different pigmentation by turning the blue RBBR plate into a brown coloured plate. Possibly, this pigment change is caused by the release of brown pigments, which overshadows the blue dye. Alternatively, the blue pigment is converted into a brown one. So far, such a conversion has not been reported in the literature. Usually, the dye loses its colour and appears white like the other tested monokaryons did (Choi *et al.*, 2020). However, the release of a brown/dark pigment has been described for several fungi in the form of melanin, which is also produced by *S. commune* (Arun *et al.*, 2015). Melanin, exposed as a dark/brownish pigment, protects the fungus from harmful environmental conditions and can be synthesized by the oxidation of dihydroxyphenylalanine (DOPA) to dopaquinone by pigment MCOs in the DOPA pathway (Langfelder *et al*., 2003; Eisenman *et al.*, 2015, Gomes *et al.*, 2003). Details of the degradation pathways still need to be defined; however, it is presumed that the lignin degrading enzymes are involved. It can be hypothesized that this monokaryon produced melanin as a protection to the toxic environment conditions due to a higher enzymatic activity of the pigment MCOs.

Furthermore, it was remarkable that monokaryotic strain *4.8a* and dikaryotic form *4.8ab* did not degrade one same dye. *4.8ab* degraded CV and MG, whereas *4.8a* degraded RBBR. It might be that *4.8b* interferes with monokaryon *4.8a*'s degradation pathway of RBBR. Additionally, it may be that either monokaryon *4.8b* individually or the combination of the monokaryons *4.8a* and *4.8ab* enhance CV's and MG's degradation path. The possible pathway interference of monokaryons on each other has been similarly observed for the *4.8ax8* and *4.8ax13* combinations. The degradation capacity of the monokaryons decreased in combination with each other. These results assume that there are different degradation pathways of RBBR, which must have interfered with each other in these combinations. The scientific literature does not provide similar findings with toxin degradation and therefore also does not provide the underlying mechanisms. However, similar results have been reported when comparing decolourisation ability of different dikaryotic *S. commune* isolates (Asgher *et al.*, 2013, Bhatti *et al*., 2008, Tang *et al.*, 2011, Selvam *et al.*, 2012).

For future research, it is suggested to perform an enzyme assay to examine the possible peroxidase activity of the RBBR degrading isolates. Additionally, it is recommended to run protein purification of the strongest degrader in this batch, including the monokaryon 9 derived by strain *228*. This experiment, possibly carried out by HPLC, could assess the degrading proteins, which can eventually be traced back to their coding genes. Conclusively, these genes

could get overexpressed to optimize the bioremediation process of organic micropollutants, ultimately.

Three out of the 16 tested strains show exudates, which differed in colour depending on the dye they were growing on. So far, coloured exudates have not been described for *S. commune*. Exudates have been described for other fungi and been correlated with their active fungal development (Colotelo, 1978; McPhee *et al.*, 2011). First biochemical analysis of *Claviceps purpurea, Myrothecium roridum, Sclerotinia sclerotiorum, Sclerotium rolfsii*, and *Thanathephorus cucumeris* exudates revealed the presence of acid phosphatase, beta-glucosidase, acid and alkaline protease, RNase polygalacturonase, cellulase enzymes, oxalic acid and ammonia, which could hint their involvement in metabolic processes. It can be hypothesized that the exudate was released due to metabolic processes whilst degrading the dye and secreted the dye in a degraded, metabolized form or released it undegraded as a by-product to protect itself from its toxicity. Therefore, it is recommended to determine the exudate's components with proteomic analysis (NMR/ HPLC/ MALDI). Additionally, it might be interesting to compare the reduction and oxidation products of the different dyes and compare them with the exudate to determine the dye's presence in the exudate.

## 4. 2 Laboratory induced evolution set-up for *S. commune*

The CV assay conducted over three weeks demonstrates that most degradation occurs within the first seven days. Therefore, it is suggested to increase the number of measurements during the first week by reading the absorbance at least once a day. In this way, the start of the enzyme activity and its development can be determined. The second approach, a real-time kinetics, indicated a shallow CV degradation speed over the exposure time of 30 minutes. It can be hypothesised, that the absorbance difference was too low or the observation time (30 min) too little to be detected by the spectrophotometer. Therefore, it is suggested to extend the exposure time to 48 hours during the first week post-inoculation to detect the precise degradational speed. The desaturation and decolourisation assessment does not demonstrate a substantial CV degradation as the CV assays do, so that it not recommended to proceed with this methodology.

The laboratory adaptive induced evolution did improve the toxicity resistance of the evolving lines since they survived higher CV concentration with later generations. However, the CV degradation analysis shows that the modified generations did not degrade the dye significantly better than the control. This outcome suggests that their improved resistance did not result because of higher degradation rates of the toxin. It can be hypothesised that the evolved lines survived these higher toxic concentrations by releasing the toxin out of the cells with the help of transmembrane proteins like the ATP-binding cassette (ABC) transport (Dassa *et al*., 2001). These transport molecules have been shown to build up a drug resistance in cancer cells by effluxing different chemical substances from the cells, which could be the case by pumping CV out of the fungal cells (Lage *et al.*, 2003; Lubelski *et al.*, 2007).
However, there are some significantly different changes in between the generations found. The spore line demonstrated a significantly lower CV degradation by the third generation, which can be interpreted as a decreasing degradation ability of spores with generation. Additionally, the first spore generation significantly decolorised and saturated CV compared to the last two generations, supporting the argument of decreasing degradation ability with further generation

within the spore line. In the homogenate line, CV concentration significantly decreased by the second generation, which was also mirrored by the significantly greater decolourisation of CV compared to its first generation. This result shows a higher resistance and faster CV degradation by the evolved homogenate generation.
In summary, the laboratory adaptive evolution set did not yield a faster CV degrader than the negative control but a higher CV resistance, and showed a tendency toward improvement in the homogenate line. This methodology improved the CV degradation for the second homogenate generation significantly, whose preparation was the most time-efficient one as well as the most practical one. Therefore, it is recommended to continue this experiment with the promising, modified homogenate line, to achieve even stronger CV degrader than the control. The sample size needs to be increased to increase the reliability of the tested samples.

Furthermore, the modified protoplast indicates an unusual mycelial development for *S. commune* WT *4.8* by developing a slight mycelial ring around the central denser mycelium. This phenotype could hint a mutation, which did not significantly improve this strain's degradation ability compared to the other samples. Therefore, it is recommended to test this modified strain to different environmental conditions to observe its possible adaptions.

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# 6. Appendix

3. 1. 1. Degradation ability and developmental stages of S. commune isolates on dyed plates

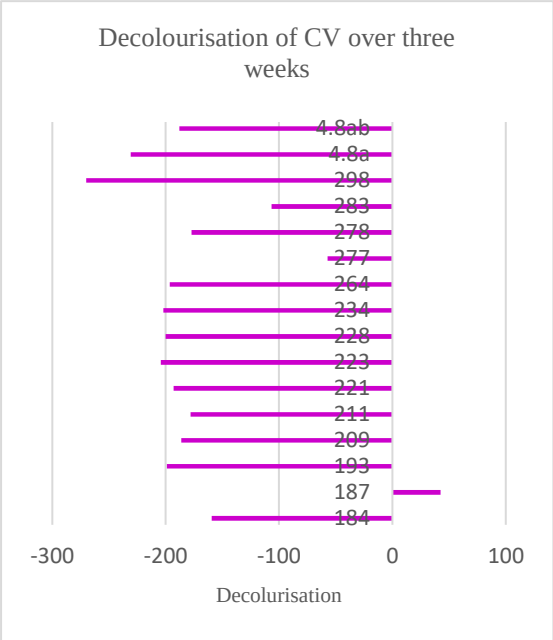


*Figure 1: Average decolourisation of CV over three weeks by 16 S. commune strains (n=3).*

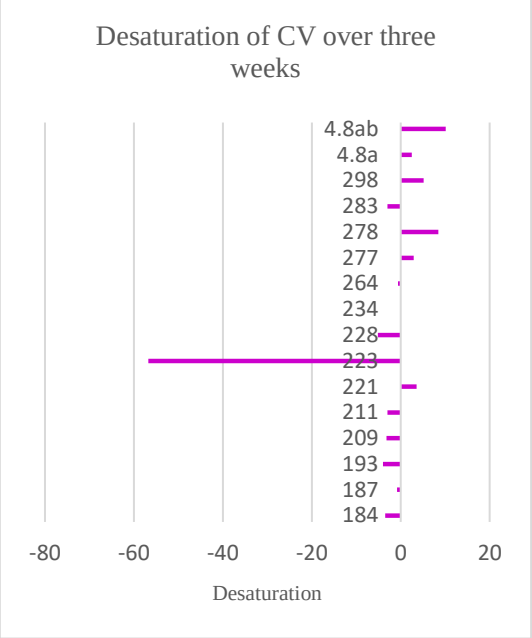


*Figure 2: Average desaturation of CV over three weeks by 16 S. commune strains (n=3).*

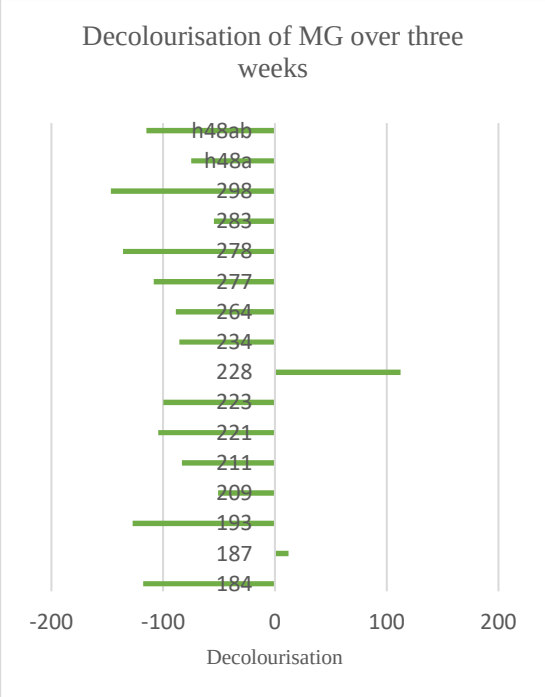


*Figure 3: Average decolourisation of MG over three weeks by 16 S. commune strains (n=3).*

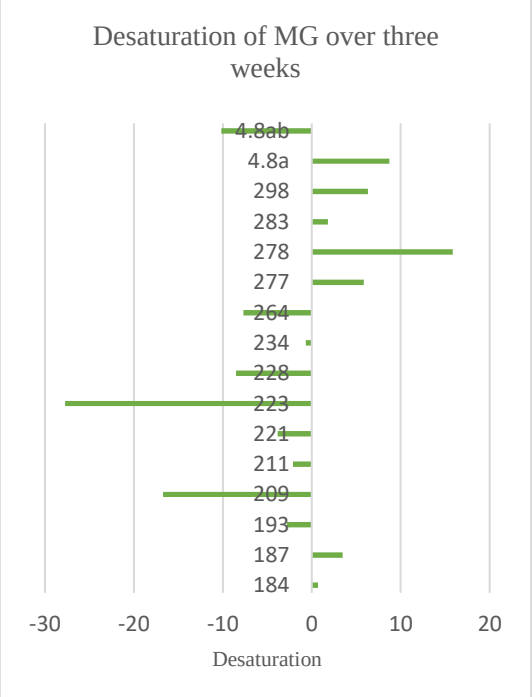


*Figure 4: Average desaturation of MG over three weeks by 16 S. commune strains (n=3).*

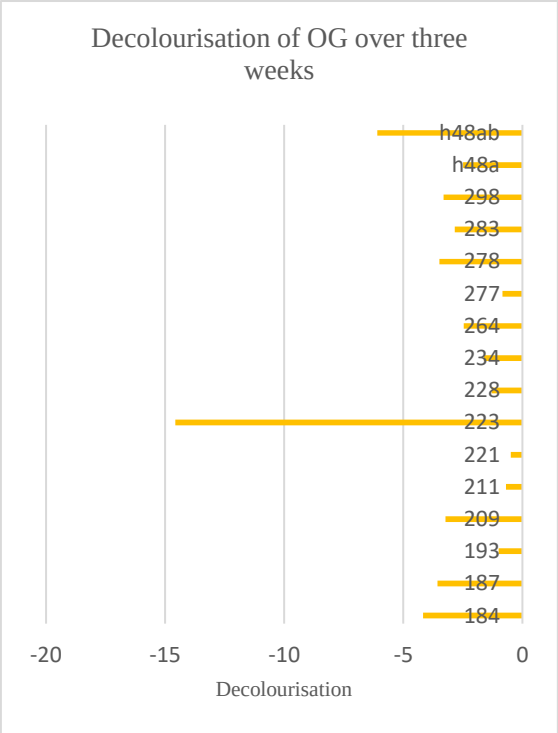


*Figure 5: Average decolourisation of OG over three weeks by 16 S. commune strains (n=3).*

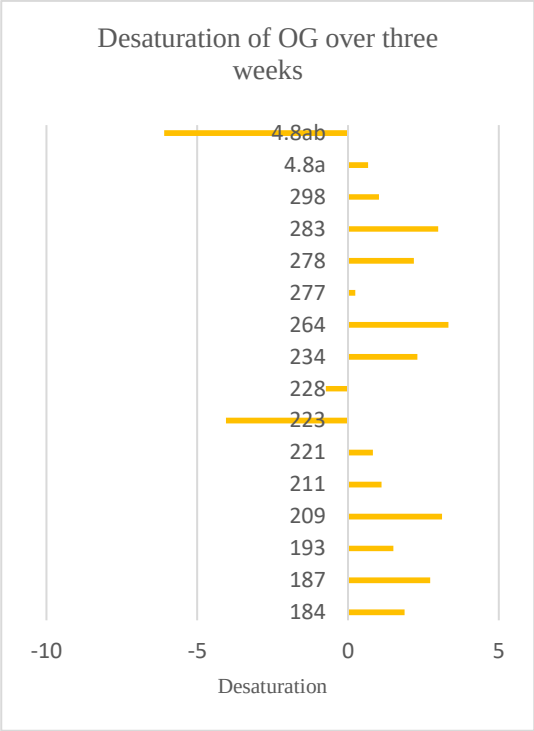


*Figure 6: Average desaturation of OG over three weeks by 16 S. commune strains (n=3).*

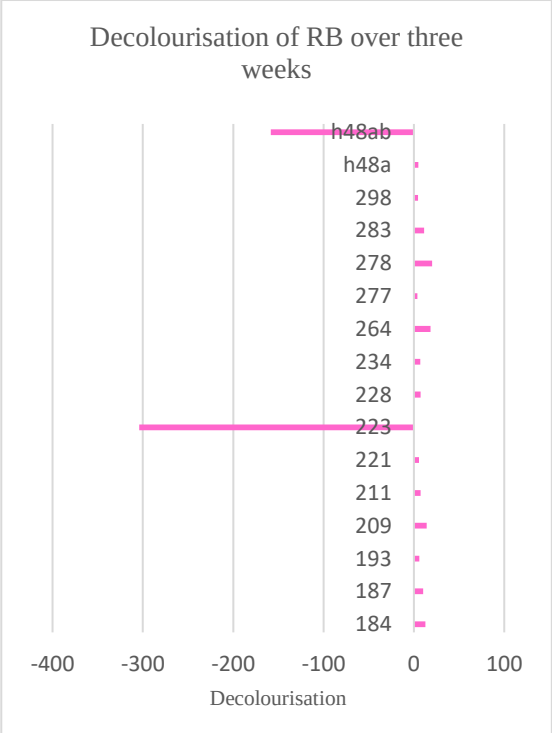


*Figure 7: Average decolourisation of RB over three weeks by 16 S. commune strains (n=3).*

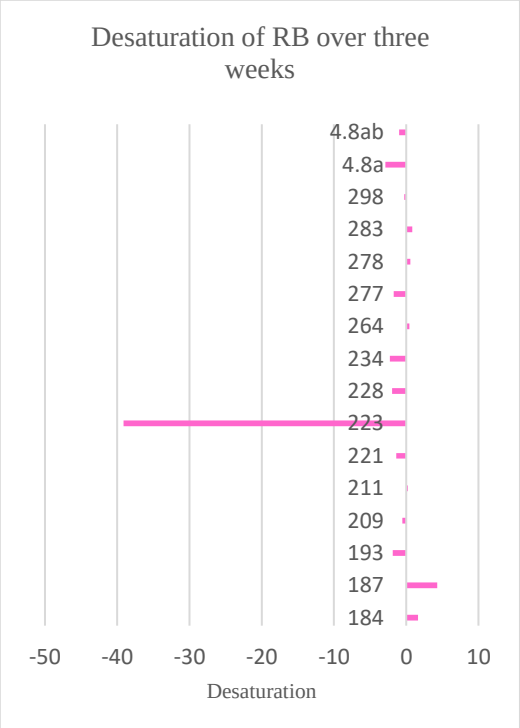


*Figure 8: Average desaturation of RB over three weeks by 16 S. commune strains (n=3).*

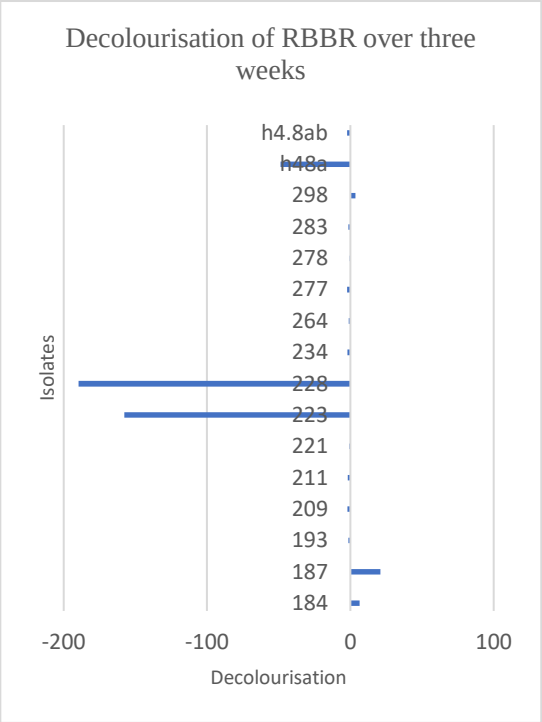


*Figure 9: Average decolourisation of RBBR over three weeks by 16 S. commune strains (n=3).*

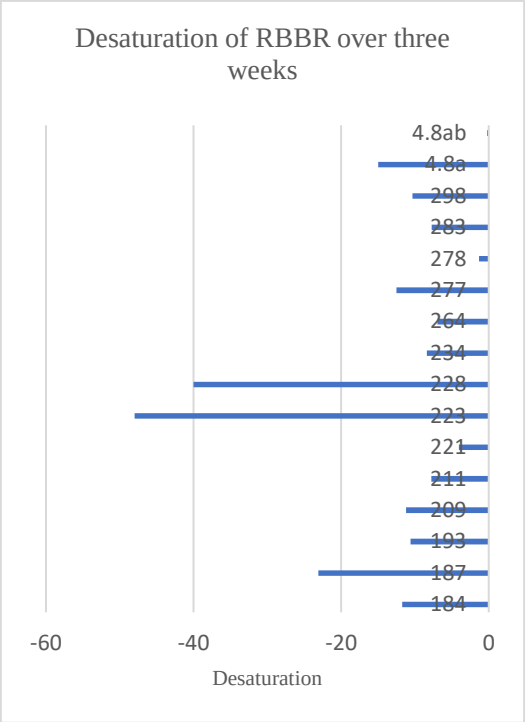


*Figure 10: Average desaturation of RBBR over three weeks by 16 S. commune strains (n=3).*

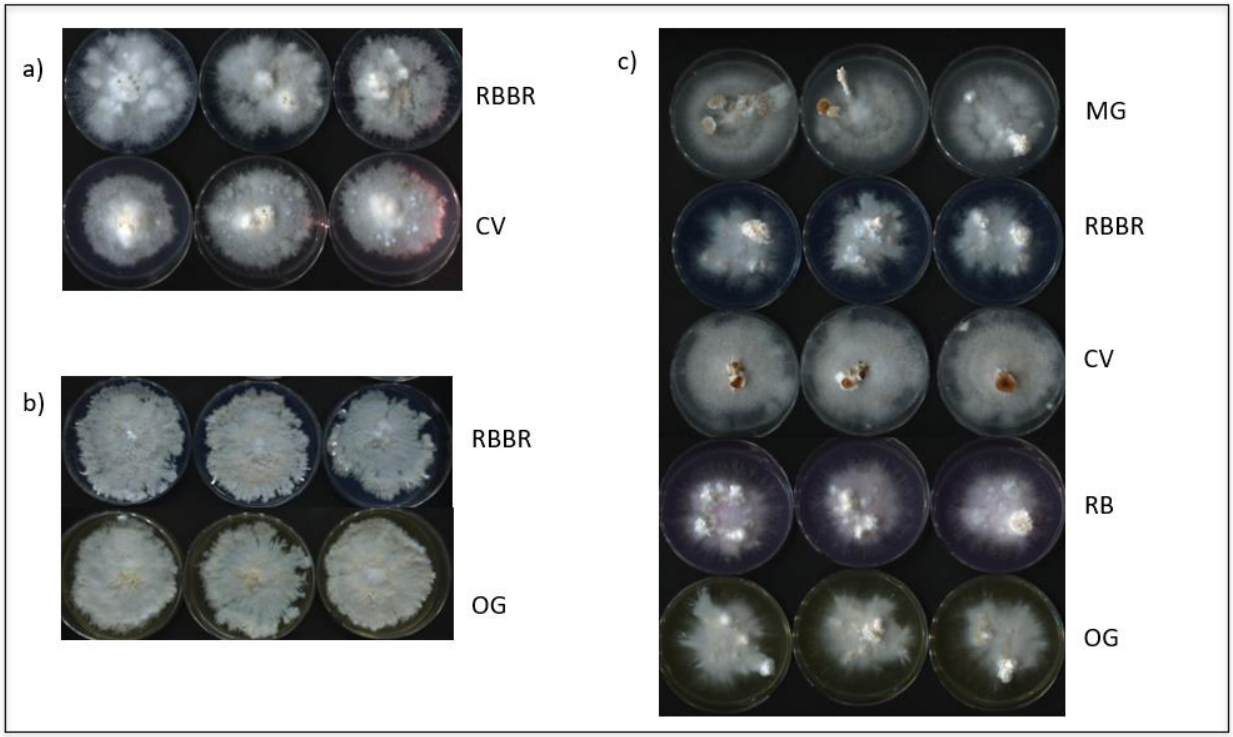


*Figure 11: Exudates of isolate 187 (a), 264 (b) and 283 (c) on differently dyed plates.*

### 3. 1. 2 Comparison of strong mono- and dikaryotic RBBR degrader

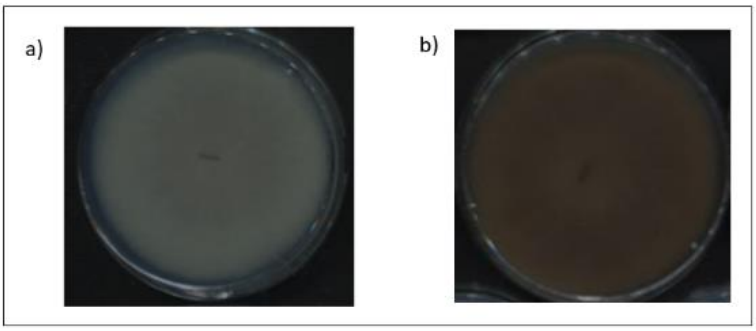


*Figure 12: Monokaryon 9 on RBBR containing MM over three weeks. a) week 1 and b) week 3(n=3).*

### 3.2.2 CV assays

Table 1: real time kinetics to detect CV degradation speed over 30 minutes (n=2).

| **Tested samples** | **Correlation (r = 0.9)** | **Degradation scale per minute** | **CV degradation speed (mol $L^{-1}$ $min^{-1}$)** |
|---|---|---|---|
| Spores f1 | -0.953368938 | -0.006022653 | -5.3774E-08 |
| Spores f1 | -0.886590917 | -0.003997236 | -3.569E-08 |
| Spores f2 | -0.981925058 | -0.00849359 | -7.58E-08 |
| Spores f2 | -0.859210597 | -0.009071185 | -8.10E-08 |
| Spores f3 | -0.382216326 | -0.000170099 | -3.04E-08 |
| Homogenate f1 | 0.141153327 | 0.000386777 | 3.45E-09 |
| Homogenate f1 | -0.944393344 | -0.015855033 | -1.42E-07 |
| Protoplast f1 | -0.847663114 | -0.002421677 | -2.16E-08 |
| Protoplast f1 | -0.481471323 | -0.00100355 | -8.96E-09 |

### 3.2.3 Decolourisation and desaturation assessment

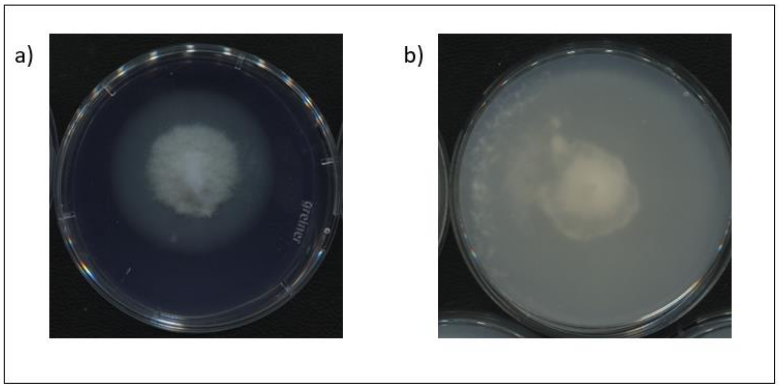


*Figure 13: Modified protoplast (generation 1) on CV containing MM showing an unusual phenotype. a) phenotype after one week, showing a ring around denser mycelium, and b) degradation after three weeks.*