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Sorghum-grown fungal biocatalysts for synthetic dye degradation

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ABSTRACT

The synthetic dye discharge is responsible for nearly one-fifth of the total water pollution from textile industry, which poses both environmental and public health risks. Herein, a solid substrate inoculated with fungi is proposed as an effective and environmentally friendly approach for catalyzing organic dye degradation. *Pleurotus ostreatus* was inoculated onto commercially available solid substrates such as sorghum, bran, and husk. Among these, *P. ostreatus* grown on sorghum (PO-SORG) produced the highest enzyme activity and was further tested for its dye biodegradation ability. Four dye compounds, Reactive Blue 19 (RB-19), Indigo Carmine, Acid Orange 7, and Acid Red 1 were degraded by PO-SORG with removal efficiencies of 93%, 95%, 95%, and 78%, respectively. Under more industrially relevant conditions, PO-SORG successfully degraded dyes in synthetic wastewater and in samples collected from a local textile factory, which reveals its potential for practical usage. Various biotransformation intermediates and end-products were identified for each dye. PO-SORG exhibited high stability even under relatively extreme temperatures and pH conditions. Over 85% removal of RB-19 was achieved after three consecutive batch cycles, demonstrating reusability of this approach. Altogether, PO-SORG demonstrated outstanding reusability and sustainability and offers considerable potential for treating wastewater streams containing synthetic organic dyes.

1. Introduction

The dyeing industry brings enormous profits to many developing countries (Góralczyk-Bińkowska et al., 2021). However, without proper legislation, manufacturers discharge nearly 11 million tons of dye polluted wastewater into aquatic systems (about 20% of textile industrial pollution) (Chauhan and Choudhury, 2021; Kant, 2012; Routoula and Patwardhan, 2020). Dye-containing wastewater is characterized by light-absorbing pigments, high biological and chemical oxygen demand, and toxic ingredients, which negatively impact receiving environments, if not properly treated (Lellis et al., 2019; Shindhal et al., 2021). While several physicochemical techniques, such as membrane filtration, ozonation, and flocculation (Shindhal et al., 2021), have been used to treat dye effluents, these methods can be inefficient, costly, and generate secondary pollutants (Agrawal and Verma, 2019). A potentially safer and cost-effective involves using microbes and their enzymes to biodegrade synthetic dyes (Lellis et al., 2019). Recent research efforts have focused on the use of fungi in wastewater treatment applications (Arikan et al., 2019; Dalecka et al., 2020; Grelska and Noszczyńska, 2020).

Wood-decaying fungi are well-suited for removing contaminants because they produce powerful oxidative enzymes (Merino et al., 2023). These extracellular ligninolytic enzymes can catalyze the degradation of many organic compounds, including dye compounds (Sen et al., 2016). One of the most commonly reported fungal enzymes is laccase. Laccase is a copper-containing oxidoreductase that uses oxygen molecules as the terminal electron acceptor to catalyze various organic reactions (Gao et al., 2022a). However, adding free fungal organisms to wastewater often clogs pipelines and limits reusability (More et al., 2010). Another approach involving fungal enzyme extracts in pollutant degradation has also been limited due to production costs, environmental stress (e.g., pH and temperature), and limited ability to recover the catalyst materials (Rodríguez Couto, 2009; Wang et al., 2019). To circumvent these concerns, fermenting solid substrates with live fungi has been proposed to produce effective and green biocatalysts. Reported solid substrates

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include rice hull (Li and Jia, 2008), sawdust (Przystaś et al., 2018), and other materials. For example, *Luffa cylindrica*, a natural biomass material, was utilized to support *Stropharia* sp. ITCC-8422, which in turn, was able to degrade and detoxify anthraquinone violet R (Agrawal and Verma, 2019).

Solid substrate-grown fungi offer many advantages including high abundance, low cost, ease of operation, and resilience. The inoculation process is simple and requires only one step: incubating the fungi and the substrate materials together. In addition, the rich and diverse nutrients in these solid substrates support fungal growth, providing sustained enzyme release during experiments. Technically, it is an immobilization process that enhances the biocatalysts' stability, making them less susceptible to environmental stresses. The solid-associated fungal biomass is also easy to separate from the reaction medium, allowing easy replacement. Furthermore, these natural materials can be sourced locally and from naturally grown plants, making them more sustainable and environmentally friendly than other supports. Collectively, fungi grown on natural solid substrates offer multiple advantages as biocatalysts (Wang et al., 2019; Zahmatkesh et al., 2018), making them good candidates for degrading dye compounds.

The objectives of this study were to develop an efficient and green biocatalyst system based on solid substrate-immobilized fungi and investigate its performance in treating individual dye compounds, synthetic textile wastewater (STWW), and a sample of real textile wastewater (RTWW) effluent. To further evaluate the biocatalyst performance, degradation products were identified, enzyme stability under a range of temperatures and pH was examined, and reusability in sequential batch experiments was assessed.

2. Results and discussion

2.1. Characterization of Pleurotus ostreatus grown on different substrates

Among the laccase activities of *P. ostreatus* grown on sorghum (PO-SORG), bran (PO-BRAN), or husk (PO-HUSK) measured over 75 days, PO-SORG showed the highest laccase activity (1.60 U/g on average) (Fig. 1A). On the other hand, PO-HUSK showed the lowest laccase activity throughout the experiment. Therefore, we selected PO-SORG and PO-BRAN to further study mycelia morphologies by scanning electron microscopy (SEM) (Fig. 1B). The hyphae of PO-SORG appeared to be straight and wired to each other, whereas PO-BRAN was characterized by flaky mycelia. These distinct structures of *P. ostreatus* could be due to the different nutrients in sorghum and bran. In addition, the healthy mycelium on sorghum suggested that the ingredients of sorghum are preferred for fungal growth. Furthermore, as shown in Fig. 1B, PO-BRAN was easily disintegrated, making it unfavorable for practical usage. More importantly, PO-SORG maintained its structure even after a 120-day storage at room temperature, superior to previously reported *Trametes versicolor* cultivated on sorghum (Fig. S2) (Zahmatkesh et al., 2018). The morphology and longevity of PO-SORG further demonstrated that it could be a robust biocatalyst for wastewater treatment applications. Transcriptional analysis has been used to study molecular mechanism of biodegradation (Zhang et al., 2021). We further profiled the transcription levels of laccase isozymes produced by PO-SORG along with growth (Fig. 1C). Among the six identified genes coding for laccase isozymes in *P. ostreatus* (*Lacc3, 5, 7, 10, 11, and 12*) (Durán-Sequeda et al., 2022), *Lacc10* showed the highest average upregulation (5.25-fold) within 40 days. Other genes showed slight upregulation or downregulation. These results indicate that the dominant isozymes for PO-SORG are *Lacc10* laccases, which further helped us characterize the PO-SORG biocatalyst.

2.2. Dye degradation by PO-SORG in laboratory buffer and STWW

Among the four dye compounds tested, Reactive Blue 19 (RB-19), Indigo Carmine (IC), Acid Orange 7 (AO-7), and Acid Red 1 (AR-1), RB-19 degradation was relatively rapid, decreasing to 10.1 mg/L within 12 h and then leveling off, as illustrated in Fig. 2A. Laccase activity gradually increased until 12 h into the experiment, when it peaked at 95.0 U/L and slightly declined for the remainder of the trial. IC was degraded to 3.91 mg/L within 48 h, and the laccase activity increased steadily to a

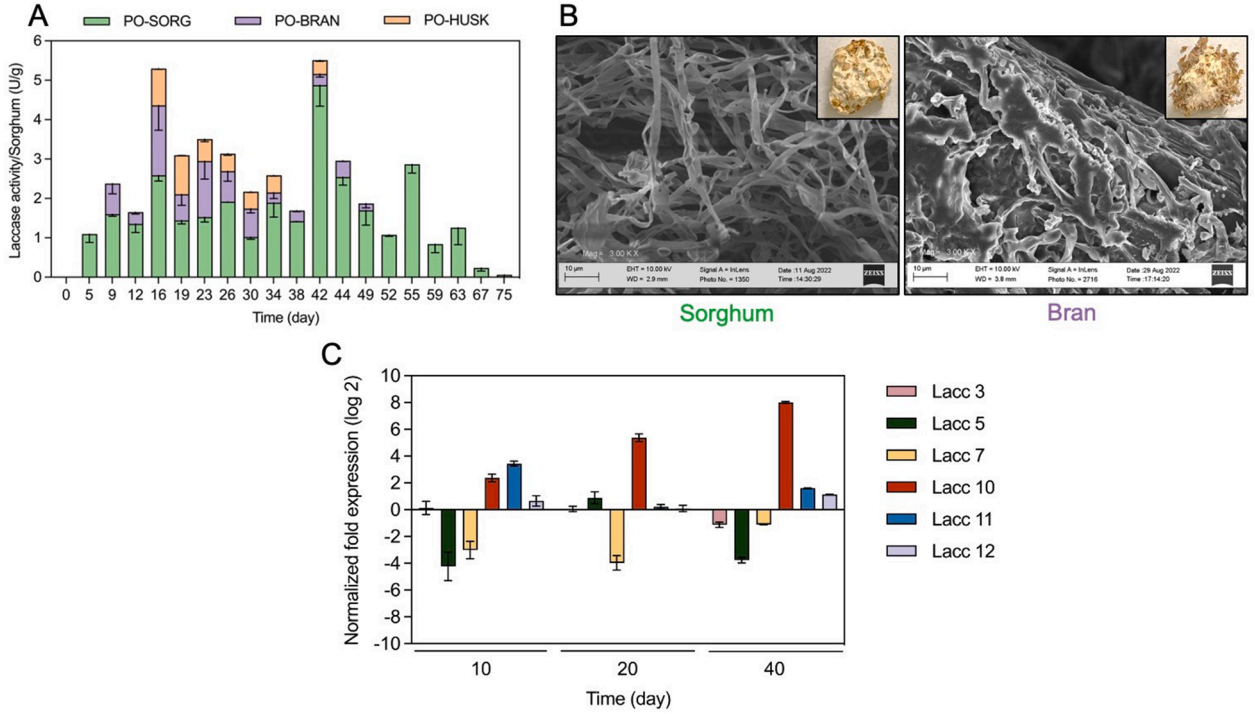

Fig. 1. A: Laccase activity of *P. ostreatus* on sorghum, bran, and husk within 75 days of inoculation.. B: SEM images of PO-SORG and PO-BRAN and corresponding photos (top right) of fungi-colonized solid materials. C: The expression of laccase isozymes produced by PO-SORG quantified at the transcription level. Error bars represent the standard deviation (n=3).

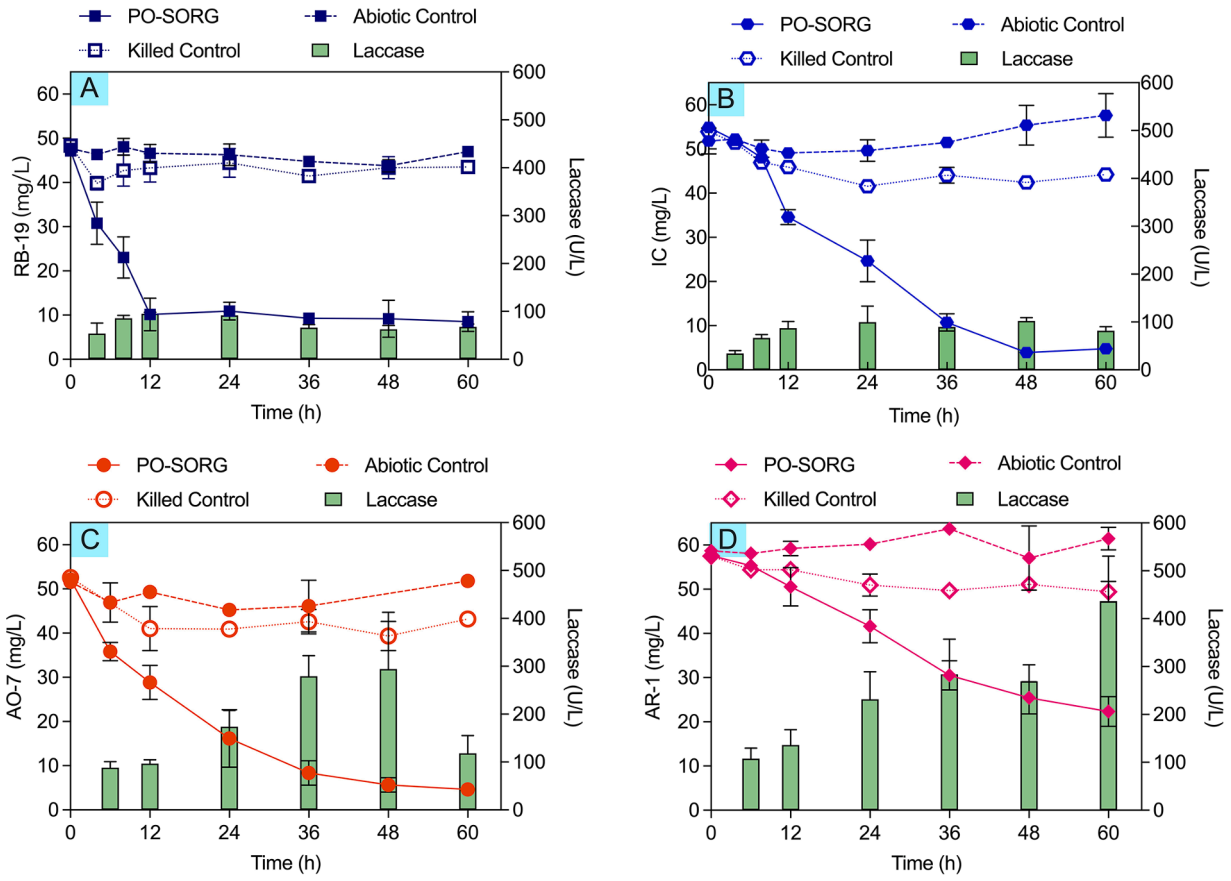

Fig. 2. Degradation of four representative dyes by PO-SORG in buffer solution: RB-19 (A), IC (B), AO-7 (C), and AR-1 (D). Results for controls and laccase activity are also shown.

maximum of 102 U/L in 24 h (Fig. 2B). AO-7 was degraded to 4.65 mg/L after 60 h, with a maximum laccase activity with 279 U/L at 48 h into the experiment, after which it declined to 118 U/L 12 h later (Fig. 2C). AR-1 was the least degraded by PO-SORG, reaching 22.4 mg/L after 60 h. Its laccase activity increased throughout the duration of the experiment, reaching 437 U/L (Fig. 2D). The killed and abiotic controls did not show observable degradation of any dyes. In descending order, the degradation efficiency of PO-SORG can be sorted as RB-19, IC, AO-7, and AR-1. Anthraquinone dye showed the most rapid decolorization, while the azo dyes were harder to degrade, and the rate of indigo dye degradation fell between these two. These results are consistent with previously reported studies (Champagne and Ramsay, 2010; Gao et al., 2022b) and can be attributed to the dye structures. AO-7 and AR-1 have an azo group (-N=N-) with higher bond energy and are less thermodynamically favorable for laccase as the substrate compared to the -C-N- bond in RB-19 and IC.

Additionally, laccase activity presented distinct profiles among dyes. In both azo dye groups, laccase activity peaked above initial activity over time, which is also reported in other studies (Han et al., 2014; Liu et al., 2021). One possible reason is that products generated from azo dye degradation serve as inducers for laccase secretion. These products might even be utilized for fungal growth. In contrast, the parent compounds or products of anthraquinone and indigo dyes play a role in fungal growth inhibition (Friedman et al., 2020; Saleem et al., 2022). It is also possible that more rapid degradation of the anthraquinone and indigo dyes prevented the need for enhanced enzyme production.

In more complex STWW samples, RB-19 degradation was initially slower but still reached a similar final dye concentration of 14.1 mg/L as that in the enzyme buffer. With respect to AR-1, degradation was much slower in STWW than in the buffer and reached a higher minimum dye concentration (Fig. 3A). The slower degradation of AR-1 could be attributed to the other constituents of the STWW such as trace metals and salts that could act as inhibitors to laccase, resulting in slower catalysis (Champagne et al., 2013). However, in the RB-19 group, laccase activity did not show apparent inhibition. This is likely due to RB-19 and formed products association with components in STWW, which lowered any inhibitory effects. It was supported by different or unidentified bisphenol A degradation products in real tertiary treated wastewater (Ozyildiz et al., 2019). These results demonstrate that PO-SORG can effectively degrade various commonly used dye classes even in complex matrices, which justifies further exploration of its applications in actual dye-containing industrial wastewaters.

2.3. *Degradation and detoxification of RTWW by PO-SORG*

As illustrated in Fig. 3B, PO-SORG was tested in a RTWW sample to assess its degradation potential under realistic textile wastewater matrices. Multiple UV-Vis spectra (200–800 nm) were collected over a 72 h period. The peak maximum at 520 nm showed no change during the first 24 h but rapidly decreased thereafter. This result may have occurred as during the first 24 h, PO-SORG expressed lower laccase activity while acclimating to complex components of RTWW, resulting in minimal dye degradation. However, after 24 h, inhibiting components might have reacted, so the degradation rate increased. The toxicity of RTWW to *Escherichia coli* was also reduced 2-fold after a 72 h treatment by PO-SORG (Fig. S3). These results indicate that PO-SORG can be applied to treatment of dye effluents under non-sterile conditions within a reasonable timeframe and that it can reduce the whole effluent toxicity of industrial dye wastewater.

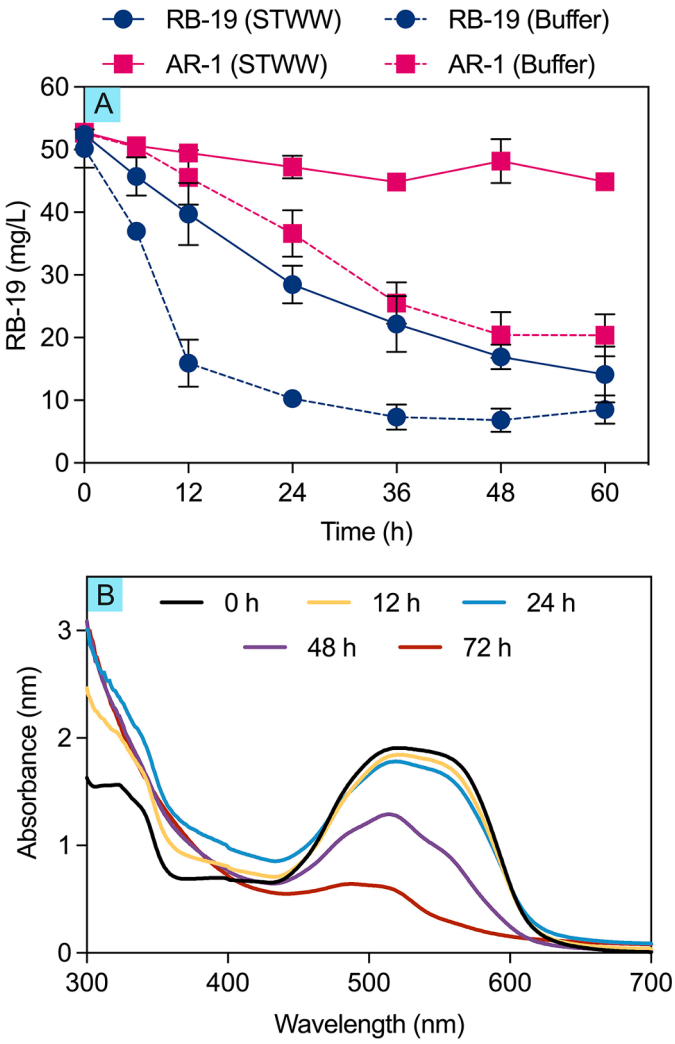

Fig. 3. STWW and RTWW treatment by PO-SORG. A: RB-19 and AR-1 degradation in buffer (lower trace of each pair) and STWW. B: UV-Vis spectra taken during RTWW treatment. A decrease in peak (near 500 nm) height over time indicates removal of colored compounds.

2.4. Biodegradation products of dye degradation

We confirmed several degradation products of RB-19 that could be generated by a previously proposed pathway (Dauda and Erkurt, 2020; Osma et al., 2010) and also identified new products (phthalic acid [$C_8H_6O_4$] and $C_6H_6SO_4$) after ring opening reactions (Fig. 4A). We also identified the formation of possible products by another pathway, pathway 2 in Fig. 4A, *via* generating an aniline group at another -C-N- bond. During AR-1 degradation pathway 1 (Fig. 4B), the hydroxyl group was converted to a phenoxy radical and sequentially oxidized to a ketone group and carbonium ion. Water then showed a nucleophilic attack, followed by C-N bond cleavage. Further, we propose another asymmetrical oxidation pathway 3, owing to observing potential products. A similar mechanism (pathway 3) was reported for methyl orange fungal laccase degradation (Telke et al., 2010). We also identified products that might be formed by azo group reduction (pathway 2), probably due to fungal reductase (Al-Tohamy et al., 2020; Goud et al., 2020; Mani et al., 2019). Another azo dye, AO-7, shares similar mechanisms as AR-1 degradation and have three possible pathways (Fig. S4). Among them, pathway 1 was previously confirmed (Ali et al., 2021; Chivukula and Renganathan, 1995; Wang et al., 2014; Zille et al., 2005), and products generated by. We also proposed degradation pathways for IC, which are shown in Fig. S5. In addition, we examined preliminary product profiles (ESI +/-) of RTWW degradation, illustrated in Fig. S6, which showed the disappearance of initial compounds and the appearance of new compounds within 48 or 60 h (e.g., ESI (+): m/z 144.08, 295.23, and 337.23; ESI (-): m/z 207.01, 313.24, and 329.23). Future research should focus on the identification of these compounds in RTWW.

2.5. Stability and reusability of PO-SORG

The temperature and pH of textile wastewater can vary greatly, even within a single factory. The stability of a biocatalyst to resist these fluctuations is directly related to the treatment performance. As illustrated in Fig. 5A, even when the temperature was increased to 70 °C after 12 h, PO-SORG still achieved around 58% removal efficiency, demonstrating its high stability towards thermal stress. However, when the pH was increased to 10, the dye removal efficiency greatly decreased. These results indicate that PO-SORG performs poorly in highly basic media, consistent with the reported favorable acidic pH range of *P. ostreatus* laccase (Vinoth et al., 2022). We hypothesize that the added stability of PO-SORG is due to the protection afforded by its attachment to the solid sorghum surface. There is a possibility that laccase secreted by *P. ostreatus* also is immobilized on the surface, which results in enhanced thermal and pH stability.

From an enconomic and environmental perspective, the reusability of a biocatalyst is a crucial factor for practical application to wastewater treatment (Zdarta et al., 2022). As illustrated in Fig. 5B, effective dye degradation was observed over three successive catalyst uses. There was no degradation observed in the control groups (Fig. S7). After three cycles, PO-SORG still degraded RB-19 from 51 mg/L to 15 mg/L after 72 h. Moreover, by using nylon mesh bags, PO-SORG was easily removed from the test solutions after each cycle, demonstrating its excellent operational potential. However, additional time was needed after each cycle to achieve maximum degradation. This could be ascribed to a decrease in laccase activity (from 105 U/L to 20 U/L), because the rate of laccase synthesis was slower than the replacement of the dye solution. Therefore, PO-SORG required a regrowth period between each round. After 1 day of recovering at 30 °C, PO-SORG could be used for up to six rounds (Fig. S8). Overall, these results demonstrate the potential of PO-SORG as a robust biocatalyst for treating dye wastewater.

3. Conclusions

An effective biocatalyst system consisting of fungi inoculated on solid sorghum, PO-SORG, was developed and demonstrated treatment of dye-containing wastewater. PO-SORG showed prolonged laccase production and robust structure, making it practical for dye treatment. Four representative dye compounds in deionized water as well as synthetic and real wastewater solutions were successfully degraded by PO-SORG to less toxic substances. The biocatalyst material performed well under temperature and pH variations typical of actual textile effluents and was shown to be reused for three successive cycles with minimal loss in degradation efficiency. Overall, our findings demonstrate the sustainability of the PO-SORG material and its potential as a practical and robust biocatalyst for treating textile wastewater streams. Further studies on process scale-up and testing are needed to assess this system's utility for various commercial applications.

4. Materials and methods

4.1. Microorganisms and chemicals

P. ostreatus (taxid:5322) was isolated from the fungal culture purchased from Root Mushroom Farm. Bob's Red Mill Sorghum, Jiva Organic Whole Psyllium Husk, and Bob's Red Mill Wheat Bran were used for fungal growth. The RTWW was obtained from an active textile factory in Vernon, California. Dye compounds, RB-19, IC, AO-7, and AR-

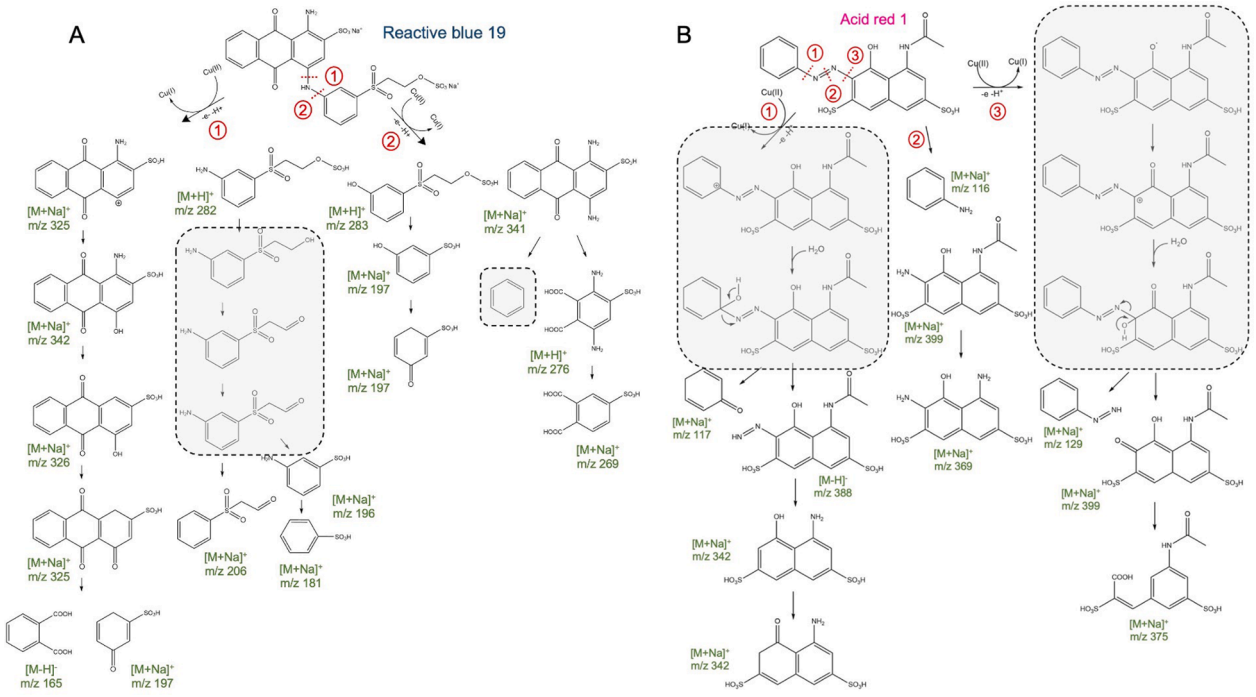

Fig. 4. Proposed dye degradation pathways. The compounds in gray squares are speculated intermediates and products (not detected by LC/MS). A: RB-19; B: AR-1.

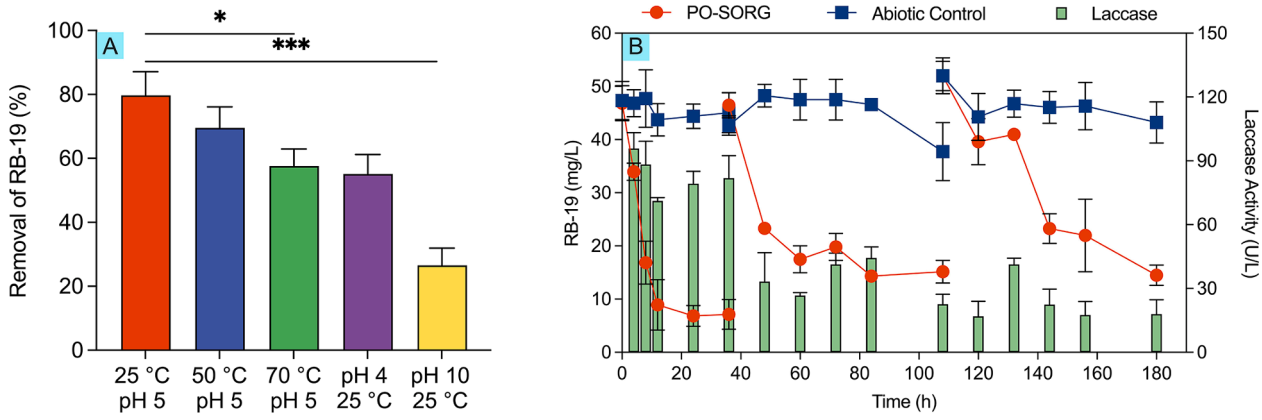

Fig. 5. Stability and reusability properties of PO-SORG. A: Extreme pH and temperature effects on PO-SORG catalyzed RB-19 degradation. B: Reusability study of PO-SORG. PO-SORG can be reused in three consecutive cycles and still achieve similar degradation level of initial cycle. The *t* test was performed between groups of 25 °C and 70 °C as well as pH 5 and 10. $*P < 0.05$ and $***P < 0.001$, N=3.

1 were obtained from Sigma Aldrich. Other chemicals were purchased from Thermo Fisher Scientific.

4.2. Preparation of solid substrate grown fungi

P. ostreatus was cultivated on Yeast Extract-Peptone-Dextrose (YPD) agar plates for 6 days at 30 °C until the entire plate was fully colonized. To prepare each bioreactor, 20 g of sorghum, bran, or husk was added to a 250-mL polypropylene Erlenmeyer flask and autoclaved. In an aseptic environment, six *P. ostreatus* mycelium agar plugs (~ 1 cm × 1 cm) collected from the YPD plates and 8 mL of liquid Tisma medium (Tišma et al., 2011) were added to each flask to initiate growth and maintain appropriate humidity level. Next, a sterile cotton ball was wrapped in an ethanol-sterilized Kimwipe to plug the top of the reactor. Each bioreactor containing PO-SORG, PO-BRAN, or PO-HUSK was placed in a stationary incubator at 30 °C. The bioreactors were mixed by gentle swirling every 3 days to ensure homogenization.

4.3. Laccase activity of solid substrate grown fungi

Laccase activity was evaluated by measuring the absorbance change at 420 nm as 2 mM 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was oxidized. In a cuvette, 10 μL of enzyme extract was combined with 490 μL of 2 mM ABTS in sodium phosphate buffer. The activity was determined by observing the absorbance change every 2 s for 2 min (ε = 36,000 L/mol × cm) on Nanodrop 2000. The activity in the supernatant (U/L) was first calculated using a previously reported equation (Lothe et al., 2020). Next, the activity was converted to units per gram of fungi grown on solid substrates (F-SS) (U/g) based on the following equation:

$$Activity\ [U/g\ F-SS] = \frac{Activity\ [U/mL] \cdot (4.5\ mL\ of\ DI\ water)}{0.5\ g\ of\ F-SS} \quad (1)$$

4.4. Characterization of solid substrate grown fungal systems

Laccase activity was quantified frequently over the incubation time. Before sampling, the PO-SORG was well mixed to homogenize the material. Subsequently, 0.5 g of the colonized sorghum was added to a falcon tube along with 5 mL of sterile deionized water. The falcon tube was inverted 5 times to mix the contents and then incubated at 30 °C for 15 min. The supernatant was then collected for laccase activity measurement (Gao et al., 2022b). Small fungal mycelia were collected after 20 days from all media using a previously reported method (Soh et al., 2021), except that the drying period was 6 h (48 °C). Samples were sputter-coated with Pt for SEM imaging. Genes coding for laccase isozymes were amplified by quantitative polymerase chain reaction (qPCR) using the method described in the SI (details in S1) to examine the expression of laccase isozymes from PO-SORG. Forward and reverse primers are listed in Table S1. The schematic of PO-SORG production and characterization is shown in Fig. S1.

4.5. Dye degradation in buffer

Four different dye compounds, RB-19, IC, AO-7, and AR-1 were selected to represent the common dye categories of anthraquinone (RB-19), indigo (IC), and azo dyes (AO-7 and AR-1). Live experimental groups were prepared in 250 mL polypropylene Erlenmeyer flasks. Each consisted of pre-grown PO-SORG and 50 mL of a 50 mg/L dye solution dissolved in a pH 5 Na-PO_4 buffer. The enzyme activity in units per gram of PO-SORG (U/g) was measured before the experiment. Subsequently, a calculated amount of PO-SORG (details in S2) was added to reach 100 U/L of the initial laccase activity. An abiotic control contained dye, buffer, and uncolonized sorghum. A killed control solution contained dye and autoclaved PO-SORG to distinguish dye sorption from degradation. All solutions were prepared and analyzed in triplicate. At each sampling event, 500 μL was taken from each flask. The dye concentration was determined by measuring the absorbance of the sample at the maximum absorbance wavelength (Table S2), and laccase activity was also measured.

The effects of temperature and pH on dye degradation in buffered solutions were also examined (details in S3) in the ranges reported for textile wastewaters (Yaseen and Scholz, 2019). The reusability of PO-SORG was tested in the buffer by using the same set of PO-SORG materials in consecutive dye degradation trials. PO-SORG was placed in sterile nylon tea bags for confinement and used in the next round. The first degradation round ceased when the dye concentrations remained unchanged after 12 h. The degraded dye solutions were removed from the live and control flasks and replaced with a fresh 50 mL RB-19 solution (50 mg/L) to begin subsequent rounds.

4.6. Dye degradation in STWW and RTWW

The STWW was prepared according to a previously reported composition (details in S4) (Yaseen and Scholz, 2019). RB-19 and AR-1 were dissolved in STWW and degraded by PO-SORG following the same conditions described above. Since the RTWW matrix was too concentrated to obtain UV-Vis spectra, it was diluted 5-fold using non-sterile tap water. PO-SORG with an initial laccase activity of 500 U/L was added to 50 mL of the diluted RTWW under non-sterile conditions. Spectra were collected over a wavelength range from 200 to 700 nm using a UV–Vis spectrometer (Lambda 365, Perkin Elmer). Whole effluent toxicity of RTWW before and after PO-SORG treatment was measured in terms of ATP content of *E. coli* using reported method (Gao et al., 2022b).

4.7. Analysis of degradation products

For RB-19 and AO-7 groups, five 2 mL samples were collected at different time stamps (6, 12, 24, 48, and 60 h). The reaction was stopped by adding 100 μL of 98% sulfuric acid, and the samples were incubated for 30 min. Then, 2 mL of ethyl acetate was added to the extract products, and the sample was vacuum concentrated into crystal, which was in turn, dissolved in 100 μL of methanol. For the IC, AR-1, and RTWW groups, 2 mL of the solution was subjected to solid phase extraction (Wang et al., 2019b) and concentrated in 100 μL of methanol. Concentrated samples were analyzed using an Agilent 1290 LC-6540 Q-TOF mass spectrometer to characterize and identify transformation products. Detailed information is provided in the SI (details in S5).

CRediT authorship contribution statement

Yifan Gao: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Benjamin Croze:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Quinn T. Birch:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Mallikarjuna N. Nadagouda:** Conceptualization, Visualization, Validation, Resources, Writing – review & editing, Supervision. **Shaily Mahendra:** Conceptualization, Methodology, Validation, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2023.100181.

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