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Solid Substrate Fermented Fungi as Biocatalysts for Degradation of Organic Dye Compounds

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Solid Substrate Fermented Fungi as Biocatalysts for Degradation of Organic Dye Compounds

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Civil and Environmental Engineering

by

Benjamin James Croze

2022

ABSTRACT OF THE THESIS

Solid Substrate Fermented Fungi as Biocatalysts for Degradation of Organic Dye Compounds

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Benjamin James Croze

Master of Science in Civil and Environmental Engineering

University of California, Los Angeles, 2022

Professor Shaily Mahendra, Chair

Synthetic dyes are used in the production of many consumer goods globally. Consequently, they are commonly released into local environments from industrial processes and mismanagement of dye wastewater. Dyes have hazardous impacts on both environment and public health owing to their chemical toxicity and pigment release into aquatic ecosystems. Historically, chemical and physical methods have been most commonly used for the treatment of dye wastewater. However, these approaches may face limitations in certain circumstances since they can be expensive, energy intensive, and not environmentally friendly. Biological treatments may provide a useful alternative or augmentation to situations when conventional methods are insufficient.

In this study, immobilized fungi were proposed as effective and sustainable agents for dye removal. To achieve this, *Pleurotus ostreatus* (*P. ostreatus*) was inoculated onto solid substrates, including sorghum, bran, husk, and their various combinations. *P. ostreatus* immobilized on sorghum (PO-SORG) produced the highest enzyme activity, and was further tested for its ability to degrade specific dye compounds. Laccase was the major enzyme produced by PO-SORG and played a central role in dye decolorization. In batch reactors,

Reactive Blue 19 (RB-19), Indigo Carmine, Acid Orange 7 (AO-7), and Acid Red 1 (AR-1) were degraded by PO-SORG by 82%, 91%, 91%, and 61%, respectively. In more environmentally relevant conditions, PO-SORG decolorized RB-19 or AR-1 in a synthetic textile wastewater (STWW) by 73% and 15%, respectively. PO-SORG was also used to treat a sample of real textile wastewater (RTWW) from a Los Angeles factory and decolorized it by 60%. In extreme conditions, PO-SORG resisted high temperatures and pHs to achieve substantial dye degradation. Furthermore, PO-SORG was successfully reused up to three consecutive batch cycles and removed 72% of RB-19 in the third round. Collectively, these results demonstrate that PO-SORG is a promising strategy for the treatment of dye wastewater under relevant industrial and environmental conditions.

The thesis of Benjamin James Croze is approved.

Jennifer Jay

Sanjay K. Mohanty

Shaily Mahendra, Committee Chair

University of California, Los Angeles

2022

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List of Acronyms

ABTS	2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AO-7	Acid Orange 7
AR-1	Acid Red 1
BOD	Biological oxygen demand
COD	Chemical oxygen demand
LiP	Lignin Peroxidase
MnP	Manganese Peroxidase
PO	*Pleurotus ostreatus*
PO-SORG	*P. ostreatus* grown on sorghum
SSF	Solid state fermentation
STWW	Synthetic textile wastewater
RB-19	Reactive Blue 19
RTWW	Real textile wastewater
TV	*Trametes versicolor*
TV-SORG	*T. versicolor* grown on sorghum
UV-Vis	Ultraviolet-Visible light
YPD	Yeast Extract-Peptone-Dextrose

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1. Introduction

The manufacture of synthetic dyes brings enormous value to many industries, but without proper legislation, it can also discharge huge volumes of harmful wastewater into aquatic systems (Chauhan et al., 2021). Generally, the dye-containing wastewater often possesses high biological oxygen demand (BOD), chemical oxygen demand (COD), toxicity, and light-absorbing pigment, which can negatively impact local environments if not properly treated (Lellis et al, 2019; Shindhal et al., 2021). For example, due to their high BOD and COD, dyes can excessively consume oxygen in the water and reduce oxygen availability for aquatic organisms (Jawad et al., 2022). They can also cloud natural water bodies which reduces the amount of penetrating sunlight and thus restricts photosynthetic function of aquatic life (Hassan and Carr, 2018). Besides these concerns, dyes themselves can cause skin irritation, cancer, and genetic mutations in humans and animals (Shindhal et al., 2021; Sen et al., 2016). Considering these harms, synthetic dyes should be eliminated from wastewater effluents before discharge in order to protect human and ecosystem health (Beltrán-Heredia et al., 2011). Historically, a variety of physicochemical techniques have been used for dye effluent treatment, including membrane filtration, ozonation, adsorption, and oxidation (Agrawal and Verma, 2019; Holkar et al., 2016). However, these methods can be limited by inefficiency, high cost, secondary pollution generation, and high energy demand. Alternatively, a more environmentally conscious, efficient, and cheaper method has been proposed, which makes use of microbes and their enzymes to biodegrade synthetic dyes (Lellis et al., 2019). In recent years, the use of fungi for dye treatment has been gaining attention.

Fungi are well-suited for contamination removal due to a suite of powerful oxidative enzymes that they produce. Specifically, extracellular ligninolytic enzymes are able to degrade many xenobiotic organic compounds, including various classes of dyes (Sen et al., 2016). Commonly implicated fungal enzymes include laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP). Laccase is a copper-containing oxidoreductase that uses molecular

oxygen as the terminal electron acceptor while MnP and LiP are heme-containing enzymes that catalyze the oxidation of dye compounds using hydrogen peroxide as electron acceptor (Gao et al., 2022). These reactions can eliminate both pigment and toxicity of dye compounds. Current research is focused on how best to produce these enzymes and add them into waste streams. One option is to add fungal flocs into effluents, but this can present safety concerns due to unmanaged fungal release into the environment and growth competition by other indigenous microbes. Another approach is to use purified fungal enzymes in pollutant degradation. However, this method faces limitations due to high production costs, low enzyme stability, and an inability to recover enzymes. Furthermore, free fungal enzyme-mediated treatment tends to be easily impacted by environmental stressors such as pH, salinity, and temperature.

To address these concerns, immobilizing fungi on solid materials has been proposed. A particular focus has been given to anchoring fungi on synthetic materials. For instance, *Aspergillus niger* was immobilized on sodium alginate and used to treat the dye Congo red (Hamad et al., 2021). In another study, *Trametes versicolor* was immobilized onto the surface of polypropylene rotating biological contactors to treat micropollutants in urban wastewater (Cruz del Álamo et al., 2020). These studies demonstrate the many advantages immobilization has over the traditional fungal treatment approaches. First, the immobilization process is simple and requires only the incubation of fungi and solid materials. Second, extracellular fungal enzymes can be continually released into wastewater while the live fungi continue to grow on solid supports. Third, the immobilized fungal biomass is easy to separate from the reaction medium, allowing for easy replacement when fungi become exhausted during treatment. However, while immobilization using synthetic supports confers many advantages, there are still shortcomings. For example, since the inert supports don't support fungal growth themselves, nutrients have to be supplied externally. This limits the applicability of the immobilized fungi since their growth and enzyme production steadily declines over time. Fungal dye degradation experiments using anchors like sodium alginate or polypropylene work well when dye solutions also contain food

for fungi, but largely fail under real wastewater conditions, especially in the long term when fungi die off.

To further overcome such limitations, natural solid substrates, which include grain, sorghum, wheat bran, and psyllium husk, have been employed for fungal immobilization. These solid substrates self-sustain fungal growth, reduce costs, lower environmental impact, and simplify application (Wang et al., 2019). Not only do solid supports immobilize fungi, but they provide rich and diverse nutrients to support fungal growth, which in turn provides sustained release of enzymes during experiments. Additionally, with the enhanced stability and growth rate, immobilized fungi can better withstand environmental stress (Zahmatkesh et al., 2018; Agrawal and Verma, 2019). Compared to synthetic materials used in immobilization, for example, metal oxides, solid substrates are a fraction of the cost and can be easily obtained from supermarkets or wholesale food suppliers. In addition, natural materials can be sourced locally and from naturally grown plants, which has an inherently lower environmental impact. Furthermore, culturing fungi on natural materials is straightforward and avoids the use of complex protocols and expensive equipment, which makes this approach more accessible and more easily applied in diverse circumstances. Overall, immobilized fungi on natural solid substrates possess many strengths, which enables them to be a good candidate for the treatment of dye compounds as well as other contaminants. Previous research by Zahmatkesh, et al. (2018) showed that *T. versicolor* immobilized on sorghum could be used for humic acid removal. Their work found 80% of humic acid removal when using immobilized fungi compared to 10% removal by free fungi (Zahmatkesh et al., 2018). Another study found that natural solid support *Luffa cylindrica* could be used to immobilize *Stropharia sp.* ITCC-8422 to degrade and detoxify the dye anthraquinone violet R (Agrawal and Verma, 2019).

The aim of the present study is to build upon past research and perform a comprehensive investigation into the treatment of dye wastewater by immobilizing *Pleurotus ostreatus* on various natural solid substrates. Sorghum, wheat bran, husk, and mixtures of these

substrates will be examined. On the basis of preliminary enzyme activity results, immobilized *P. ostreatus* on sorghum (PO-SORG) will be selected for comprehensive studies of decolorization of simple dye solutions, laboratory synthetic textile wastewater (STWW), and a sample of real textile wastewater (RTWW). Findings from this study will be valuable in demonstrating that fungi can be grown on affordable solid substrates to produce high amounts of target enzymes over extended periods of time and that immobilized fungi can decolorize solutions of model anthraquinone and azo dyes under a variety of conditions.

2. Materials and Methods

2.1 Microorganism and chemicals

P. ostreatus (taxid: 5322) that was previously isolated from the fungal culture purchased from Root Mushroom Farm. Anthony's Premium Sorghum Grain and Bob's Red Mill Sorghum were used interchangeably for fungal growth. Jiva Organic Whole Psyllium Husk and Bob's Red Mill Wheat Bran were also used for fungal growth. Reactive Blue 19 (RB-19), Indigo carmine, Acid Orange 7 (AO-7), Acid Red 1 (AR-1), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Yeast Extract-Peptone-Dextrose (YPD) agar were obtained from Sigma Aldrich. Other chemicals were purchased from Thermo Fisher Scientific. All chemicals were ACS grade or higher.

2.2 Preparation of immobilized fungi

P. ostreatus was cultivated on YPD agar for 6 days at 30 °C. To prepare each bioreactor, 20 g of sorghum was added into a 250 mL dried polypropylene Erlenmeyer flask. Flasks were then capped and sterilized at 15 psi and 121 °C for 30 minutes. In an aseptic environment, six *P. ostreatus* mycelium agar plugs (~2 cm diameter) from YPD plates and 8 mL liquid Tišma medium were added into bioreactors. Liquid Tišma medium was made according to Tišma et al. (2011) and was added to initiate growth and maintain humidity levels. Next, a sterile cotton ball was wrapped in an ethanol-sterilized Kim wipe and used to plug the top of the

reactor. Each bioreactor was placed into a stationary incubator at 30 °C. Bioreactors were then left to grow for 5 days and were regularly mixed. Bioreactor solid state fermentations (SSF) were mixed every 3 days to ensure homogenization. SSF bioreactors were maintained for up to 11 weeks in the stationary incubator to monitor enzyme activity during growth.

2.3 Sample extraction for enzyme activity assays

Before each sampling, PO-SORG was mixed to ensure homogeneity in the reactor. Subsequently, 0.5 g of colonized sorghum was added to a falcon tube along with 4.5 mL of sterile deionized (DI) water. The falcon tube was inverted 5 times to mix and then placed in a 30 °C stationary incubator. The tube was left in the incubator for 15 minutes and inverted at timepoints of 5, 10, and 15 minutes. The supernatant was then collected for enzyme assays.

2.4 Enzyme activity assays

Laccase activity was evaluated by following protocols from Robles et al. (2000). Absorbance change at 420 nm was measured as 2 mM ABTS was oxidized. In a cuvette, 20 µL of enzyme extract was combined with 980 µL of ABTS in sodium phosphate buffer. Activity was determined by observing the absorbance change every 2 seconds for 2 minutes (ε = 36000 L/mol×cm). MnP activity was evaluated by following protocols from Petrucciolo et al. (2009) and Wariishi et al. (1992). Absorbance change at 270 nm was measured every 2 seconds for 1 minute. To run the assay, 250 µL of 50 mM malonate buffer (pH 4.5), 125 µL enzyme extract, 25 µL of 5 mM $MnCl_2$, and 25 µL of 4 mM H_2O_2 are combined in a cuvette (ε = 2860 L/mol×cm). LiP was evaluated by following protocols from Ramírez et al. (2010). Absorbance change at 310 was measured every 2 seconds for 2 minutes. The LiP assay contained 250 µL of 20 mM citrate buffer (pH 3), 125 µL enzyme extract, 50 µL of 10 mM veratryl alcohol and 25 µL of 4 mM H_2O_2 (ε = 9300 L/mol×cm). All enzyme activities (U/L) were calculated by previously reported equations from Michel et al. (1990). Then enzyme activities were further calculated to U per gram of PO-SORG based on following equation:

$$Activity\ [U/g\ SSF] = \frac{Activity\ [U/mL] \cdot (4.5\ mL\ of\ DI\ water)}{0.5\ g\ of\ SSF}$$

2.5 Dye concentration measurements

RB-19 was selected as a model anthraquinone dye. AO-7 and AR-1 were selected as model azo dyes. Indigo Carmine was selected as a representative indigo dye. For decolorization experiments, a NanoDrop 2000 Spectrophotometer was used to measure the decrease in dye absorbance at a maximum wavelength of 592 nm for RB-19, 490 nm for AO-7, 506 nm for AR-1, and 569 nm for Indigo Carmine. Decolorization is expressed as the ratio between the measured value and the initial concentration. An Ultraviolet-Visible light (UV-Vis) spectrophotometer (PerkinElmer) was used to measure the RTWW spectra from 200-700 nm. Spectra were recorded before incubation with fungi and after incubation with fungi at desired time points.

2.6 Decolorization experiments

Experimental (designated as live) flasks were prepared in 250 mL polypropylene Erlenmeyer flasks and consisted of pre-grown PO-SORG and 50 mL of 50 mg/L dye solution dissolved in pH 5 Na-PO_4 buffer. The enzyme activity of PO-SORG was measured before the experiment to determine the enzyme activity units per gram. Based on the measured activity, enough PO-SORG was added so that the live flasks had 100 U/L of initial laccase activity. Abiotic or non-fungal control flasks consisted of 50 mL of 50 mg/L of dye solution and uncolonized sorghum. Biosorption or killed control flasks consisted of 50 mL of 50 mg/L of dye solution and autoclaved PO-SORG.

For single round batch decolorization experiments, live flasks and control flasks were made in triplicate and then subjected to desired conditions. Flasks were sampled at time points of 4, 8, 12, 24, 36, and 48 hours. At each sampling event, 500 µL was taken from each flask. First, the dye concentration was determined by measuring the absorbance of the sample at the appropriate wavelength. Next, the enzyme activity was measured using the previously mentioned assays.

The reusability of PO-SORG was tested by using the same set of immobilized fungi in consecutive dye degradation trials. For these experiments, PO-SORG was added into sterile nylon tea bags and then placed into the dye solution bioreactors. Tea bags were used to handle the fungi more easily during replenishment of dye solutions in each reusability round. The first round of reusability experiments followed the same protocol as for single round decolorization. Triplicate live groups were made whereby PO-SORG was added into bioreactors containing 50 mL of 50 mg/L RB-19 dye dissolved in pH 5 Na-PO_4 buffer. PO-SORG was added to the bioreactors to obtain an initial laccase activity of 100 U/L. Triplicate non-fungal controls containing only uncolonized sorghum were created in parallel. The non-fungal groups had equal amounts of sorghum as the live group by weight. The first decolorization round was concluded when dye concentrations remained unchanged after 12 hours. To begin subsequent rounds, the dye solutions were removed from the live and control flasks and replaced with a fresh 50 mL of 50 mg/L RB-19 solution. Time points for subsequent rounds were taken every 12 hours. These experiments were conducted at pH 5 and 20 °C.

2.7 High temperature and pH effects on dye decolorization

To test dye degradation under various temperatures, 50 U/L PO-SORG was added to 30 mL of 50 mg/L RB-19 solution in falcon tubes and incubated at 4, 20, 50, 60, and 70 °C. Temperature trials were conducted at a pH of 5. To test dye degradation at different pH, 50 U/L PO-SORG was added to falcon tubes containing 30 mL of 50 mg/L RB-19 solutions buffered to pH 4, 5, 7, and 10 and incubated at 20 °C. The pH of the buffers was adjusted using HCl and NaOH. Samples for temperature and pH testing were taken at 0, 4, 8, 12, 24, 36, and 48 hours to measure dye concentration and enzyme activity.

2.8 STWW preparation and decolorization

A synthetic wastewater was made based on the recipe described in the comprehensive review paper by (Yaseen and Scholtz, 2019). This review compiled data from numerous reports on the composition of real textile dye wastewaters and determined a representative recipe to

make synthetic dye wastewater. Overall, the recipe described includes 107.1 mg/L of sodium benzoate (C_6H_5COONa), 204.9 mg/L sodium acetate (CH_3COONa), 27.1 mg/L ammonium nitrate (NH_4NO_3), 7 mg/L sodium chloride (NaCl), 3.4 mg/L magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 4 mg/L calcium chloride dihydrate ($CaCl_2 \cdot H_2O$), 36.7 mg/L potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), and 1 mL of trace element stock. The trace element stock consists of 150 mg/L boric acid (H_3BO_3), 1500 mg/L ferrous chloride ($FeCl_2$), 120 mg/L zinc chloride ($ZnCl_2$), 120 mg/L manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 30 mg/L copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), 150 mg/L cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 60 mg/L sodium molybdate (Na_2MoO_4), and 30 mg/L potassium iodide (KI). RB-19 and AR-1 were dissolved in STWW and PO-SORG was used to decolorize these compounds in batch reactors. Experiments were conducted at pH 5 and 20 °C.

2.9 RTWW decolorization

RTWW was obtained from an active textile factory in Vernon, California. Vernon borders Downtown Los Angeles and consists mainly of industrial and factory areas. The RTWW contained dyes from the Dharma Trading Company. The raw RTWW was too concentrated to obtain spectra on the UV-Vis, so it was diluted 5-fold using non-sterile water. To initiate the experiment, 50 mL of RTWW was added to a polypropylene Erlenmeyer flask and then PO-SORG was added to obtain an initial laccase activity of 500 U/L. A control abiotic flask was created which contained 50 mL of RTWW and an equal amount of sorghum as the live PO-SORG group by weight. The experiment was carried out under non-sterile conditions. UV-Vis was used to take 200-700 nm spectra readings at 0, 24, 48, and 72 hours to observe changes in absorbance. Experiments were conducted at 20 °C.

3. Results & Discussion

3.1 Characterization of enzyme production of PO-SORG

Enzyme activity was investigated for *P. ostreatus* grown on sorghum, shown in Figure 1. Other substrates including bran, psyllium husk, and combination mixtures were tested as well

and can be found in Appendix C. PO-SORG showed laccase activity over the course of an 11-week experiment. PO-SORG reaches a maximum activity of 4.9 U/g at day 42. PO-SORG shows MnP and LiP activity from day 5 to day 46. PO-SORG has a much higher laccase activity than MnP and LiP, thus laccase is the primarily active enzyme under these conditions. The structural integrity of PO-SORG was much better than previous reported fungi *T. versicolor* immobilized sorghum (TV-SORG) as seen in Appendix B. PO-SORG was more suitable for dye decolorization work compared to other solid media and *T. versicolor* grown on sorghum because it better retained its structure and did not leach color when added to solution. As such, PO-SORG was further investigated for dye biodegradation applications.

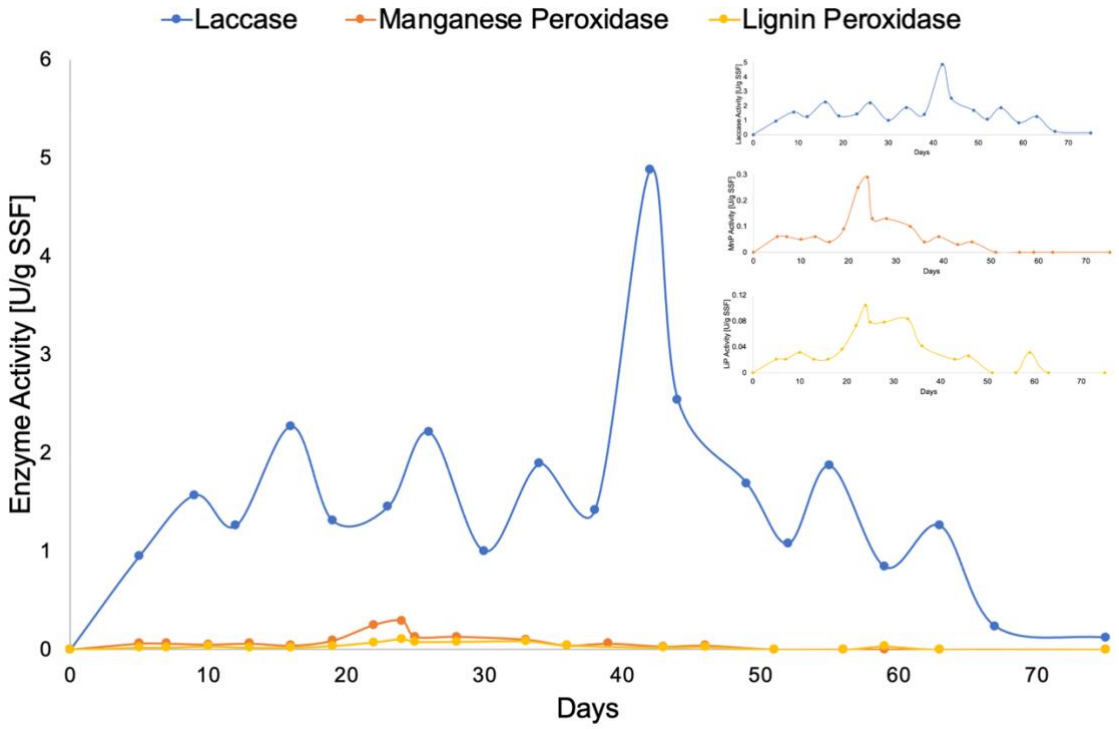


Figure 1: Laccase, MnP, and LiP activity of PO-SORG over 11 weeks.

3.2 Single dye compounds decolorization by PO-SORG in batch experiments

PO-SORG was used to decolorize four different dye compounds: RB-19, Indigo Carmine, AO-7, and AR-1 (Figure 2). These dyes represent the common anthraquinone, indigo, and two azo dyes, respectively.

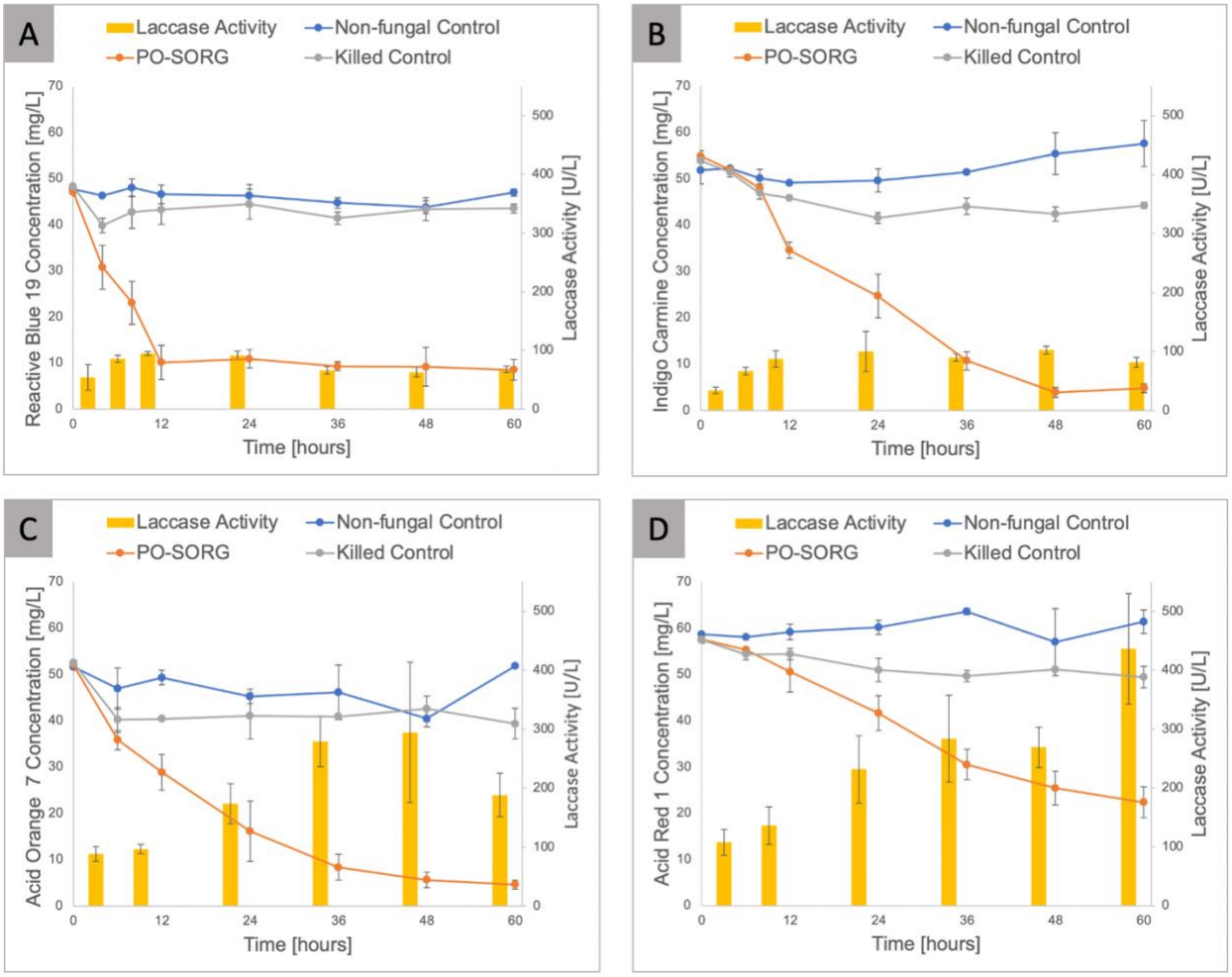


Figure 2: Dye degradation (shown on the left axis) and enzyme activity of laccase (shown on the right axis) from PO-SORG. [A] RB-19 decolorization, [B] Indigo Carmine decolorization, [C] AO-7 decolorization, and [D] Acid Red 1 decolorization.

RB-19 was degraded rapidly within 12 hours and leveled off with a minimum dye concentration of 10 mg/L. Laccase activity in the RB-19 trial gradually increased from the time of inoculation until the 12-hour time point where it peaked at 100 U/L and then slightly declined for the remainder of the trial. Indigo Carmine was degraded to 5 mg/L within 48 hours. Laccase

activity increased steadily from time zero to a maximum of 100 U/L in 24 hours and then fluctuated only slightly for the remainder of the experiment. AO-7 was degraded to a final concentration of 5 mg/L within 60 hours. Laccase activity gradually increased from time zero until it reached a maximum of 300 U/L at 48 hours, after which it declined to 100 U/L at the 60-hour time point. AR-1 was the least degraded by PO-SORG, reaching a minimum dye concentration of 22 mg/L after 60 hours. Laccase activity increased throughout the duration of the experiment, reaching a maximum activity of 420 U/L at the 60-hour time point. The biosorption control, containing killed fungi immobilized on sorghum, did not show significant dye decolorization in any of the trails. The non-fungal control, containing uncolonized sorghum, did not show dye decolorization for any of the four dye compounds tested. The pH in the bioreactors remained constant during the experiment, thus any pH-dependent color changes did not occur.

The four dyes in order of most to least readily degradable dye by PO-SORG were RB-19, Indigo Carmine, AO-7, and AR-1. This is influenced by the different dye structures. Anthraquinone dye showed the most rapid decolorization while azo dyes were harder to be degraded. Indigo dye decolorization rate was degraded faster than the azo dyes and slower than RB-19. These results are consistent with previously reported studies (Champagne and Ramsay, 2010; Gao et al., 2022). Besides the difference in decolorization rate, laccase activity presented distinct profiles between dyes. In both azo dye groups, laccase activity peaked above initial activity over time. One possible reason is that products generated from azo dye decolorization enhanced the laccase production in PO-SORG. These products might even be utilized for fungal growth. The azo dyes may also induce enzyme activity as the fungi seeks to remove an apparent persistent toxin. In contrast, the parent compounds, or products of anthraquinone and indigo dye groups play a role in fungal growth inhibition. Hence, instead of rising activity, we observed stable or even leveling off laccase activity. It’s also possible the more rapid degradation of the anthraquinone and indigo dyes prevented the need for enhanced

enzyme production. These results demonstrate that PO-SORG can effectively decolorize various commonly used dye classes. The similarity between the killed control and the non-fungal controls suggests that fungal biosorption does not play a role in dye removal. Additionally, these data could be used to modify treatment approaches and better target treatments depending on what dyes exists in a given wastewater.

3.3 Decolorization of RB-19 and AO-7 in STWW by PO-SORG

In order to investigate the potential effects of complex components in dye effluent on PO-SORG decolorization, RB-19 and AR-1 decolorization were prepared in STWW solutions (Figure 3). STWW solutions mimic a more real-world environment by introducing salts, metals, and trace elements to the bioreactors (Yaseen and Scholtz, 2019).

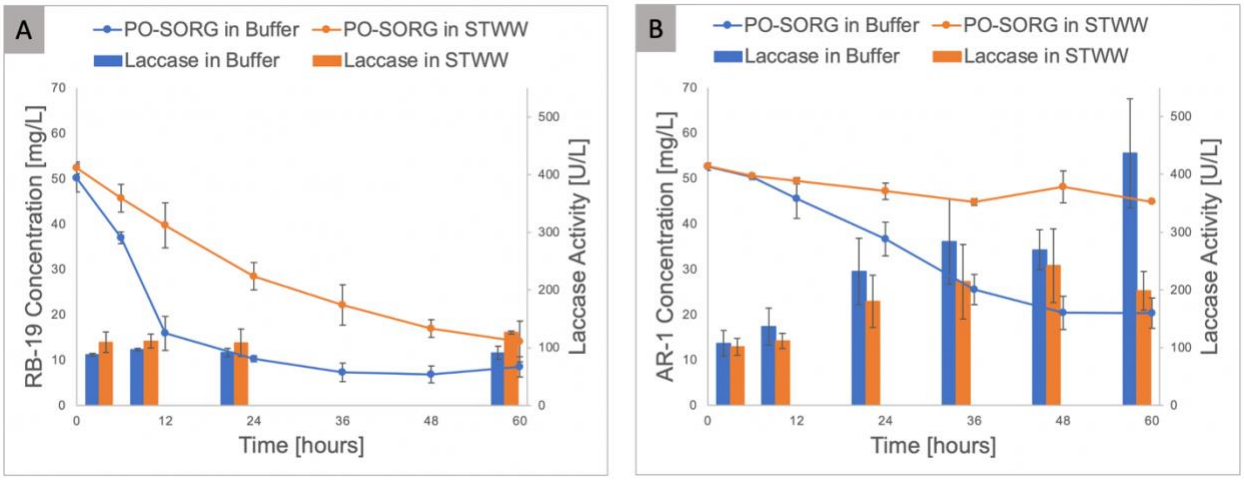


Figure 3: Comparisons of dye degradation by PO-SORG in $NaPO_4$ buffer and a synthetic textile wastewater recipe. Dye degradation (shown on the left axis) and enzyme activity of laccase (shown on the right axis). The two graphs show [A] RB-19 and [B] AR-1 decolorization. Insets show laccase activity under each condition.

As shown in Figure 3, RB-19 was initially slower in STWW than in buffer, but both ultimately reached approximately the same minimum dye concentration. Degradation in the buffer occurred rapidly and reached the minimum dye concentration within 12 hours while degradation in STWW occurred more slowly and reached the minimum dye concentration at the 60-hour time point. Laccase activity followed the same trend in both conditions and was on

average 22 U higher in the STWW bioreactors. Activity was consistent throughout the 60-hour experiment. These results demonstrate that despite slower initial rates, PO-SORG can degrade RB-19 dye in the presence of salts, metals, and trace elements typically discharged during textile processing. AR-1 degradation was noticeably slower in STWW than in buffer and did not reach as low of a minimum dye concentration. Degradation in the buffer occurred gradually over 60 hours and was reduced from 53 mg/L to 20 mg/L. Degradation in STWW also occurred gradually over 60 hours but was only reduced from 53 mg/L to 45 mg/L. Laccase activity in the buffer generally increased over time and reached a maximum of 437 U/L at 60 hours. Laccase activity of STWW increased steadily until 48 hours when it reached a maximum of 241 U/L, after which it declined slightly to 200 U/L. The results show that the components of the STWW significantly inhibit the degradation of AR-1 and may reduce laccase effectiveness. Slower degradation rates could be attributed to the components of the STWW. For example, trace metals and salts in STWW could act as inhibitors to fungal growth or laccase production as it tries to degrade AR-1, resulting in slower catalyzation (Champagne et al., 2013). The STWW, AR-1, and AR-1 degradation products likely interact to reduce biodegradation as compared to the buffer trial. In the RB-19 group, laccase activity did not show apparent inhibition in the presence of STWW. This is likely due to RB-19 and formed products associated with components in STWW, which did not produce any inhibitory effects. Clearly, in order to achieve successful PO-SORG decolorization, the components in textile wastewater must be considered.

3.4 Decolorization of RB-19 under extreme conditions by PO-SORG

In textile factories, the temperature and pH of textile wastewater can vary greatly, even within a single location. To test the potential of immobilized fungi to withstand variable and harsh environmental conditions, PO-SORG was used to decolorize RB-19 at a series of extreme temperatures and pHs.

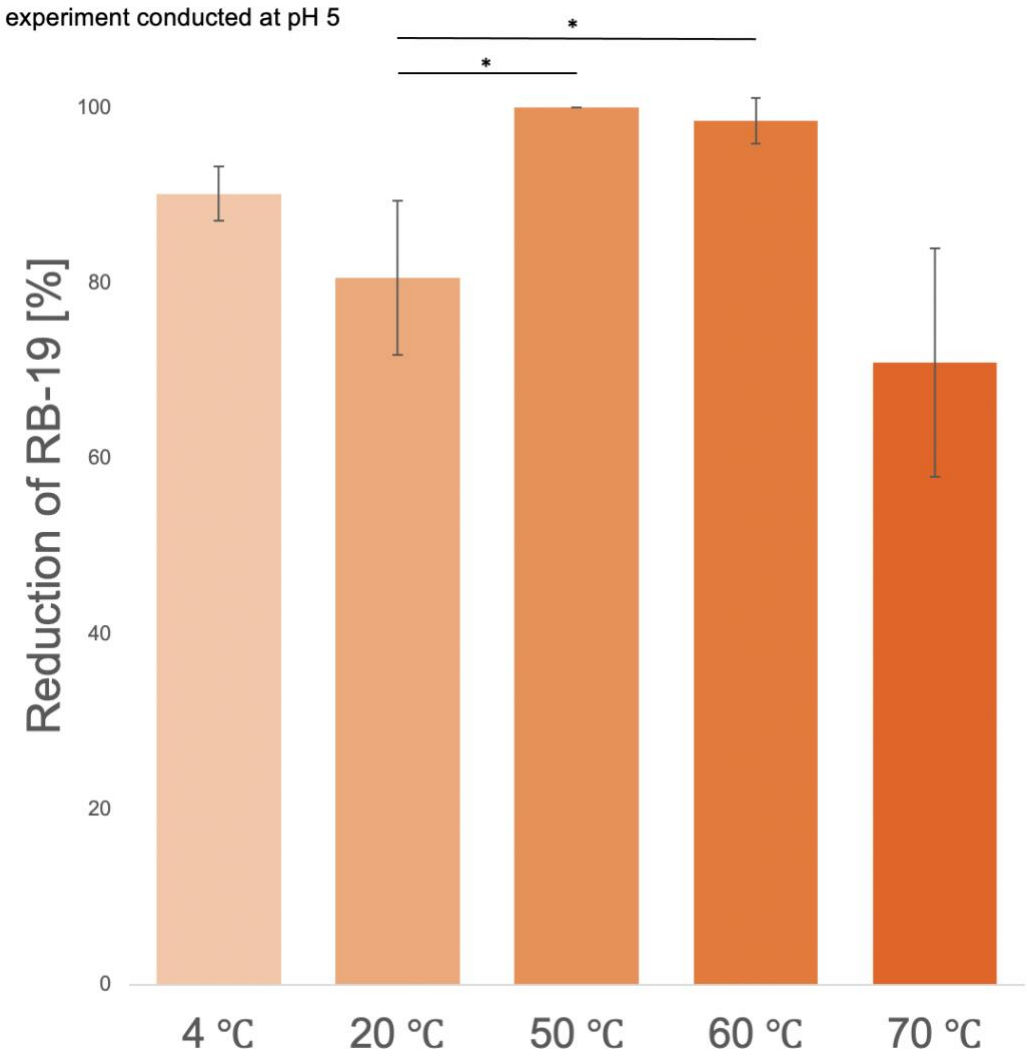


Figure 4: Bar graph showing the percent reduction of RB-19 from PO-SORG exposure at temperatures of 4, 20, 50, 60, and 70 °C. A P value of < 0.05 is indicated by one asterisk (*).

As shown in Figure 4, among tested temperatures, decolorization was not affected except at the highest temperature of 70 °C, which showed a reduced rate of degradation and a higher minimum dye concentration at the termination of the trial. Statistical comparisons are based off of the ambient temperature trial at 20 °C. The only statistically significant change in RB-19 reduction occurred 50 and 60 °C, where there was an increase in decolorization. Temporal biodegradation graphs can be found for each temperature in Appendix E. RB-19 decolorization at 4, 20, 50, and 60 °C follow nearly identical trends. The dye was degraded rapidly from an initial concentration of 50 mg/L to below 10 mg/L within 12 hours. At 70 °C the

RB-19 decolorization was slightly inhibited and reached a minimum dye concentration of 13 mg/L by 48 hours. In general, temperature did not greatly inhibit biodegradation of RB-19 and in fact promoted decolorization at 50 and 60 °C. As seen in Figure 5, decolorization was affected by pH, which is expected since enzyme activity is highly dependent on pH.

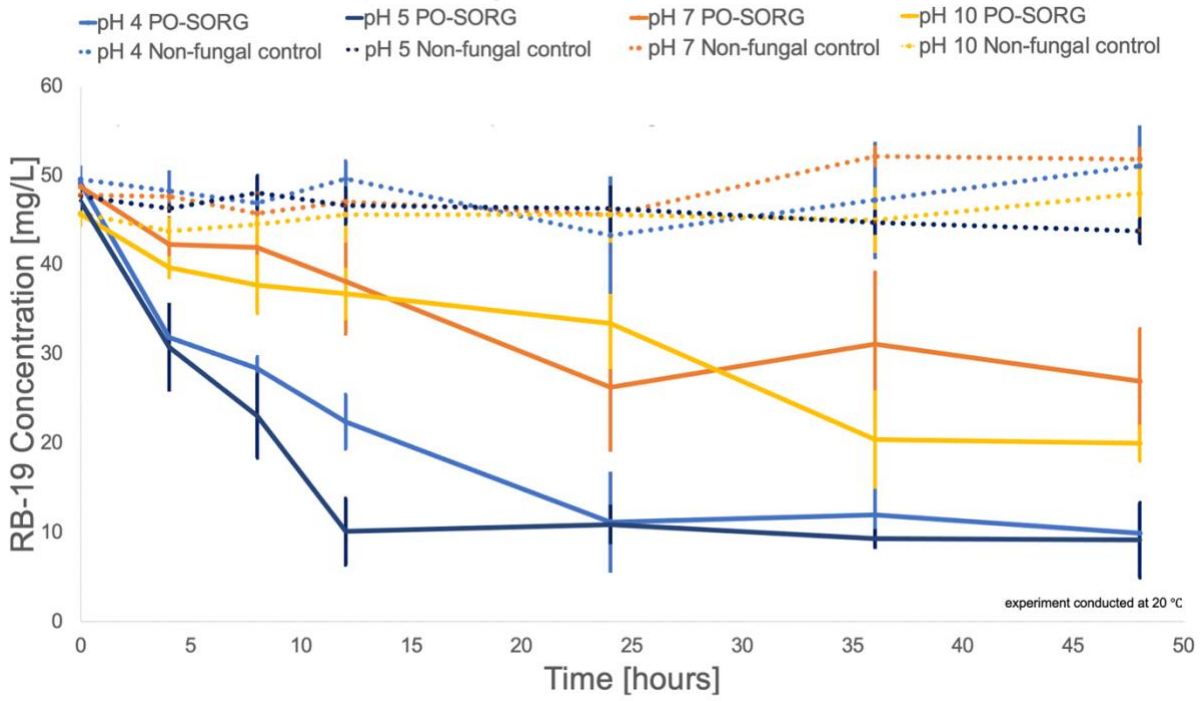


Figure 5: RB-19 dye degradation using PO-SORG at pH 4, 5, 7, and 10.

The slowest decolorization rate occurred at pH 10. At pH 4, decolorization was less impacted, although it was still slower than the rate at pH 5. This agrees with the general properties of laccase, which is most active around pH 5 (Datta et al., 2017). At pH 4 and 5 total decolorization was at 80% and 82% respectively. Though decolorization did occur at pH 7 and 10, it was noticeably impaired. At neutral pH 7 decolorization reached only 45% while at a more basic pH 10 decolorization was 56%. According to a t-test, the inhibition of decolorization observed at pH 7 and 10 was a statistically significant relative to the baseline of pH 5 (Appendix D). While temperature did not greatly affect biodegradation, pH outside of the optimal range for

laccase demonstrated greatly reduced dye removal. Overall, these results show that immobilized fungi can withstand a wide range of temperatures and pH and still achieve some decolorization even in extreme conditions. Cold or hot conditions (4-70 °C) had only minor effects. A range of pH 4-10 produced varying results, but PO-SORG was not fully inhibited in any condition. The added stability of *P. ostreatus* owes to the protection it gains by its attachment to the solid surface of the sorghum. This has real-world relevance for treating dye wastewater that may be discharged from textile facilities in a variety of climates (Yaseen and Scholtz, 2019).

3.5 Reusability of PO-SORG in batch experiments

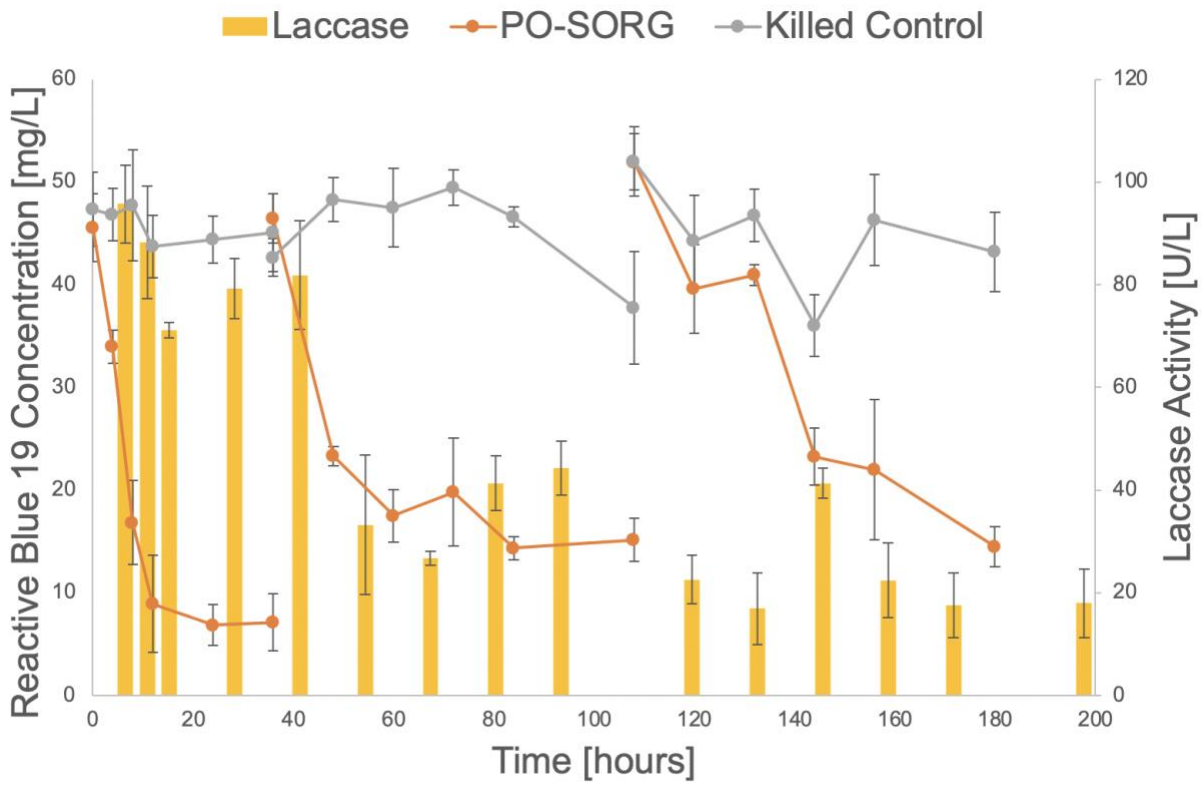


Figure 6: RB-19 dye degradation (shown on the left axis) and enzyme activity of laccase (shown on the right axis) from PO-SORG in three consecutive trials.

The reusability of agents is an important economic and operational factor regarding practical wastewater treatment. Hence, the reusability of PO-SORG was tested by using the

immobilized fungi in consecutive dye degradation trials (Figure 6). Dye decolorization was observed across three sequential rounds. The PO-SORG was not given any time to regenerate between rounds and was used successively.

In round one, total laccase activity was at its highest, between 75-95 U/L, and RB-19 was degraded from 46 to 7 mg/L within 24 hours. In round two, laccase activity was reduced roughly by half, compared to round one, to a range of 27-44 U/L, and RB-19 was degraded from 46 to 15 mg/L within 48 hours. In the third and final round, laccase activity again decreased, to a range of 17-47 U/L RB-19 and was degraded from 52 to 15 mg/L within 72 hours. Throughout the duration of the three rounds the non-fungal control, containing uncolonized sorghum, did not show any dye decolorization. These data demonstrate that RB-19 was successfully degraded in three rounds by the same sample of PO-SORG even without regrowth in an incubator. However, it was observed that decolorization rates slowed in each successive round, taking 24 hours longer in each round to reach the maximum degradation. A t-test found that the rate reduction between the first round and the following two was statistically significant. However, the change in decolorization rate between rounds two and three was not relevant (Appendix F). This could be ascribed to the decrease in laccase activity after each round and slowed growth rate within the dye solution. Reduction of laccase activity was likely due to the loss of activity between cycles and slower laccase production. Also, during pre-experiment growth in the incubator PO-SORG was allowed to build up laccase for release. Upon exposure to the dye solution in round one, the stored extracellular enzyme was released into solution by diffusion. At the end of the round when the dye solution is replaced, the laccase (and other enzymes including MnP and LiP) that were released remain in the old dye solution and were discarded. When the subsequent round began, the PO-SORG had to generate new laccase for release. Thus, it is expected that enzyme activity declines in each subsequent round. Further study is required to determine how to better sustain enzyme rates. In terms of practical application, due to being packaged in a porous container, nylon tea bags, PO-SORG could be more easily

removed from the solutions after each cycle, which demonstrates operational advantages for future treatment. Altogether, these results found that PO-SORG has great reusability and can achieve dye decolorization after at least three rounds, albeit at successively slower rates.

3.6 RTWW treatment by PO-SORG

As demonstrated in Figure 7, PO-SORG was used to treat RTWW containing Dharma Trading Company dyes to evaluate decolorization in a real-world textile wastewater condition. The UV-Vis scanned absorbances across 200 nm to 800 nm over 72 hours.

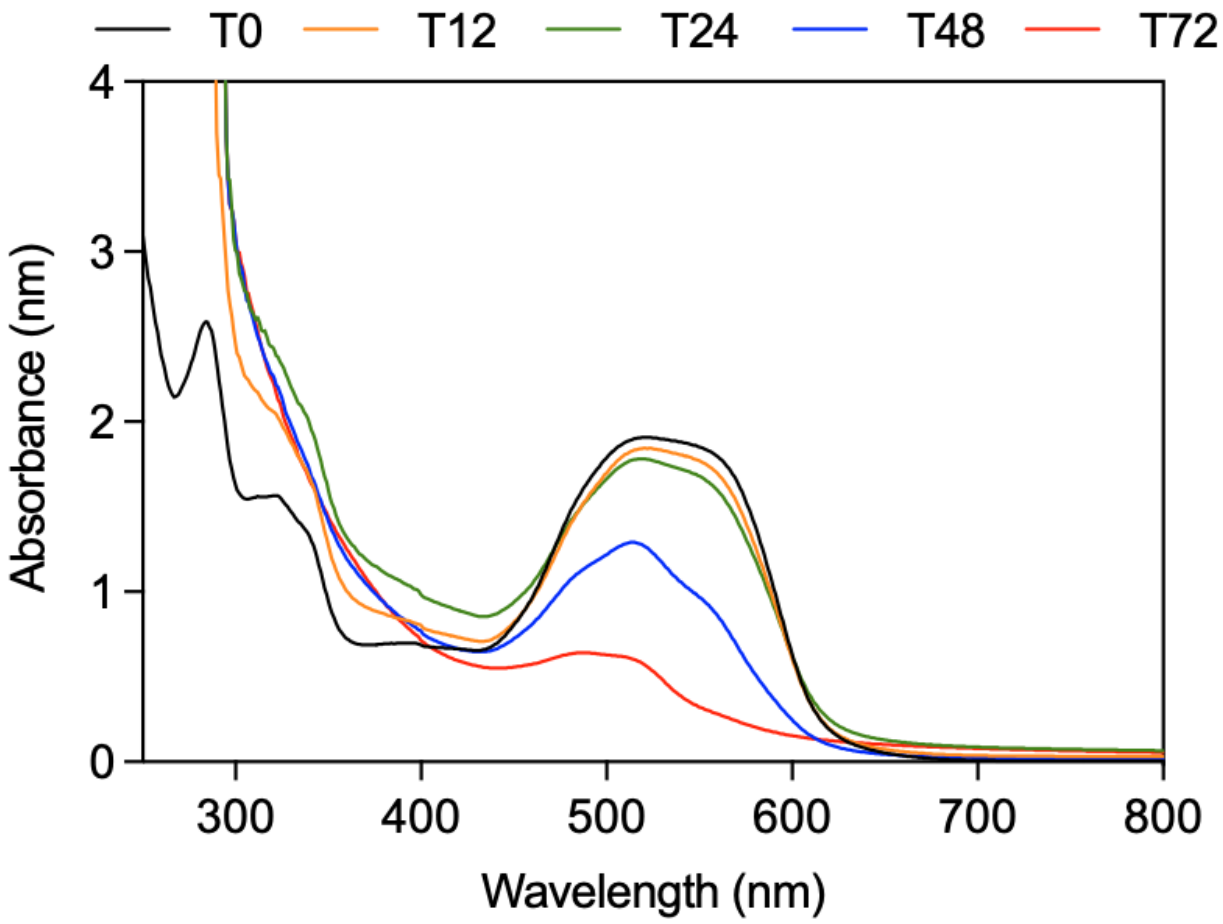


Figure 7: UV-Vis spectrum from 200-700 nm showing absorbance change of RTWW over time when applied with PO-SORG.

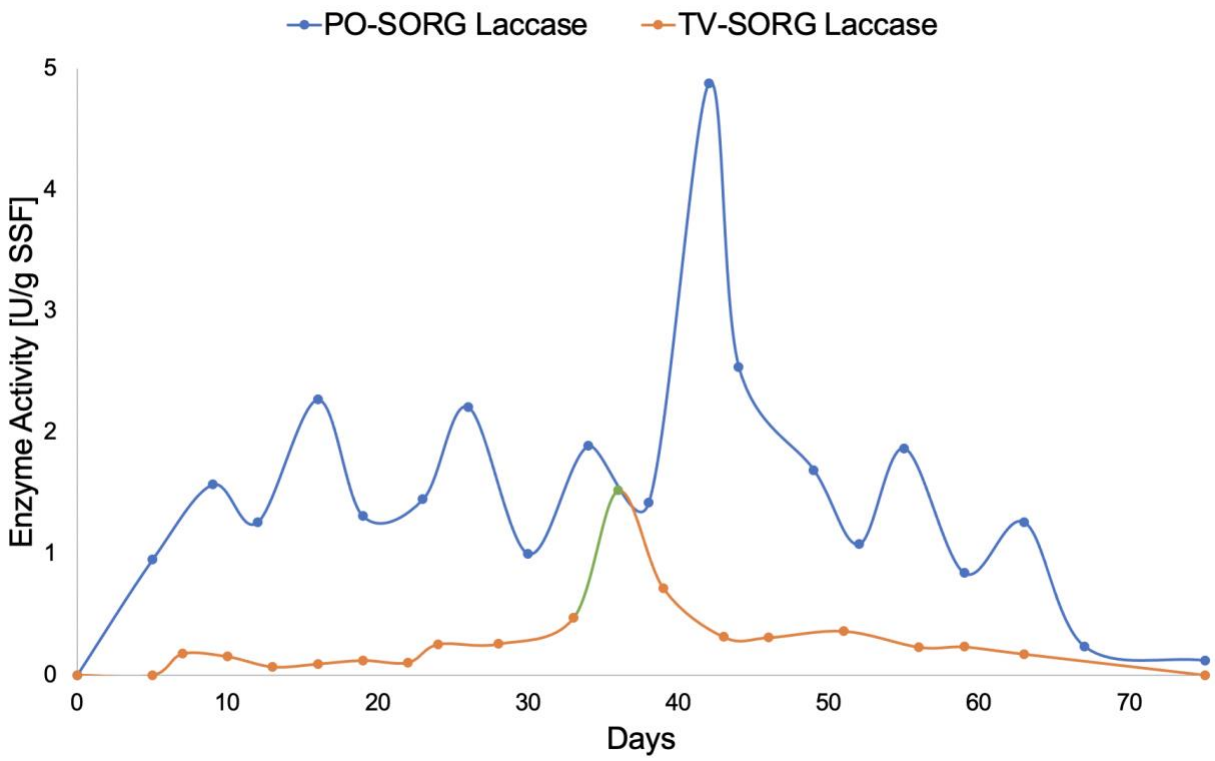
The main absorbance peak at 520 nm was only reduced by 8% within the first 24 hours. From 24 to 48 hours, it was reduced by 33% and by 72 hours the peak had been reduced by 60%, at which point the experiment was terminated. The initially slow degradation may have

been due to complex components of RTWW that inhibited the laccase from acting on the dyes, resulting in minimum decolorization. After 24 hours, inhibiting components may have been degraded so the decolorization rate increased. Regardless, these results indicate that PO-SORG is able to decolorize mixtures of dye compounds under non-sterile conditions within a reasonable timeframe.

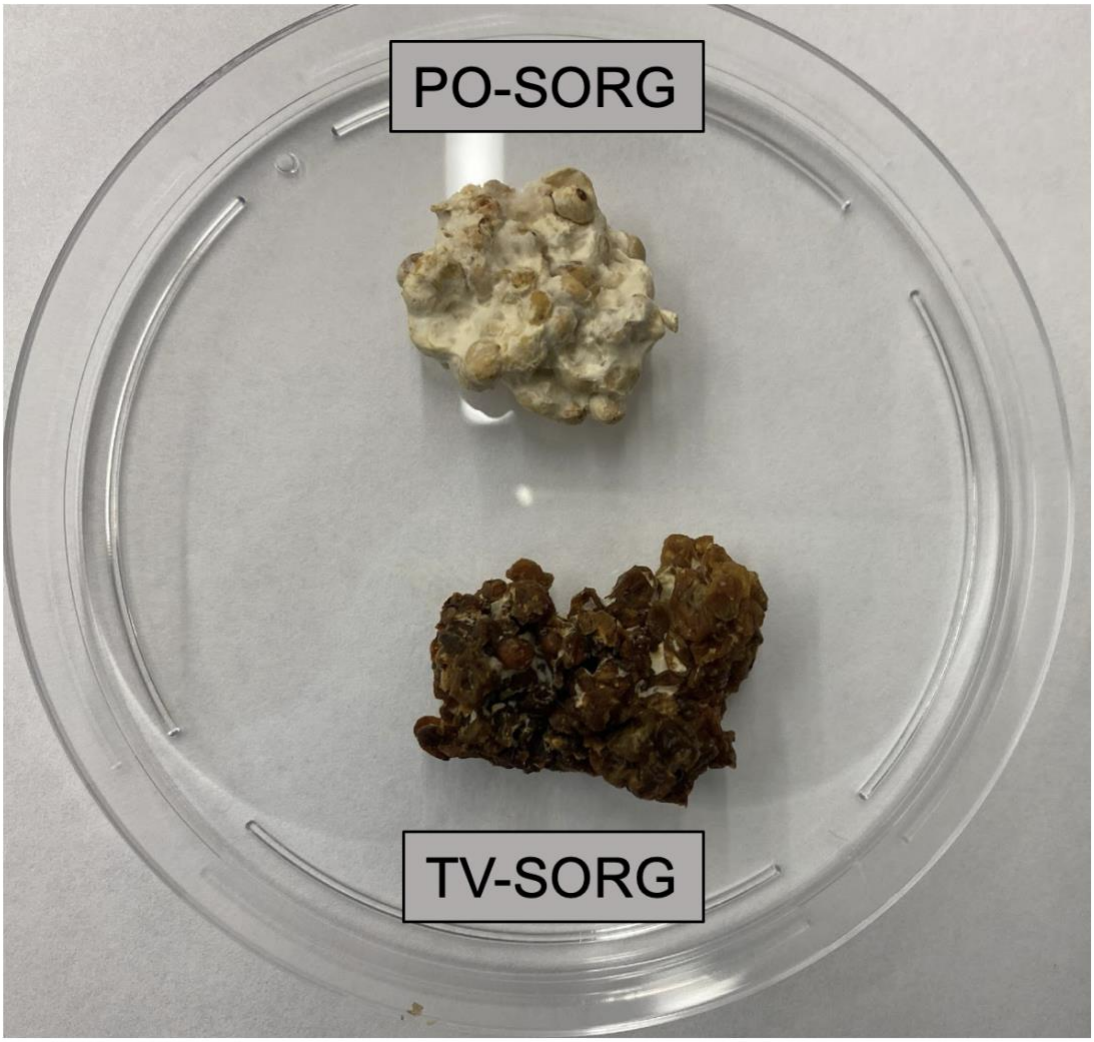
4. Conclusion

This study developed an effective and green technology using fungi immobilized on a solid substrate, PO-SORG, to achieve dye-containing wastewater treatment. The PO-SORG system produced laccase with high activity even after nine weeks of growth, which demonstrates PO-SORG could have continuous activity for real dye biotreatment. Here, PO-SORG was used to degrade dyes in batch reactors containing laboratory buffers, STWW, or RTWW. The immobilized fungi successfully degraded various dye compounds in a range of settings and experimental conditions. Even at extreme pH (4-10) and temperature (4-70 °C) that may occur in actual textile effluents, PO-SORG was able to noticeably decolorize stable dyes. Furthermore, PO-SORG was reused up to three successive cycles, rendering more merits regarding practical usage. Overall, PO-SORG shows great potential to be used in textile wastewater treatment, which highlights the need for more scale-up research, comprehensive risk assessment, and commercial evaluation for future development of this sustainable approach.

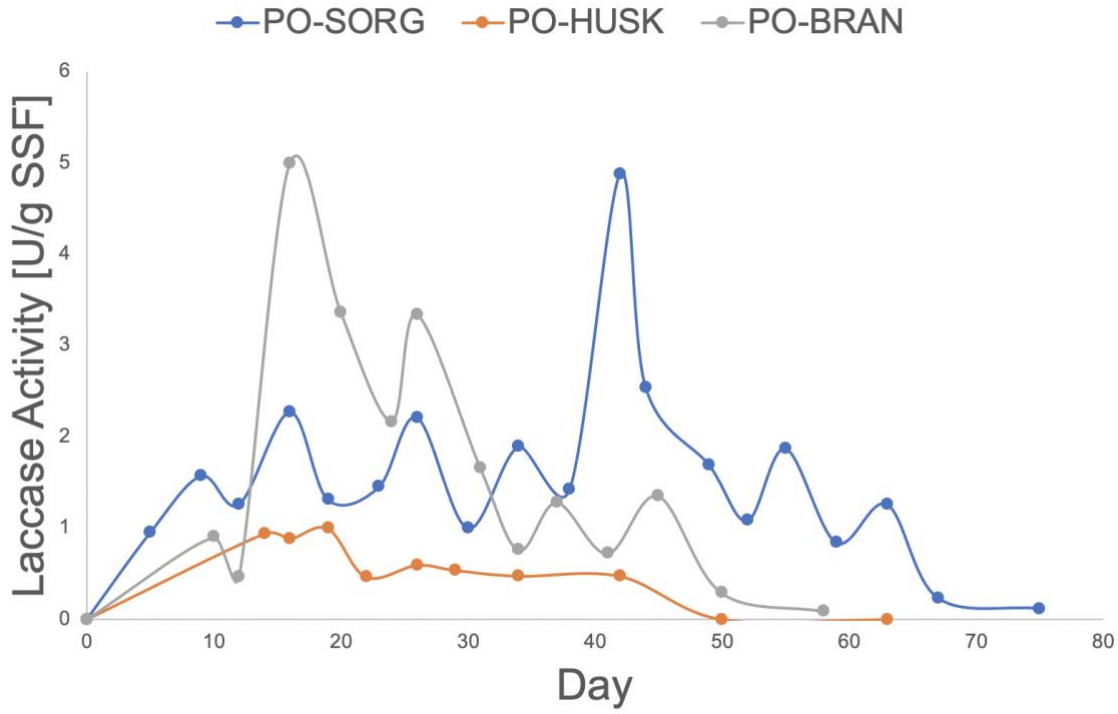
Appendix A: Activities of Laccase produced by *T. versicolor* and *P. ostreatus*

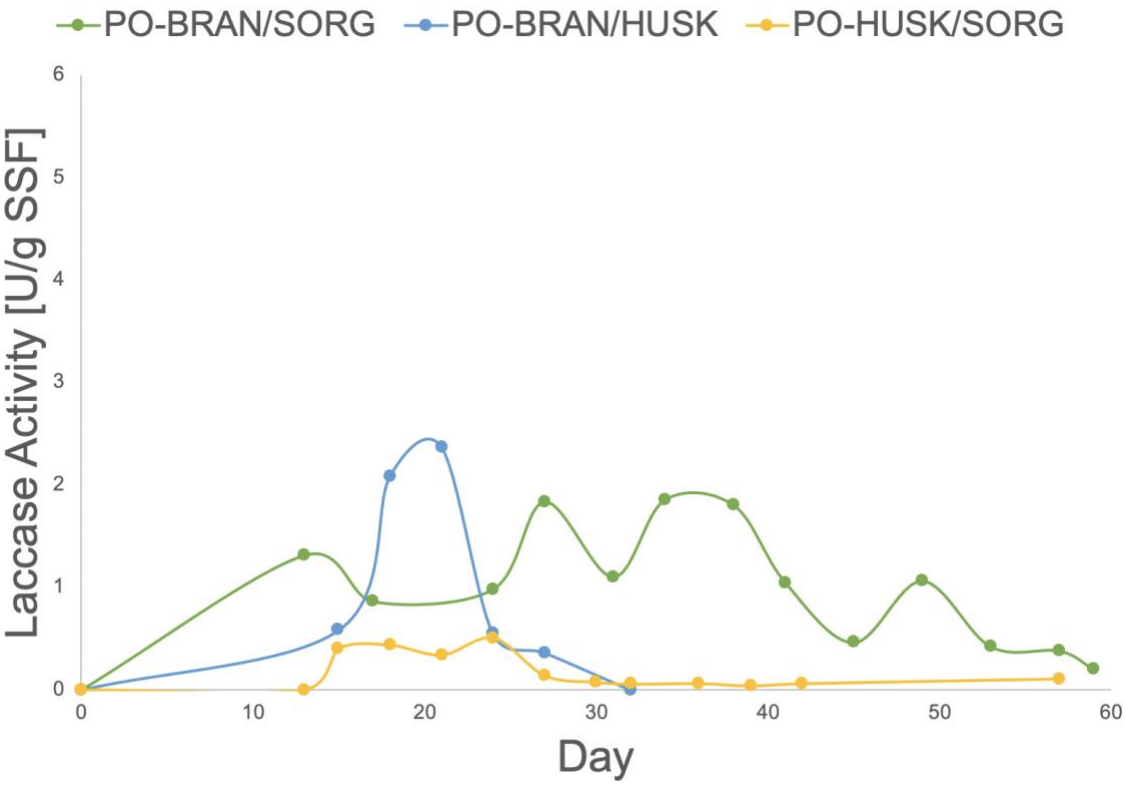


Appendix B: Photograph showing the color and structural differences between PO-SORG and TV-SORG

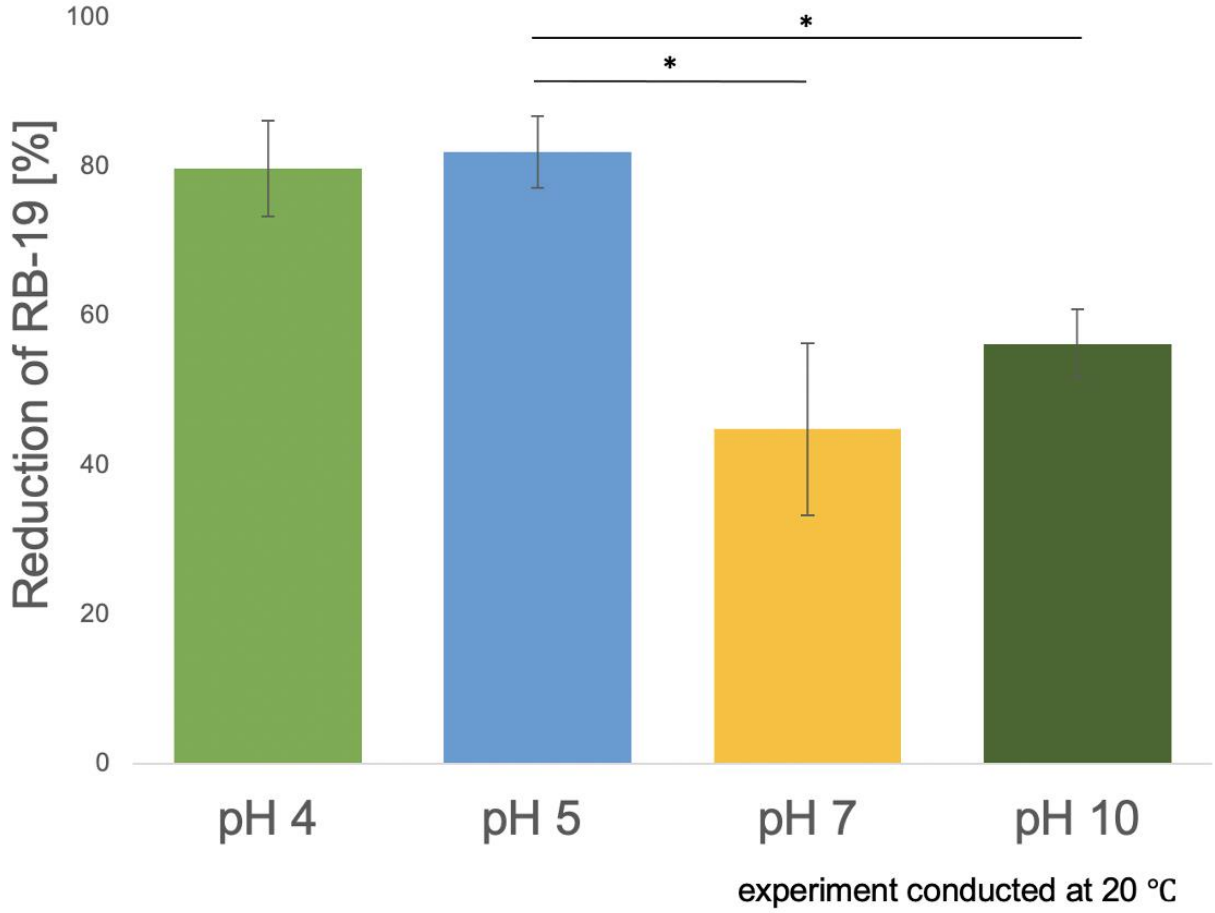


Appendix C: Laccase activity of *P. ostreatus* grown on different solid substrates



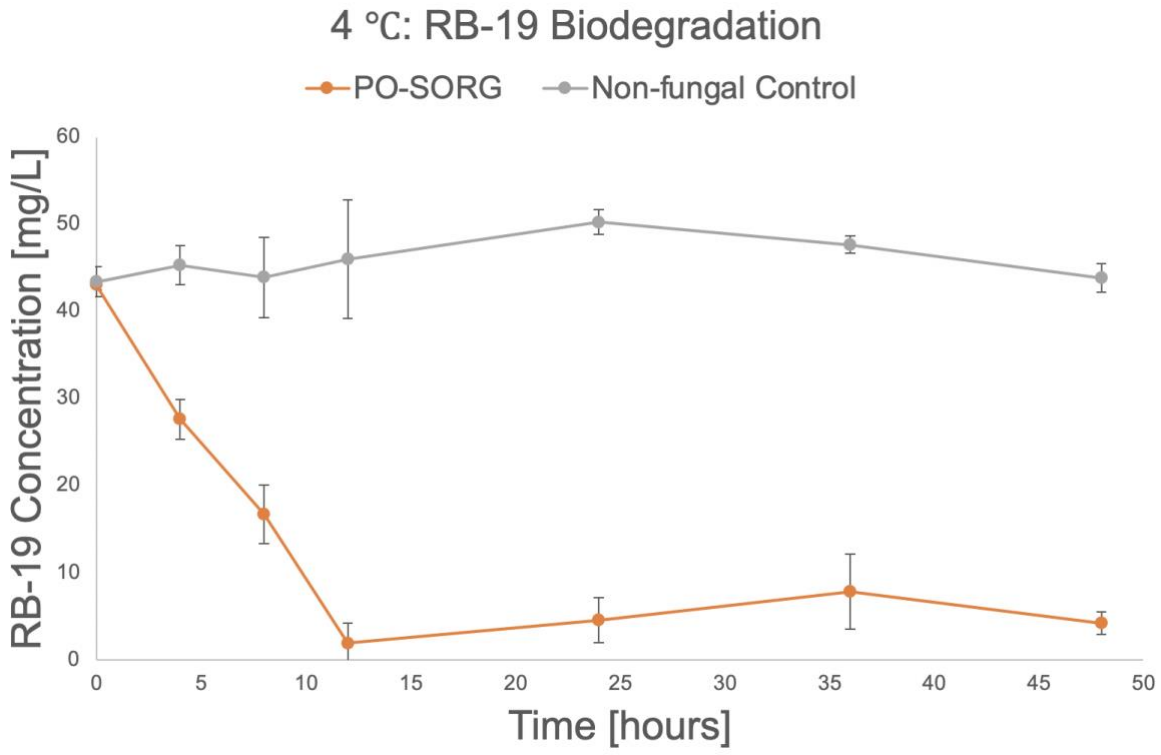


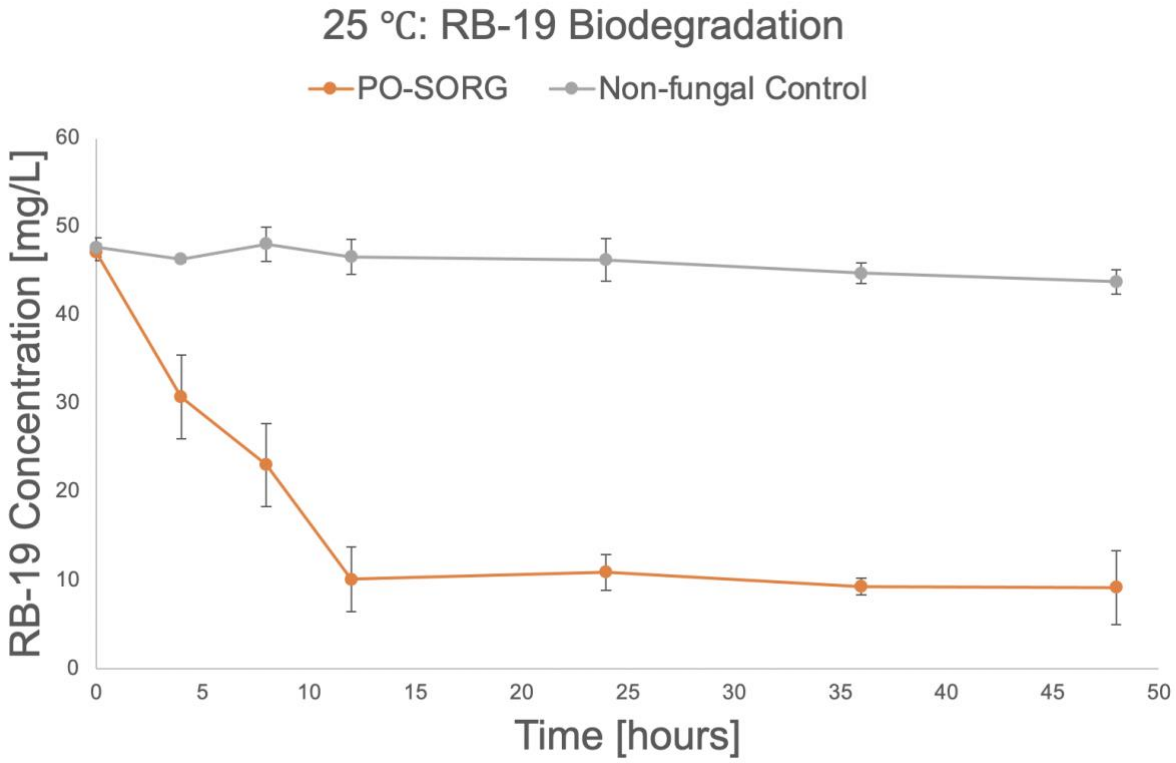
Appendix D: Percent reduction of Reactive Blue 19 at various pH values after exposure to PO-SORG

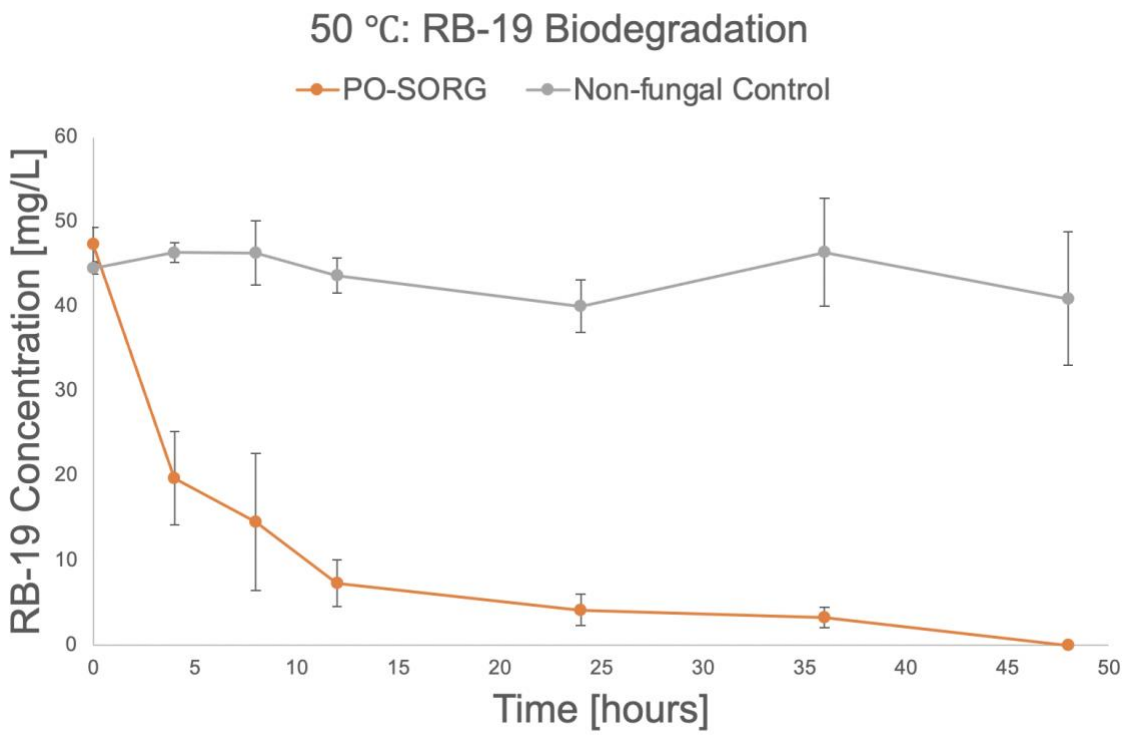


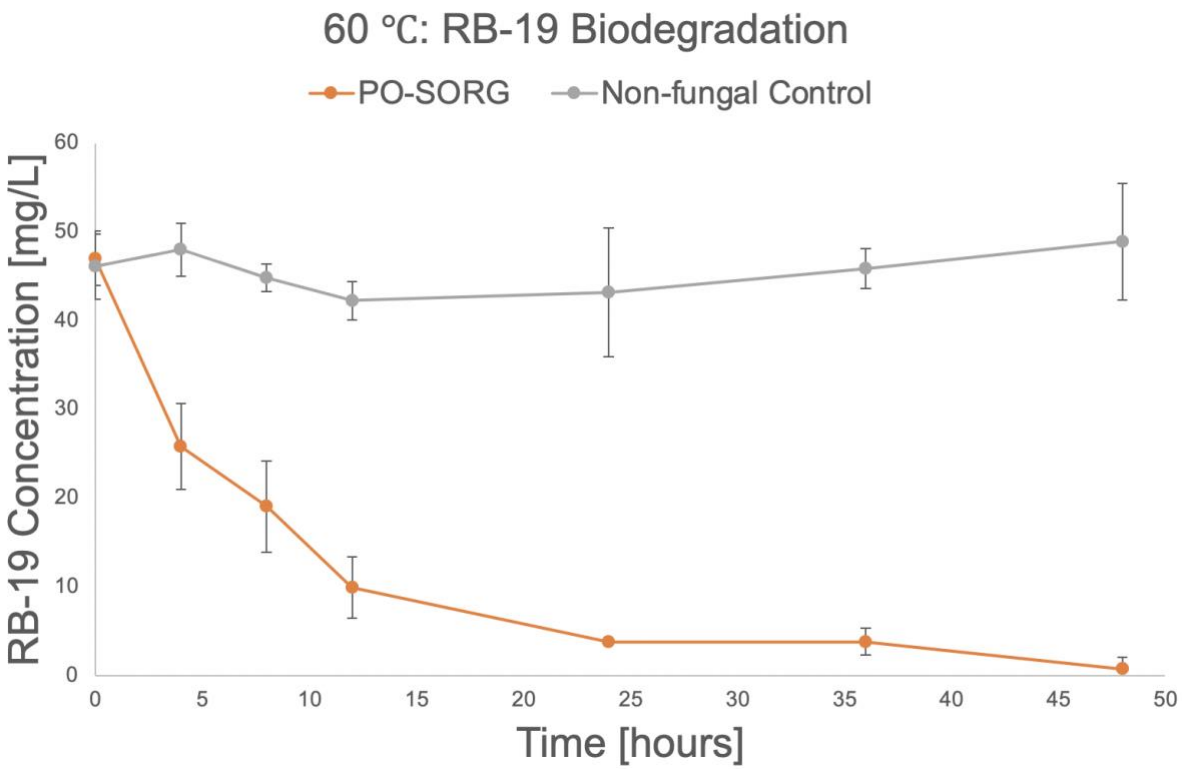
A P value of < 0.05 is indicated by one asterisk (*)

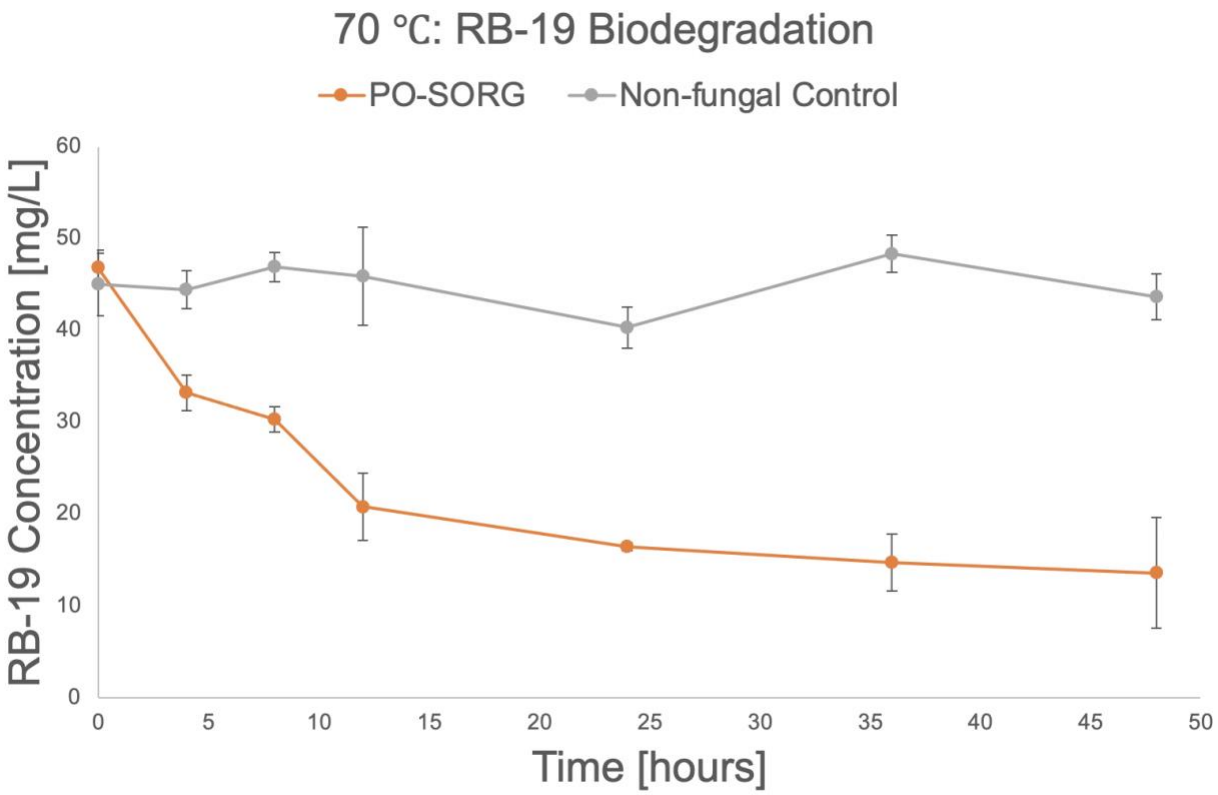
Appendix E: Reactive Blue 19 biodegradation at various temperatures






50 ℃: RB-19 Biodegradation
PO-SORG
Non-fungal Control
RB-19 Concentration [mg/L]
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
45
50
Time [hours]

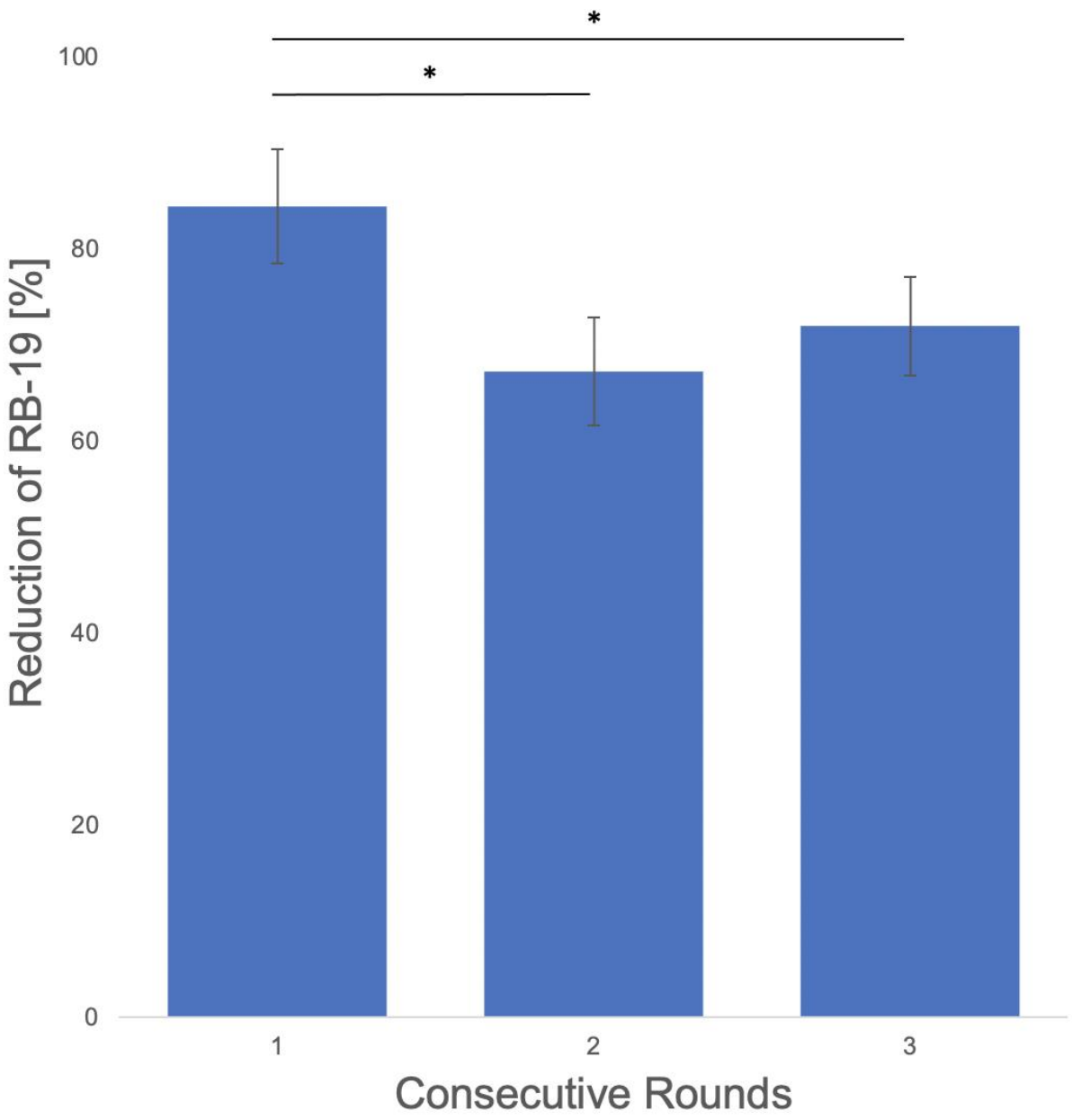

60 ℃: RB-19 Biodegradation
PO-SORG
Non-fungal Control
RB-19 Concentration [mg/L]
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40
45
50
Time [hours]


70 ℃: RB-19 Biodegradation
PO-SORG
Non-fungal Control
RB-19 Concentration [mg/L]
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
Time [hours]

Appendix F: Percent reduction of Reactive Blue 19 after consecutive cycles of exposure to PO-SORG.

A P value of < 0.05 is indicated by one asterisk (*)

experiment conducted at pH 5 and 20℃



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