Pharmaceutical and personal care product removal and microbial community succession in fungal wheel reactor using solid-state fermentation

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Abstract

Wood-rotting fungi and their enzymatic systems represent promising biocatalysts for the removal of pharmaceuticals and personal care products (PPCPs) from wastewater. We designed a fungal wheel reactor (FWR) based on solid-state fermentation (SSF) of *Trametes versicolor* and a lignocellulosic substrate, which was used as an immobilization carrier for fungal biomass and the sole initial nutrient source for producing fungal oxidative enzymes. Three pharmaceutical and personal care products, acetaminophen, bisphenol A and carbamazepine, were spiked into the synthetic wastewater and the treatment was carried out under non-sterile conditions. Acetaminophen was completely removed from the FWR until laccase was observed. The acetaminophen removal efficiency was retrieved by replacing the fungal wheel with fresh SSF products. Bisphenol A and carbamazepine were removed via enzymatic activity and adsorption. In FR30, where the fungal wheel was replaced, remarkable removal of acetaminophen was observed, even after laccase depletion. The microbial community analysis indicated that the continuous removal of acetaminophen was mainly due to the high proportion of *T. versicolor*. The relative abundance of the co-occurring microbial community might be responsible for the divergence in acetaminophen removal between FR30_R1 and FR30_R2. Overall, FWRs are promising tools for the removal of PPCPs by highly reactive enzymatic mechanisms as well as adsorption on the carrier surface. By replacing SSF and settled microbial communities, FWRs may continuously contribute to bioremediation over a long-term period.

Keywords: Pharmaceutical and personal care products, wood rotting fungi, fungal wheel reactor, solid-state fermentation, microbial community analysis

1. Introduction

Pharmaceuticals and personal care products (PPCPs), which are widely used by humans, are a group of biologically active pollutants. The risk of PPCPs in aquatic environments was first reported in the late 1970s, and their adverse effects on ecosystems are still being revealed (Ebele et al., 2017). PPCPs can easily bioaccumulate in living organisms, disrupt the endocrine system, and cause both acute and chronic diseases depending on their concentration (Keerthanan et al., 2021). Despite their potential risk, the consumption of PPCPs has been rapidly increasing, and high amounts of PPCPs have been detected in wastewater treatment plants (WWTPs). Conventional WWTPs were originally designed for the removal of pollutants that occur at high load concentrations, such as diverse carbon, nitrogen, and phosphorus compounds (Helbling et al., 2012). As only trace concentrations of PPCPs (in the range of ng to μg/L) are found in WWTPs, their bioavailability is substantially lower compared to that of "conventional" substrates of biological systems (Zarei-Baygi et al., 2019). Consequently, partially removed PPCPs are released through effluents and threaten aquatic environments by exerting undesirable effects (Petrie et al., 2015).

Wood-rotting fungi have been extensively studied as cost-effective solutions for PPCP removal (Harms et al., 2011). These microorganisms harbor a suite of extracellular enzymes that are secreted to colonize diverse lignocellulosic substrates but also contribute to PPCP removal (Goodell et al., 2020). Laccase (EC 1.10.3.2) is a well-studied extracellular enzyme that can degrade a broad range of aromatic compounds and requires only oxygen as the final electron acceptor (Baldrian, 2006). The successful removal of PPCPs has been achieved using a batch system with purified laccase (Xu et al., 2000), a laccase mediator system with chemical compounds (Niladevi and Prema, 2008), and crude laccase induced by lignocellulosic substrates (Wang et al., 2014). Nevertheless, shortcomings remain in the long-term operation of fungal bioreactors. If laccase is considered a key enzyme for PPCP removal in bioreactors,

it should ideally be present throughout the operating period. To maintain laccase activity in continuous operation, many studies have performed reactor inoculation with fungal species rather than permanently injecting laccase solution (Rodríguez-Rodríguez et al., 2012). However, this practice necessitates the supplementation of substrates (e.g., glucose and ammonium tartrate) for fungal growth and laccase production, which increases the operational costs in real application settings (Mir-Tutusaus et al., 2018). An adequate oxygen supply is another important prerequisite to provide sufficient environmental conditions for fungal species and to initiate reactions of oxidative enzymes such as laccases.

In real-world application scenarios, reactors and matrices are non-sterile, and bioaugmented fungal species would have to cope with the presence of other microorganisms, which can compete for substrates and overtake fungal species that are alien to wastewater and reactor environments (Badia-Fabregat et al., 2017). Some studies have also demonstrated that synergistic effects occur when combining fungal and bacterial communities (Badia-Fabregat et al., 2016). Nevertheless, the fungal-bacterial interactions in complex microbial communities for wastewater treatment performance are still poorly understood. In this context, the priority should be to provide internal conditions favorable for the desired fungal species to address the aforementioned complications.

Hence, we designed a lab-scale fungal wheel reactor (FWR) based on the immobilization of the wood-rotting fungus *Trametes versicolor* on lignocellulosic substrate. *T. versicolor* was fermented in a solid state with ash wood chips, which were used as preferred substrates for fungal growth, thereby concomitantly stimulating the production of lignin-modifying enzymes, such as laccase, by the presence of lignin-related wood constituents. Rotation of the fungal wheel offers sufficient aeration for both fungi and laccases. Synthetic wastewater was spiked with acetaminophen (ACE), bisphenol A (BPA), and carbamazepine (CBZ), and their removal was assessed in a time series during the operation of the FWR for two months. To explore the

significant parameters related to PPCP removal, water quality parameters [e.g., pH, dissolved oxygen (DO), total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD), and colony forming units (CFUs)] were analyzed. Finally, fungal and bacterial communities from the FWR were evaluated to gain a deeper understanding of the potential fungal-bacterial interactions.

2. Materials and methods

2.1. Materials

ACE and CBZ; Anti-inflammatory analgesic drug, and BPA; a primary chemical compound for manufacturing fields were selected as target compounds for this study because of their frequent detection in the effluent as well as the influent of WWTPs in many countries (Bahlmann et al., 2014; He et al., 2020; Lassouane et al., 2019).

ACE, BPA, CBZ, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were purchased from Avantor J. T. Baker (Radnor, PA, USA). Polypropylene filter bags (25 mm in diameter), shafts (2 mm in diameter), rotating motors (3 rpm), and other materials for manufacturing the FWR were purchased from a domestic online market (Seoul, Republic of Korea).

2.2. Fungal incubation and solid-state fermentation

The white-rot fungus *T. versicolor* KCTC 26203 (obtained from the Korean Collection for Type Cultures, Jeongeup, Republic of Korea) was maintained by subculturing on potato dextrose agar at 25 °C in the dark. Five agar plugs from the mycelium-grown plate were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract medium (ME:20 g/L malt extract, 1 g/L peptone, pH 4.5). The fungal inoculum was prepared by homogenizing seven-day-old mycelial cultures. Ash wood chips were cut into fine sections (0.7–1.0 cm),

washed three times with distilled water, and autoclaved at 121 °C for 15 min. Sterile ash chips and 5% (v/v) of fungal inoculum were well mixed then incubated at 25 °C in dark until ash chips were completely colonized by fungal mycelium to be used as solid-state fermentation (SSF) products (~ 4 weeks).

2.3. PPCP removal by free laccase and SSF products in batch

Laccase was produced by inoculation (1% fungal inoculum; v/v) of 100 mL of peptone solution (1 g/L) containing 20 g/L of sterile ash chips in addition (pH 4.5), and subsequent incubation at 25 °C and 120 rpm for 10 days. The cell-free supernatant was obtained by filtering the culture broth (0.2 μm) and stored at −20 °C until use as a source of free laccase. PPCP removal assays employing free laccase were conducted in 50 mL glass vials containing 10 mL of McIlvaine buffer (pH 4.5) with a mixture of ACE, BPA, and CBZ at 20 mg/L (McIlvaine, 1921). The reactions were initiated by adding 100 μL of free laccase (~ 270.0 U/l of laccase activity), and the vials were mixed well and incubated at 24°C in the dark for 24 h. To assess the effect of SSF products, PPCP-spiked McIlvaine buffer was supplemented with 1 g of SSF products. Non-inoculated ash chips were used as control conditions for the SSF. Incubation under dark conditions was performed for 24 h for ACE and BPA and 96 h for CBZ.

During the respective incubation times, 500 μL of supernatant was collected at different time points (0, 0.5, 1, 2, 4, 6, 12, and 24 h for ACE and BPA and an additional 48, 72, and 96 h for CBZ considering its recalcitrance), mixed with 500 μL of methanol, and supplemented with 10 μM sodium azide to stop the enzyme activity. One milliliter of the prepared sample was centrifuged at 14,000 rpm for 15 min. The supernatant was then analyzed via reverse-phase liquid chromatography coupled with a photodiode array detector (HPLC-PDA; Nexera XR, Shimadzu, Kyoto, Japan). For chromatographic separation, a YMC-Triart C18 column (4.8

mm × 250 mm; particle size, 3.5 μm; YMC, Kyoto, Japan) was used following the method described in detail by Kang et al. (Kang et al., 2019).

2.4. FWR setup and operation

The trapezoidal reactors were composed of acrylic material with a 250 mL operation volume capacity (Fig. 1; base length = 12 mm; top length = 19 mm; height = 8.5 mm; width = 30 mm). The upper ceiling of the reactor was opened to mimic the conditions of real WWTPs, and two holes were drilled on each side of the reactor to fit the shaft. The forepart of the reactor was connected to a peristaltic pump (L/S® Variable Speed Analog Console Pump, Masterflex, Gelsenkirchen, Germany) equipped with a multichannel pump head and platinum-cured silicone tubing (0.8 mm inner diameter). The fungal wheel was prepared by filling a round polypropylene filter bag (25 mm diameter) with 20 g of one-month-old SSF products. A mounted shaft (2 mm) penetrating the middle of the fungal wheel horizontally was connected to a rotating motor operated at a speed of 3 rpm.

Synthetic wastewater was adjusted to half the concentration of the standards used for effluent water quality in domestic wastewater treatment in the Republic of Korea and contained 20 mg/L COD, 10 mg/L T-N, and 0.1 mg/L T-P (Ministry of Environment, 2019). The concentrations of the carbon, nitrogen, and phosphorus sources corresponded to 18.6 mg/L glucose, 60.6 mg/L $NaNO_3$, and 0.4 mg/L K_2HPO_4, respectively. In addition, 7 mg/L NaCl, 4 mg/L $CaCl_2 \cdot 2H_2O$, and 2 mg/L $MgSO_4 \cdot 7H_2O$ were injected to provide micronutrients. The pH was adjusted to 6.0 based on both the optimum pH reported for *T. versicolor* (pH 4.5) and an average wastewater pH value of 7.8 from WWTPs (Margot et al., 2013a; Popa et al., 2012). The synthetic wastewater was sterilized by filtration at 0.2 μm. The flow was fed through the tubing at a rate of 34 μL/min, and a hydraulic retention time of 2 d was applied. The working volume was set at 100 mL, thus allowing the overflow to leave the reactor.

To modulate the optimal rate of laccase secretion and ensure appropriate aeration during the continuous operation of the reactor, several strategies were applied: (A) submerging the fungal wheel by half and rotating continuously; (B) submerging the fungal wheel by half and rotating intermittently (succession of 1 h rotation and 1 h rest, respectively); (C) submerging the fungal wheel at a depth of 10 mm and rotating continuously; (D) submerging the fungal wheel at a depth of 10 mm and rotating intermittently (1 h rotation and 1 h rest, respectively); and (E) submerging the fungal wheel completely and aerating continuously at 0.08 L/min. The details of each condition are listed in Table S1.

The removal efficiencies of single compounds and mixtures of PPCPs (ACE, BPA, and CBZ) during the continuous operation of FWRs were tested separately (referred to as operation 1 and 2, respectively). Uninoculated sterile ash chips were used in the control reactor. During operation 1, three PPCPs were added to the synthetic wastewater at 20 mg/L in independent sequential phases. Between the sequential injections of target compounds, periods without PPCP addition of 13 to 18 days were used to avoid the coexistence of different PPCPs in the FWR. Two control reactors and three FWRs were operated for two months, respectively. In operation 2, a mixture of three PPCPs in synthetic wastewater (concentration 20 mg/L) was treated in two parallel replicates of non-inoculated controls (NC60) and four parallel replicates of the FWR (F30 and F60, respectively). After 30 days, the fungal wheels from two replicates (F30) were replaced with fresh SSF products (FR30). Thereafter, the operation of the reactors (F60, FR30, and NC60) continued until day 60. During the process, 5 mL of effluent samples was collected every two–three days. Samples were centrifuged at 8,000 rpm for 20 min and filtered through a 0.2 μm. Aliquots of 200 and 500 μL were used to determine the laccase activity and PPCP concentration, respectively. The remaining 4 mL of the samples was stored at −20 °C until the chemical property analysis. The cell pellets obtained from centrifugation

and SSF products of F30, F60, FR30, and NC60 were stored at −80 °C for further sequencing analysis.

2.5. Assays

2.5.1. Laccase activity

Laccase activity was measured spectrophotometrically by monitoring the oxidation of 0.5 mM of 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at 420 nm using 0.1 M acetate buffer (pH 4.5) (ε_{420} = 36,000 M^{-1} cm^{-1}) (Margot et al., 2013b). One unit of laccase activity (U) was defined as the amount of laccase that oxidized 1 µmol of substrate per min.

2.5.2. Chemical properties

The pH and DO values were monitored directly from the reactor supernatant using a portable multiparameter meter (Orion Star™ A329, Thermo Scientific, MA, USA). Total organic carbon was determined using a TOC analyzer (TOC-V CPH, Shimadzu, Japan). The analysis of N (NH_4^+-N, NO_2^--N, NO_3^--N, and TN) and P (PO_4^{3-}-P and TP) followed the standard methods of the U.S. Environmental Protection Agency, although they were downscaled and optimized to fit a 96-well plate format (Baird and Bridgewater, 2017). Microplates were analyzed spectrophotometrically using a multimode microplate reader (Spark®, Tecan, Switzerland). All measurements were performed in triplicate.

2.5.3. Enumeration of suspended bacteria in the reactor

For each time point of sampling, 200 µL of supernatant was collected and serially diluted (10^{-1} to 10^{-2}) using a PBS buffer, and then 100 µl of the dilutions were plated on nutrient agar (BD Biosciences, NJ, USA), followed by incubation at 30 °C for 24 h. Viable colony numbers were counted and expressed as CFU/ml.

215

216 **2.6. DNA extraction and amplicon sequencing**

217 Genomic DNA from wood and supernatants was extracted using the FastDNA™ SPIN Kit
218 for soil (MP Biomedicals, CA, USA) according to the manufacturer's instructions. Bacterial
219 16s rRNA genes and the ITS1 region of the fungal rRNA gene were amplified using the primers
220 518F (5′- CCAGCAGCYGCGGTAAN-3′) - 926R (5′- CCGTCAATTCNTTTRAGT-3′) and
221 18SF (5′- GTAAAAGTCGTAACAAGGTTTC-3′) - 5.8SR (5′-GTTCAAAGAYTCGATGAT
222 TCAC-3′). Illumina Nextera adaptors (Illumina, CA, USA) were attached to each primer
223 (Glenn et al., 2019). Primers were synthesized by Macrogen Inc. (Seoul, Republic of Korea).
224 PCR assays were carried out in a total volume of 25 µL, which contained 12.5 µL of 2x KAPA
225 HiFi HotStart ReadyMix (Roche, Switzerland), 0.2 µM of each primer, and 15 ng DNA
226 template.

227 The thermal cycle conditions for 16s rRNA were initialized for 3 min at 95 °C, followed by
228 25 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C, 1 min of extension at 72
229 °C, and a final elongation for 10 min at 72 °C. Cycling conditions for the fungal ITS gene were
230 as follows: 3 min at 95 °C, followed by 33 cycles of 30 s of denaturation at 95 °C, 30 s of
231 annealing at 55°C, 1 min of extension at 72 °C, and a final elongation for 10 min at 72 °C.
232 Triplicate amplicons from each condition were pooled for clean-up using AMPure XP beads
233 (Beckman Coulter, UK). Sequencing libraries were generated using the Nextera XT Index Kit
234 v2 (Illumina) and unique dual Nextera indices according to the manufacturer's
235 recommendations. The PCR conditions were as follows: 95 °C for 3 min, followed by 8 cycles
236 of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, and finally 5 min at 72 °C. Libraries were cleaned
237 with AMPure XP beads and the quality of the enriched libraries was evaluated using a
238 microplate reader. The libraries were sequenced using the Illumina MiSeq platform
239 (Macrogen).

240

241 **2.7. Sequencing data analysis**

242 The raw paired sequences were analyzed using Quantitative Insights into Microbial Ecology
243 (QIIME2 v2020.11) (Bolyen et al., 2019). The imported forward and reverse sequences were
244 assembled, demultiplexed, and filtered using the plugins implemented in QIIME2. Near-error-
245 free sequences were obtained using the Deblur plugin (Amir et al., 2017). The sequences were
246 clustered into a single OTU using open reference OTUs at a level of 97% identity. All
247 representative sequences per OTU were retrieved and used for classification at the different
248 taxon levels. Taxonomic classification was performed using scikit-learn multinomial naïve
249 Bayes methods trained with reference sequences (Bokulich et al., 2018). Operational
250 taxonomic units (OTUs) were referenced from the SILVA v132 and UNITE fungal ITS
251 databases (version 8.3) for 16s rRNA and fungal ITS, respectively (Nilsson et al., 2019; Quast
252 et al., 2012). The sequences were deposited in the NCBI Sequence Read Archive under
253 accession number PRJNA749881.

254

255 **2.8. Statistical analysis**

256 All statistical analyses were performed using the R platform (Development Core Team, 2013).
257 Normal data distributions were evaluated using the Shapiro-Wilk normality test. Non-metric
258 multidimensional scaling (NMDS) was performed to visualize differences in fungal and
259 bacterial communities in F30, F60, FR30, and NC60 using the Bray-Curtis dissimilarity index.
260 The microbial community composition was analyzed at the OTU level with the hclust and
261 metaMDS functions using the vegan package. A Venn diagram was constructed to visualize
262 the overlap of OTUs detected under different conditions. The differences between the relative
263 abundance of microbial communities in the reactor sets were considered significant at P values
264 < 0.05.

3. Results

3.1. PPCP removal by SSF products in batch systems

To evaluate the PPCP removal efficiency by SSF products, these products were applied to the treatment of ACE, BPA, and CBZ in batch systems. The ACE concentration decreased by 35% within the first 30 min, and it was completely removed after 2 h (Fig. 2A). In addition, 82% of the initial BPA was removed during the first 30 min (complete removal after 12 h; Fig. 2B). In the control, 28% and 78% of ACE and BPA were removed during the first two hours, respectively, and then relatively stable levels remained until the end of the reaction. This result suggests that the enzymatic system is the primary factor for the removal of ACE, while adsorption is the primary factor for the removal of BPA. In the case of CBZ, approximately 45% was removed during the first two hours from both the control and SSF products, implying that most of the CBZ was adsorbed on the surface of lignocellulosic substrates (Fig. 2C). The removal mechanism of BPA and CBZ mainly appeared to be adsorption, and compared to the reaction with free laccase, the removal of BPA and CBZ was improved by SSF as much as 15.3 and 2.3 times in the first two hours, respectively (Fig. S1). Taken together, the results confirmed that the removal of PPCPs, especially those that were hardly removed by the enzymatic system alone, could be improved by combination with adsorption through SSF products.

3.2. Behavior of laccase in FWR experiments

As the produced laccase could be easily extracted and flowed out with the inflow of synthetic wastewater, various heights and rotation rates of the fungal wheel were configured to modulate appropriate laccase activity during the continuous operation of the FWR (Table S1). Laccase activity decreased over time, especially at a lower position on the fungal wheel (conditions A

and B, Fig. S2). When the fungal wheel was completely submerged (condition E), the initial laccase activity was higher than that under other conditions, although it decreased rapidly, as shown in condition D. Additional aeration for condition E did not seem to be effective in maintaining laccase activity. Condition C showed an increase in laccase activity after day 1, which might indicate presumptive laccase reproduction. Finally, condition C was selected for further reactor setup.

Laccase activity during operation 1 of the FWR under condition C was monitored for 61 days (Fig. S3). Until day 10, 60% of the initial laccase activity was maintained, whereas after day 33, the activity decreased below 5 U/l. From operation 2, a parallel trend of laccase activity was indicated with operation 1, while the SSF product-replaced reactor (operation 2_R) showed distinct behavior (Fig. S4). When SSF products were replaced, the laccase activity reached 88.5 U/l at day 2, which was 1.5 and 1.9 times higher than the initial laccase activity from operation 1 and operation 2, respectively. It was then rapidly exhausted over the first 12 days.

TOC is an indicator of the aging of lignocellulosic substrates in this study, and it was compared to the laccase activity results (Fig. S5). Similar to the laccase activity, the initial secretion of TOC from operations 1 (1156 mg/L) and 2 (803 mg/L) was comparable, followed by similar multiple R-squared values between the laccase activity and TOC (0.8076 for operation 1 and 0.8095 for operation 2). However, a relatively lower initial TOC concentration (489.2 mg/L) was shown in the SSF-replaced reactor, which led to a low multiple R-squared value (0.6682). In this case, the replaced SSF products might have a greater age and thus laccase secretion was not maintained continuously owing to the lack of substrates.

3.3. Removal of PPCPs and chemical properties during continuous operation of the FWR

From operation 1, the FWR showed higher removal efficiency compared to the non-inoculated control (Fig. 3). Complete removal of ACE was noted from day 7 to day 15 when

laccase activity remained from 37.5 of 21.3 U/l. ACE in the control showed accumulation from 73.5 to 110.3% because it was continuously injected into the reactor. The earlier instantaneous decrease in ACE was consistent with that of batch studies because the marginal percentage of ACE (under 30%) was eliminated by adsorption (Fig. 2A). BPA was well removed from both the FWR and control and remained below the maximum of 15% through the whole treatment. CBZ decreased rapidly when injected into the reactor, thus implying a strong effect of adsorption in the early stage, although the contents soon increased.

When a mixture of the three PPCPs was continuously injected into the reactor at operation 2 (Fig. 4), ACE was completely removed from the FWR (F30; magenta) until day 18, while it accumulated in the control, as previously shown in operation 1 (NC60; grey). Unexpectedly, the residual concentration of ACE from the FWR increased after day 18 and reached 37.1% on day 30. However, partial removal of ACE was detected in the control after day 12, when laccase was slightly produced. BPA was scarcely detected during the entire operation period under both the conditions. Residual BPA (≈ 20%) was eliminated after 10 days, indicating that most of the BPA was captured on the surface of SSF products. The concentration of CBZ was maintained at approximately 40% until day 18, when ACE was reactively eradicated. After the fungal wheel was replaced, the removal of CBZ was slightly enhanced, although it soon returned to its previous status.

ACE removal also improved as the fungal wheel was replaced (FR30, yellow). Complete removal of ACE was maintained from days 33 to 44 in FR30. Interestingly, ACE started to increase from the first replicate of FR30 (FR30_R2), which was similar to that of F30, whereas it was constantly removed from another replicate (FR30_R1). ACE was removed from FR30_R1 by as much as 96% to 99%, even after laccase activity was depleted. This trend was not observed for F30 or F60. Among the reactor parameters, considerable differences between FR30_R1 and FR30_R2 were observed for DO and CFUs, which are related to microbial

activity (Fig. S6). The other parameters (pH, TN, TP, and TOC) did not show significant differences between the two replicates.

3.4. Microbial community analysis

Differences in fungal communities between the FWRs (F30, F60, and FR30) and non-inoculated control (NC60) were illustrated by non-metric multidimensional scaling (NMDS) ordinations (Fig. 5A). Fungal communities were mainly diverged by wood and supernatant, except in the case of F30 supernatant and NC60 wood (Fig. 5B). Focusing on the wood, *T. versicolor* was predominant in FR30 wood, and it was clustered together with the SSF inoculum. At the class level, the dominance of the class Agaricomycetes, to which *T. versicolor* belonged, was highly maintained in FR30 wood (78.3%), followed by F30 wood (36.3%) and F60 wood (16.2%). At the species level, *Naganishia globosa* was dominant in most samples, with the exception of FR30 wood (Fig. 5B). It was more abundant in the supernatant than in the wood, especially in the late period (e.g., F60, FR30, and NC60). A comparison of the relative abundance from two replicates of FR30 showed that the proportion of *T. versicolor* was 56.2% from FR30_R1 and 35.9% from FR30_R2 (Fig. 5C). *Slooffia tsugae* was a unique species observed in FR30_R2.

To investigate the co-occurrence with *T. versicolor*, the bacterial and fungal communities were analyzed at each OTU level in a Venn diagram (Fig. 6). In total, 79 OTUs were recovered from both the fungal and bacterial communities of F30, F60, FR30_R1, and FR30_R2 and 25 OTUs were universally present in all conditions as the core microbial taxa. A total of 79.4% and 83.6% relative abundance of F30 was observed in the core community from the wood and supernatant, respectively, and the abundance of core taxa decreased after 30 days (F60, 60.8% and 50.5% from wood and supernatant, respectively). After replacing the fungal wheel, the relative abundance of core microbial taxa was recovered in FR30_R1 (75.6% and 67.3% from

wood and supernatant, respectively), whereas it was not conserved in FR30_R2, which presented unique taxa of fungi with higher dominance.

Of the 56 OTUs of fungal and bacterial communities from FR30_R1, 8 OTUs showed significantly higher abundance from FR30_R1 compared to F30, F60 and FR30_R2 (Fig. S7). Fungal genera *Tulasnella* was highly dominated from both wood and supernatant. Another fungal genera *Chalara* was less abundant but it was solely observed in FR30_R1. Other six OTUs (*Herbaspirillum, Caulobacter, Edaphobaculum, Filimonas, Chryseobacterium*, and *Pedobacter*) were bacterial genera, and mainly detected from wood compartment except the case of *Chryseobacterium*.

4. Discussion

SSF is a microbial process that involves solid materials in the absence of a free aqueous phase while maintaining sufficient moisture content (Hölker et al., 2004). For the past 50 years, SSF has been somewhat masked by submerged fermentation technology, and it has been partly ignored in various fields of application. Nevertheless, SSF may provide tremendous benefits for fungal growth because it exhibits environmental characteristics that are similar to those of natural fungal habitats. To colonize woody materials, most filamentous fungi need a slight watery matrix with moisture content below 30% (Goodell et al., 2020). Excess water levels can reduce colonization efficiency through a decline in the available oxygen needed for growth, necessitating sufficient artificial aeration in submerged fermentation systems. Owing to its beneficial characteristics, SSF may substantially inspire fungal research, as has previously been demonstrated for the production of useful fungal metabolites. (Hölker et al., 2004).

Many studies have evaluated SSF processes, although these works have also carried out additional extraction steps to obtain useful crude products (Liu et al., 2020). Instead of using the crude extract from SSF, we attempted to apply the SSF products directly within the reactor.

In this sense, lignocellulosic substrates can not only represent a source of nutrients but also a solid support to immobilize fungal products in the reactor (Mir-Tutusaus et al., 2018). Water and air are the most important factors for wood and filamentous fungi (Goodell et al., 2020), thus implying that SSF products should contact both water and air simultaneously. Considering these aspects, we designed an FWR as shown in Fig. 1. Rotation of the fungal wheel allows filamentous fungi to be exposed to oxygen and air in the reaction mixture. We optimized the operational parameters by controlling the height and rotation rate of the SSF products and confirmed that additional aeration was not required in our design, which can further reduce the operation cost (Table S1 and Fig. S2).

Additional advantages may be expected when sorptive and biocatalytic/enzymatic removal processes are combined during exposure of the SSF products to PPCP mixtures. BPA has been suggested to represent a sufficient model compound to demonstrate such benefits. It possesses a strong electron-donating OH group and sufficient hydrophobicity, making it prone to removal by both adsorption and enzymatic systems (Yang et al., 2013). Indeed, the removal of BPA was improved using SSF products compared to free laccase (Fig. S1B), and notable removal was observed during the continuous operation of the FWR (Figs. 3 and 4). This result corroborates previous studies investigating the removal of BPA by adsorption combined with microbial activity (Mita et al., 2015; Zhang et al., 2016). Our previous study showed that ACE was well-removed, mainly because of an enzymatic system, while adsorption was less important (Fig. 2A). After radicalization by laccase, ACE dimers are produced by follow-up polymerization (Wang et al., 2014). Spontaneous polymerization continues to form oligomers (e.g., trimers and tetramers) at a much faster rate than general oxidation and easily removable polymers from the ACE precipitate. As ACE was completely removed in only 2 h from the previous batch test, it was difficult to detect a decrease in ACE during operation 1 (Fig. 3). Rapid ACE removal was also observed in operation 2 (Fig. 4). ACE was completely removed

from day 1 to day 18 and then accumulated as laccase activity decreased from FWR. At the same time, the residual concentration of ACE in NC60 slightly decreased because laccase production continued under this condition. These results demonstrated that ACE responds sensitively to laccase activity.

As bioreactor efficiency often decreases with increasing operation time, many efforts have been made to maintain the long-term operational stability of fungal bioreactors. Corresponding measures include the renovation of fungal cells (Mir-Tutusaus et al., 2019), proper adjustment of the C/N ratio (Badia-Fabregat et al., 2016), and feeding with a substrate (Cruz-Morató et al., 2013). In the present study, we also observed a decrease in the removal efficiency owing to a lack of laccase activity, and we were able to overcome this drawback by replacing the fungal wheel with fresh SSF products (Fig. 4). The replacement of the fungal wheel could provide not only fresh *T. versicolor* biomass but also fresh substrates to microbial communities concomitantly present in the bioreactor. Rapid depletion of laccase upon SSF product replacement was unexpected (Fig. 4). This observation may indicate the importance of optimal aging of fungal SSF to provide sufficient laccase levels during continuous operation. Nevertheless, continuous removal of ACE was observed from FR30, especially from one replicate of FR30 (FR30_R1), even after laccase was depleted on day 47. The removal of CBZ by FR30_R1 and FR30_R2 showed similar behavior and was not due to a physical difference in the fungal wheel. This behavior of ACE was unique to FR30_R1 and was not detected in F30 or F60, implying that such activity would occur at a relatively late period of operation and be promoted through the introduction of new substrates.

One possible explanation might be that intracellular fungal enzymes, such as cytochrome P450, are involved in the removal of ACE during this period (Haroune et al., 2017). The cytochrome P450 family consists of a group of monooxygenases related to several reactions responsible for the removal of PPCPs, including hydroxylation, heteroatom oxygenation, and

dealkylation (Díaz-Cruz et al., 2014). The fungal genus *Trametes* is also known for its intracellular enzymatic system; thus, considerable removal of ACE may have been due to intracellular transformation derived from inoculated *T. versicolor*. Indeed, the abundance of *T. versicolor* was highly maintained in FR30_R1 wood, whereas its initial abundance was lost in the wood of F30, F60, and FR30_R2 (Fig. 5). Even at the class level, FR30_R1 wood possessed the highest proportion of Agaricomycetes, which includes numerous well-studied fungal degraders such as *Stropharia rugosoannulata* (Castellet-Rovira et al., 2018), *Bjerkandera adusta* (Shahi et al., 2016) and *Pleurotus ostreatus* (Golan-Rozen et al., 2011). This result again demonstrates the benefit of using SSF products combined with whole fungal cells rather than free laccase.

Another explanation could be the effect of co-occurring microbial communities (Badia-Fabregat et al., 2016; Mir-Tutusaus et al., 2017). The positive interaction between resident fungi and bacteria can promote the survival of each and stable community adaptations (Zupancic et al., 2018). The supernatants of F30 and FR30 were spatially equal, although FR30 was more mature. The difference in ACE removal between FR30_R1 and FR30_R2 was consistent with the decrease in CFUs at FR30_R2 (Fig. S6). Indeed, the relative abundance of core communities was recovered in FR30_R1 by implanting new nutrient sources with fresh SSF products, while it decreased in FR30_R2 (Fig. 6). The microbial communities of FR30_R2 represented a relatively unique community that was not shared with F30. These results might explain why communities from FR30_R1 were relatively stable during succession in the presence of previous microbial communities of F30 while those from FR30_R2 were not. These dynamics might be reflected by the fluctuation in ACE removal from FR30_R2.

Additionally, a synergistic effect of the settled microorganisms in FR30_R1 can be expected. Of the 8 genera whose relative abundance from FR30_R1 was significantly higher than the others, the fungal genera *Chalara* and *Tulasnella* were remarkable for their characteristics of

host cell wall attack (Fig. S7; Adamo et al., 2020). Contrary to *T. versicolor*, *Chalara* and *Tulasnella* are known to secrete hydrolytic enzymes to degrade the polysaccharide proportion of plant cells, and these unique taxa may serve as supporting degraders of wood material, similar to ACE and even laccase. The bacterial genus *Herbaspirillum* was another dominant taxon that was abundant in FR30_R1 in the wood. Given that bacteria were not present in the initial SSF inoculum, the bacterial community observed in wood suggested the presence of biofilm communities (Desiante et al., 2021). In addition, *Herbaspirillum* has been reported as a candidate for the removal of ACE as a biofilm community (Badger et al., 2006; Tuleski et al., 2019). These results provide fundamental knowledge of the microbial succession associated with the operation of fungal bioreactors and emphasize the importance of monitoring microbial dynamics to understand the stability of bioreactor performance.

5. Conclusions

A fungal wheel reactor based on solid-state fermentation was designed and operated for 60 days to remove the target PPCPs. Solid-state fermentation products enhance the removal efficiency compared to free laccase when combined with adsorption. ACE was successfully removed in the FWR, and the removal efficiency of ACE was replenished by replacing the fungal wheel. Microbial community analysis revealed that the maintenance of removal efficiency was mainly controlled by *T. versicolor* and its enzymatic system and can be supported by co-occurring microbial communities. This research offers a fundamental understanding of the long-term operation of fungal reactors, which have great potential for use in water treatment.

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CRediT authorship contribution statement

Bo Ram Kang: Conceptualization, Investigation, Formal analysis, Methodology, Data analysis, Writing - original draft, Writing - review & editing. **Jin Ju Kim:** Formal analysis, Methodology. **Jin-Kyung Hong:** Data analysis, Methodology. **Dietmar Schlosser:** Methodology, Writing - review & editing. **Tae Kwon Lee:** Conceptualization, Supervision, Project administration, Writing - review & editing, Funding acquisition.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Figure captions

Fig. 1. Conceptual schematic of the fungal wheel reactor (FWR). Solid-state fermentation products were contained in a polypropylene filter bag (25 mm in diameter). Non-inoculated wood chips were used as a negative control, and they showed initial adsorption effects. The shaft penetrated the middle of the fungal wheel horizontally and was connected to a rotating motor.

Fig. 2. Residual concentration of (A) ACE, (B) BPA, and (C) CBZ from the batch reaction (colored circle). Dashed line corresponds to the non-inoculated control. Symbols and error bars represent means and standard deviations from triplicate determinations, respectively.

Fig. 3. Laccase activity and removal of ACE, BPA, and CBZ (C/C_0) during operation 1 of the FWR and non-inoculated control. The continuous grey line represents laccase activity from FWR (solid line) and control (dashed line). Colored boxes show the ACE (red), BPA (blue), and CBZ (green) added in independent sequential phases. The dashed line indicates the control. Symbols and error bars represent the means and standard deviations from triplicate determinations, respectively.

Fig. 4. Timeline of the experiment, laccase activity, and ACE, BPA, and CBZ removal during operation 2 of the FWR (F30, F60, and FR30) and non-inoculated control (NC60). Area and line with magenta color represent the values from the original reactor (F30 and F60), and yellow color indicates the values from the fungal wheel-replaced reactor (FR30). Grey color-area and dashed line are from NC60. Symbols and error bars represent means and standard deviations from triplicate determinations, respectively.

Fig. 5. Clustering analysis of fungal communities from FWR (F30, F60, and FR30) and non-inoculated control (NC60) in (A) NMDS ordination and (B) phylogenetic assignments at the class level. Fungal taxa *Tramates*, *Naganishia*, and *Slooffia* were demonstrated at the genus

level due to their remarkable dominance. (C) Distributions of fungal community from FR30_R1 and FR30_R2 according to the wood and supernatant proportion.

Fig. 6. Quadro Venn diagram representing the intersection of the fungal and bacterial communities from F30, F60, FR30_R1, and FR30_R2. Most of the OTUs contained in the core community (25) also belonged to the shared community except for F30 (11). The bar graph shows the sum of relative abundance of fungal and bacterial community from FR30_R1 and FR30_R2 according to the wood and supernatant proportion.

Table.S1 Details of the operating conditions for the FWR setup.

Fig. S1. Residual concentration of (A) ACE, (B) BPA, and (C) CBZ from the batch reaction treated by SSF products (circle) and free laccase (triangle). The dashed line corresponds to the non-inoculated control. Symbols and error bars represent the means and standard deviations from triplicate determinations, respectively.

Fig. S2. Relative laccase activity (% of initial) from each operating condition over ten days of operation. Symbols and error bars represent the means and standard deviations from triplicate determinations, respectively.

Fig. S3. Laccase activity during operation 1 of the FWR (red line and symbols) compared to the non-inoculated control (dashed line). Symbols and error bars represent the means and standard deviations from triplicate determinations, respectively.

Fig. S4. Comparison of laccase activity from the operation of the FWR. Operation 1 (red) and operation 2 (magenta) proceeded over 60 days. Fungal wheel-replaced reactor (operation 2_R; yellow) was maintained from day 31 to day 60 and drawn together at the initial time point (day 0) for a comparison with operation 1 and 2.

Fig. S5. Correlation between laccase activity and TOC from the operation of the FWR. Dots from operation 1 (red) and operation 2 (magenta) were obtained from day 1 to day 60, while dots from operation 2_R (yellow) were from day 31 to day 60. Solid lines are based on the linear correlation fit, and corresponding R2 values are shown in the upper left part of the figure.

Fig. S6. Removal of ACE, laccase activity, and biochemical parameters from the operation of FR30. Two replicates of FR30 were drawn separately and named FR30_R1 (yellow triangle) and FR30_R2 (green round). Laccase was depleted after day 47 (faint yellow box).

Fig. S7. Relative abundance of the bacterial and fungal community, for which the abundance was significantly higher in FR30_R1 compared to F30, F60, and FR30_R2.