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RESEARCH PAPER

A proposed stepwise screening framework for the selection of polycyclic aromatic hydrocarbon (PAH)-degrading white rot fungi

Aslan Hwanhwi Lee[1,2,3] · Hanbyul Lee[1] · Young Mok Heo[1] · Young Woon Lim[4] · Chang-Mu Kim[5] · Gyu-Hyeok Kim[1] · Wonjae Chang[2] · Jae-Jin Kim[1]

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Abstract

This study suggests a simple three-step screening protocol for the selection of white rot fungi (WRF) capable of degrading polycyclic aromatic hydrocarbons (PAHs), which combines easily applicable bioassay techniques, and verifies that protocol by evaluating the PAH degradation activity, ligninolytic enzyme secretion, and relevant gene expressions of the selected PAH-degraders. Using 120 fungal strains, a sequence of bioassay techniques was applied: Bavendamm's reaction (Step 1), remazol brilliant blue R (RBBR) decolorization (Step 2); assays for tolerance to four mixed PAHs—phenanthrene, anthracene, fluoranthene, and pyrene (Step 3). This stepwise protocol selected 14 PAH-degrading WRF, including *Microporus vernicipes*, *Peniophora incarnata*, *Perenniporia subacida*, *Phanerochaete sordida*, *Phlebia acerina*, and *Phlebia radiata.* Of these, *P. incarnata* exhibited the highest PAH degradative activity, ranging from 40 to > 90%, which was related to the time-variable secretions of three extracellular ligninolytic enzymes: laccase, manganese-dependent peroxidase (MnP) and lignin peroxidase (LiP). Laccase and MnP production by *P. incarnata* tended to be greater in the early stages of PAH degradation, whereas its LiP production became intensified with decreasing laccase and MnP production. *Pilc1* and *pimp1* genes encoding laccase and MnP were expressed, indicating the occurrence of extracellular enzyme-driven biodegradation of PAH by the fungal strains.

Keywords White rot fungi · Polycyclic aromatic hydrocarbons · Screening · Biodegradation · Ligninolytic enzyme · Gene expression

Introduction

Polycyclic aromatic hydrocarbons (PAHs) with two or more fused benzene rings include environmental toxicants and carcinogens [1, 2]. With an increase in molecular weight, PAHs tend to have low aqueous solubilities, decreased vapor pressures, and increased hydrophobicity [3]. PAHs can accumulate over long periods of time in soil and water environments, which may adversely influence diverse environmental receptors in nature [3, 4]. Common anthropogenic sources of PAH contamination are fossil fuels, coal tar, wood, lubricating oil, crude oil, and solid municipal wastes [5]. Natural PAHs can be released by forest fires, oil seeps, volcanic eruptions, tree exudates, and so on [3]. Various physical, chemical and biological remediation processes, technologies and strategies for dealing with PAH-contaminated soils, sediments and waters have been extensively suggested in the literature [6, 7]. Bioremediation has often been considered a minimally destructive, less energy-intensive, and

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✉ Jae-Jin Kim
jae-jinkim@korea.ac.kr

[1] Division of Environmental Science and Ecological Engineering, Korea University, 145, Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea

[2] Department of Civil, Geological and Environmental Engineering, University of Saskatchewan, Saskatoon S7N 5A9, Canada

[3] School of Earth and Environmental Sciences and Research Institute of Oceanography, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea

[4] School of Biological Sciences and Institute of Microbiology, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea

[5] Microorganism Resources Division, National Institute of Biological Resources, Incheon, Republic of Korea

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cost-effective remediation approach for soils and water impacted by hydrocarbons [8]. Identifying PAH-degrading microorganisms (bacteria, algae, and fungi), as well as optimizing the environmental conditions for enhancing metabolic activity of PAH-degraders (e.g., nutrients, oxygen, substrates, pH, bioavailability, etc.), are key elements for successful bioremediation of PAH-impacted soils and water [9].

White rot fungi (WRF) are ligninolytic fungi classified as basidiomycetes and cause the white rot decay of wood [10]. It is known that WRF are able to degrade lignin, a highly complex aromatic polymer, thorough the ligninolytic enzyme-induced oxidation of lignin [10, 11]. The major ligninolytic enzymes secreted by WRF are laccase, manganese-dependent peroxidase (MnP) and lignin peroxidase (LiP) [12–14]. These enzymes are involved in the oxidation of phenol and/or non-phenolic organic compounds [4, 15–21]. The ligninolytic enzymes achieve a one-electron radical oxidation, producing cationic radicals from contaminants (e.g., PAHs) followed by the appearance of quinines [22]. These oxidative enzymes generally exhibit flexible substrate affinity, which is significantly advantageous for developing bioremediation strategies adapted to lignin-like organic contaminants [23]. The ligninolytic enzymes are extracellular and may diffuse into smaller, less accessible pores within soil or sediment matrices to reach entrapped PAHs. WRF have been considered as an important microbial resource for environmental biotechnologies such as bioremediation, dye decolorization, biopulping, and biobleaching [3, 24].

Diverse WRF species have been assessed to promote enzyme-driven biodegradation of PAHs, petroleum hydrocarbons, phenolic compounds, dioxins, polychlorinated biphenyls (PCBs), munitions wastes (e.g., trinitrotoluene), industrial dye effluents, herbicides, pesticides, and chloroorganic [5, 23, 25, 26]. Screening procedures for selecting such environmentally significant WRF have been increasingly found in the literature, mainly for identifying WRF strains effective in degrading wood lignin, rice straw lignin and organic contaminants (e.g., dyes and PAHs) [10, 11, 27–30]. In general, screening methods have been developed by means of culture-dependent approaches specific to the representative chemical mixtures and/or target compounds. The screening approaches vary in their fungi types, contaminants, environmental matrices, growth conditions, laboratory techniques, and screening scopes (e.g., the initial number of fungal species to be screened). Yet, there remains a demand for various high-throughput screening techniques specific to PAH-degrading WRF.

Prior to detailed PAH biodegradation assessments, molecular characterization of WRF, and comprehensive characterization studies for bioremediation feasibility, systematic stepwise screening procedures may be useful or required for rapidly selecting efficient PAH-degrading WRF strains from environmental samples or large existing collections of WRF isolates. Diverse screening options and verification techniques that are specific to PAH-degrading WRF are needed by researchers and practitioners. The objective of the present study is therefore to suggest a simple, stepwise culture-based screening protocol for the selection of PAH-degrading WRF strains, as well as to verify the degradative activity of the selected WRF for large PAHs (phenanthrene, anthracene, fluoranthene, and pyrene). This verification was performed through assessments of PAH biodegradation and lignin enzyme secretion, and genetic analysis at the transcriptional level.

The key concept for the screening method proposed in this study is a sequential combination of easily applicable culture-based reaction bioassays: Bavendamm's reaction (Step 1), RBBR decolorization assays (Step 2), and PAHs tolerance tests (Step 3). Starting with 120 native WRF, Bavendamm's reaction first broadly selects WRF exhibiting strong oxidative activity for phenolic lignin-like compounds. The RBBR decolorization assays then narrows down the selected WRF to those exhibiting oxidative activity for aromatic compounds. PAH tolerance tests finally select WRF capable of growing in the presence of four large PAHs.

Materials and methods

Fungal species and identification

Indigenous white rot fungi (WRF) were randomly collected from various forested areas in South Korea and identified by the Korea University Culture (KUC) collection. The WRF were aseptically maintained as pure cultures by the KUC. From the forest samples collected for this screening study, a total of 120 WRF were identified, including 63 species belonging to 41 genera. Detailed protocols were followed for fungal DNA extraction and polymerase chain reaction (PCR) amplification for WRF identification, as described by Lim et al. [31]. The 28S rDNA sequences representing the partial large subunit (LSU) and the internal transcribed spacer (ITS) regions were amplified through PCR analyses using the universal fungal primers LR0R/LR3 [32]. The PCR products were sent to Macrogen Inc. in Seoul, South Korea, and the resultant sequences were processed for fungal identification and classification using the GenBank databases and the tools available in BLASTIN 2.3.1 [33], aligned with DNA sequences of related fungi using ClustalX algorithm [34], and PAUP*4.0b10 software [35].

Screening protocol

For the screening protocol proposed in this study, the three following tests were sequentially combined: Bavendamm's reaction assay (Step 1), the dye decolorization test (Step 2) and the PAH tolerance test (Step 3). The details of the screening protocol are described in the following sections.

Bavendamm's reaction assay (Step 1)

Bavendamm's reaction assay indicates the lignin degradation potential of fungi. It has been consistently reported that there is a significant correlation between the lignin-degrading ability of basidiomycetes and their strong oxidative capability for gallic and tannic acids [36–38]. Gallic acid (3,4,5-trihydroxybenzoic acid, $C_7H_6O_5$) is a naturally occurring triphenolic compound and has been indicated as a strong antioxidant [39]. Tannic acid ($C_{76}H_{52}O_{46}$) is a polyphenolic compound, which is heavier and more complex than gallic acid.

The 120 fungal isolates were aseptically inoculated, in duplicate, onto 1.5% MEA (Malt extract agar) plates supplemented with 5 g L^{-1} of gallic acid or tannic acid, and the plates were incubated at 27 °C. The inoculated plates that turn brown are a visual indication of the oxidation of gallic acid or tannic acid, as well as the formation of quinones. The Bavendamm's reaction plates were observed up to two weeks for the 120 WRF. The colors of the plates were ranked as dark brown (DB), brown (BR), yellowish brown (YB), yellow (YE), or no reaction (N). In terms of the selection criteria, WRF exhibiting DB and BR were selected for the next screening step.

Dye decolorization tests (Step 2)

The Remazol Brilliant Blue R (RBBR) decolorization tests were then performed to further select the fungal species screened by Bavendamm's reaction assay. The fungi were aseptically inoculated in duplicates onto 2% MEA plates amended with 100 mg L^{-1} of RBBR. The inoculated MEA plates were incubated at 27 °C and were inspected every day for 15 days to observe decolorization from blue to yellowish white and the growth of fungal hyphae until the petri dishes were fully covered. The RBBR decolorization assays were categorized based on decolorization timing, as (A) within 5 days, (B) within 6 to 10 days, and (C) within 11–15 days. The WRF isolates screened into categories A and B were selected for the next step.

PAH tolerance tests (Step 3)

The fungi selected through the RBBR and decolorization tests were screened further with respect to their PAH tolerance. The fungal isolates were cultured on 2% MEA plates amended with a mixture of PAHs to obtain 25 mg L^{-1} each of phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), and pyrene (PYR) (all analytical grade from Aldrich Chemical Co., St. Louis, USA). The PAHs were dissolved in acetone (Samchun Chemical Co. Ltd., Pyeongtaek City, Korea) before being added to the cooling agar solution. Each of the fungal cultures was aseptically inoculated onto the PAH-amended plates in triplicates and were incubated at 27 °C for 7 days. As previously suggested by Lee et al. [40], the PAH tolerance (TR) was estimated in this study based on the following equation, in which TR is the tolerance rate (%), FGR is the radial growth rate (mm/day) of the hyphae on the PAH-spiked MEA plates, and GRC is the radial growth rate (mm/day) of the same isolate on PAH-free MEA plates. Both FGR and GRC were determined using a caliper to measure the diameters (mm) of growing fungal colonies.

$$TR(\%) = \frac{FGR}{GRC} \times 100(\%)$$

The tolerance rates were ranked as + (0–30%), + + (30–50%), + + + (50–70%), + + + + (70–90%), and + + + + + (90–100%), and the WRF strains landing in the latter three categories (> 50%) were selected for the PAH biodegradation assessment. The remaining WRF exhibiting PAH tolerances below 50% (+ and + +) were re-screened based on their radial growth rates on PAH-spiked MEA plates (1 to 6 mm/day). WRF exhibiting radial growth rates over 5 mm/day were also selected for the following biodegradation assessment.

PAH biodegradative capability, ligninolytic enzyme activity, and gene expression

The fungal species screened through the sequential screening process were assessed in detail, to verify their PAH biodegradative capability. A series of experiments and analyses were performed: PAH biodegradation experiments, ligninolytic enzyme activity assessments and gene expression analyses.

PAH biodegradation microcosm experiment

The PAH biodegradation microcosm experiments were performed using a PAH mixture containing phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), and pyrene (PYR). The PAH mixture was prepared by dissolving 3 mg of each PAH in 1 mL of acetone for a total PAH concentration of 12 mg L^{-1}. In a sterilized 250 mL-Erlenmeyer flask, 100 mL of malt extract (ME) liquid media (2% w/v) was spiked with the 1-mL filter-sterilized PAH mixture. The selected fungal isolates were pre-cultured on 2% ME-amended agar plates; each cultured isolate was aseptically

inoculated as ten agar plugs (5 mm in diameter). The cultured plugs of each isolate were used to inoculate triplicates of the PAH-spiked ME media flasks. The flasks were then placed on an incubated rotary shaker at 150 rpm and 27 °C for 14 days. The negative control flasks (uninoculated PAH-spiked nutrient media) were also incubated to estimate the abiotic loss of PAHs under the same conditions. Positive control flasks, which were inoculated with two known PAH-degrading fungus species, *Phanerochaete chrysosporium* KCTC 6293 [1] or *Peniophora incarnata* KUC8836 (KACC53476) [40], were also incubated under the same conditions.

The PAHs were extracted at the end of the experiments using 100 mL of dichloromethane (HPLC grade, ≥ 99.9%, Duksan, Korea). The solvent extraction was repeated three consecutive times for each flask, producing 300 mL of extract solution. Each of the bulk extracts (300 mL) was then concentrated into a 5-mL PAH extract solution using a vacuum rotary evaporator (Eyela N-1000 series, Japan). Concentrations of PAHs in the extract solutions were determined by gas chromatography with mass spectrometry (GC–MS, Agilent Technologies, Model 7890, Santa Clara, CA, USA). The extract injection volume was 1 µL and the GC column was a DB-5MS column (0.25 mm in diameter, 30 m in length, with a film thickness of 0.25 µm). The detailed GC–MS analysis was followed according to a previous study [29]. Statistical analyses of the GC datasets were performed using the Statistical Analysis Systems package (SAS 9.2, SAS Institute Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) followed by a Turkey's test were adopted for the statistical data analysis ($p < 0.05$).

Tracking ligninolytic enzyme activity

Extracellular ligninolytic enzyme activity was tracked every 2 days over the 14-day PAH biodegradation experiments using the spectrophotometry-based enzyme assay method, as described elsewhere in Lee et al. [41]. To do that, an identical set of WRF-inoculated, PAH-amended flasks (triplicates) were used in separate experiments run in parallel with the PAH biodegradation microcosms. Fungal biomass samples obtained from the microcosms were filtered to remove mycelium and spores (0.45-µm syringe filter). The crude filtrates were added to prepared enzyme assay solutions specific to laccase, manganese-dependent peroxidase (MnP) and lignin peroxidase (LiP). The enzyme reaction activities were then measured using a UV–Vis spectrophotometer. For the laccase assay solution, 0.1 M sodium acetate (pH 4.5) and 1.5 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used. For the MnP assay solution, 0.5 M sodium malonate (pH 4.5), 5 mM $MnSO_4$, 1 mM 2,6-dimethoxylphenol, and 1 mM H_2O_2 were used. For the LiP assay solution, 0.25 M sodium tartrate (pH 2.5), 10 mM veratryl alcohol, and 5 mM H_2O_2 were used [14, 42–44]. One unit of ligninolytic enzyme activity corresponds to the production of 1 µmol of reaction product per minute at room temperature; the activity was expressed in U/mL [29]. One unit of ligninolytic enzyme activity is equivalent to the production of 1 µmol of reaction product per min at room temperature, and the activity is expressed in U/mL, as described elsewhere [29].

Gene expression analyses

A set of pyrene-spiked microcosm experiments (triplicate) was set up in the same manner as described above for the gene expression analyses. After seven days of incubated shaking, supernatant filtrates were collected to measure ligninolytic enzyme activity, as described in "Tracking ligninolytic enzyme activity" section. Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, USA) and immediately frozen at − 80 °C. First strand cDNA was synthesized from 1 µg of total RNA using the SuperScript® III First-Strand Synthesis System for real-time PCR (RT-PCR), according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The transcript abundances of the genes encoding laccase and MnP were determined using a real-time quantitative reverse transcription PCR system (CFX96™ Real-Time PCR Detection System, Biorad, CA, USA). To normalize the relative quantification of the cDNA, Actin 1 (*act1*) was selected as an endogenous internal control gene (GenBank accession No. AF156157). RT-qPCR amplification was performed using a CFX96™ Real-Time PCR Detection System (Biorad, CA, USA) and performed in a total volume of 20 µL containing 10 µL of SYBR® Premix (Qiagen, Hilden, Germany) with the primers listed in Table 1. The mixture was subjected to an annealing temperature of 52 °C for the laccase gene and 51 °C for the MnP gene, according to the manufacturer's default operating procedures. All the reactions were independently performed on separate biological triplicates. The relative expression levels of the genes were calculated by Pfaffl's method [45], which is based on the gene expression (E) and threshold cycle (Ct) deviation of the target genes *pilc1* (laccase gene) and *pimp1* (MnP gene) [41], compared to a reference (*act1*) [46]. The relative expression levels are therefore expressed with respect to the reference gene, as shown in the equation below, where E_{target} and E_{ref} are the gene expressions of the target and reference gene transcripts, respectively, and ΔCt_{target} and ΔCt_{ref} are the Ct deviations of control-treated samples for the target and reference gene transcripts, respectively.

Table 1 Oligonucleotides used in this study

Target gene (GenBank Accession No.)	Gene information	Primer	Nucleotide sequence	Amplicon Length
pilc1 (KJ622360)	Laccase gene	Pi_lac1F	5′-GATCACAGGCAAGAAGGGC-3’	95 bp
		Pi_lac1R	5′-GAAGGACCATCAGCCCAG-3’	
pimp1 (KJ622361)	MnP gene	Pi_mnp1F	5′- CCGGAGATCAATACCGC-3’	111 bp
		Pi_mnp1R	5′- GAAGGGCTCGGGAATG -3’	
act1 (AF156157)	Actin gene	CY_Sc_act1_F	5′-CGATCTCGATATCCGTCGT-3’	57 bp
		CY_Sc_act1_R	5′-GCTCCTTCTGCATACGGTC-3’	

$$\text{Relative expression ratio} = \frac{\left(E_{target}\right)^{\Delta Ct_{target}}}{\left(E_{ref}\right)^{\Delta Ct_{ref}}}$$

Results and discussion

Overall quantitative screening results

The screening protocol proposed in this study sequentially combines in three biochemical reaction tests for a large number of WRF strains: Bavendamm’s reactions (Step 1), dye decolorization tests (Step 2), and PAHs tolerance tests (Step 3). Figure 1 presents the overall stepwise results of the quantitative screening for PAH-degrading WRF. Out of the 120 WRF belonging to 63 species, the screening protocol ultimately selected 14 WRF stains belonging to 6 species.

In Step 1, Bavendamm’s reaction was carried out over 14 days to select 31 WRF belonging to 15 species that exhibited more oxidative activity for either gallic or tannic acid. In Step 2, the RBBR decolorization test was conducted for 15 days to further select 28 WRF belonging to 13 species capable of *ligninolytic activity*. In Step 3, the PAH tolerance tests selected 10 WRF strains belonging to 5 species that exhibited over 50% of the percent tolerance estimates from the 7th day samples. Among the non-selected 18 WRF (exhibiting less than 50% of tolerance estimate), 4 WRF strains were additionally selected based on their observed growth rates over 5 mm/day in the presence of 100 mg L^{-1} of the PAH mixture.

As a result, a total of 14 WRF strains were selected through the screening protocol for PAH biodegradation assessment. The details about the WRF selected at each step of the screening protocol are presented in the following (Bavendamm’s reaction (Step 1) section and RBBR decolorization and PAH tolerance Section). The PAH biodegradative capabilities of the 14 selected WRF strains were further assessed (“PAH biodegradation assessment in liquid cultures” Section).

During the three-stepwise screening, seven species from the 31 WRF selected in Step 1 (total of 15 species) have previously exhibited biodegradation potential for some phenolic compounds: *I. lacteus*, *P. crassa*, *P. sordida*, *P. acerina*, *P. radiata*, *M. vernicipes*, and *P. incarnata*. For example, *I. lacteus* degrades phenols, phenolic textile dyes, and trichloroethylene [47]. *P. crassa* degrades chlorinated phenols and pentachlorophenol [48]. *P. sordida*, *P. acerina*, and *P. radiata* exhibit degradation activity for dioxin, PCB, and/or chlorobenzene [47, 49, 50]. *M. vernicipes* has been reported as a lignin degrader [51]. *P. incarnata* exhibits significant oxidative activity for gallic acid [40]. Three of the genera screened by Bavendamm’s reaction assay, *Phanerochaete*, *Irpex*, and *Phlebia*, have been reported as PAH-degrading WRF [15, 19, 29], and other remaining genera, *Gymnopus*, *Hymenochaete*, *Perenniporia*, *Porostereum*, and *Trametes*, have been reported as dye-degraders [52]. However, some of the other screened genera, *Fuscoporia*, *Hyphoderma*, and *Oudemansiella*, have not been extensively identified as biological oxidizers of phenolic compounds. Bavendamm’s reaction assay broadly covers PAH-degrading WRF, including oxidizers of phenolic compounds, and the results in this study are in good agreement with the reported PAH-degrading WRF in the literature.

Bavendamm’s reaction (Step 1)

The WRF isolates were selected based on the intensity of their oxidative activity for gallic acid (G) and tannic acid (T), which are phenolic compounds that oxidize to quinonic acid derivatives. The results were ranked using the five different color intensities exhibited in the Bavendamm’s reaction plates inoculated with the WRF strains. As stated earlier, these color rankings are labelled dark brown (DB), brown (BR), yellowish brown (YB), yellow (YE), and no reaction (N). Table 2 lists the 120 WRF isolates that were screened using the Bavendamm’s plate response and shows the 31 selected WRF that exhibited DB and BR for the next screening steps. The 9 WRF strains that exhibited dark brown (DB) on the plates belong to the genera *Peniophora incarnata*, *Porostereum crassum*, *Phanerochaete crassa*,

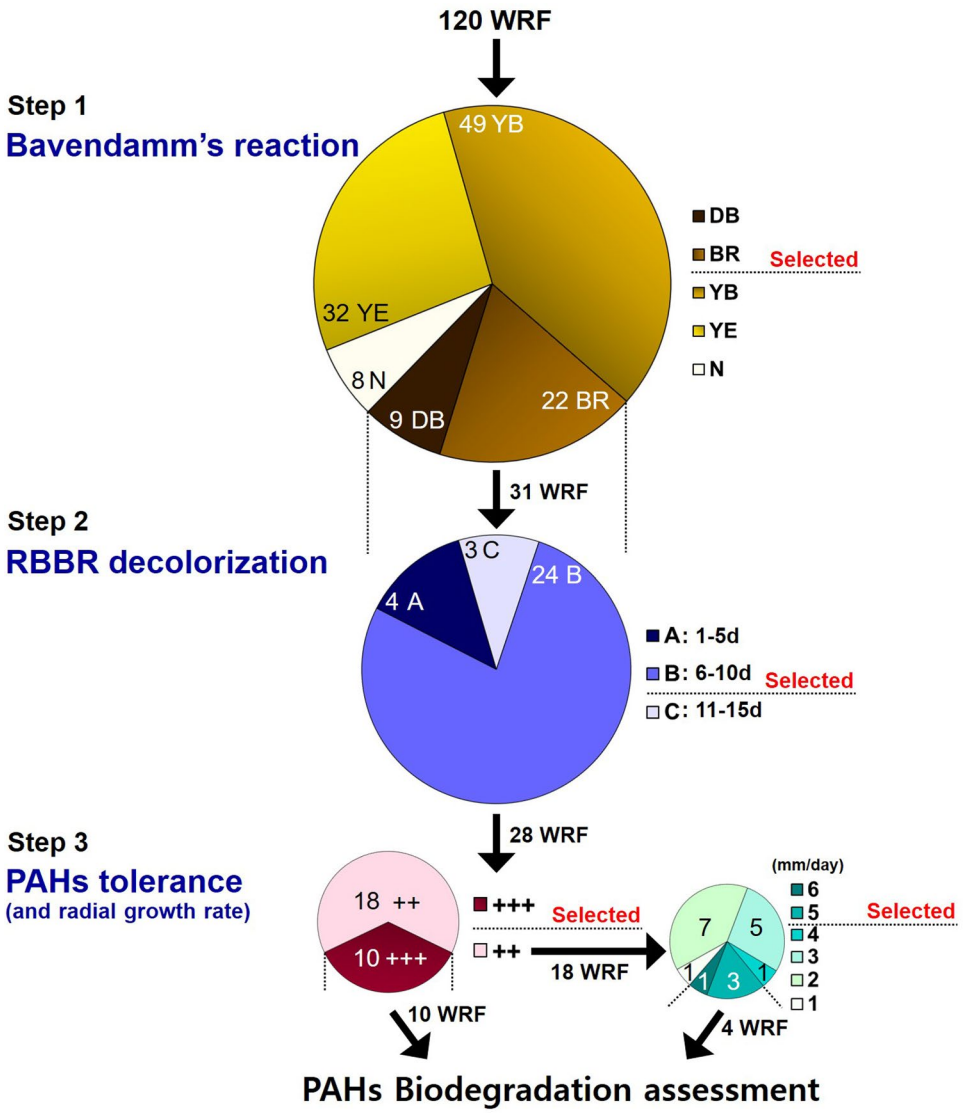

Fig. 1 The sequential screening procedure for PAH-degrading white rot fungi (WRF) for subsequent PAH biodegradation assessment. The PAH-degrading WRF strains selected at each step are presented

and *Phanerochaete sordida*, and the 22 fungal isolates that exhibited brown (B) colonies belong to the genera *Fuscoporia*, *Gymnopus*, *Hymenochaete*, *Irpex*, *Microporus*, *Oudemansiella*, *Phanerochaete*, and *Phlebia*. Among the non-selected 89 WRF strains, 49 and 32 WRF strains exhibited yellowish brown and yellow colonies, respectively.

RBBR decolorization and PAH tolerance

Table 3 presents the detailed results of the RBBR decolorization tests (Step 2), which screened 28 WRF strains out of 31 WRF, based on the time needed for RBBR decolorization. As explained earlier, decolorization times are categorized as (A) within 5 days, (B) 6–10 days, and (C) 11–15 days.

Among the selected strains for Step 2, 4 WRF strains belonging to *P. sordida* and *P. crassum* rapidly decolorized the RBBR assays within 5 days. Casieri et al. [52] also reported that these strains rapidly decolorize textile dyes such as RBBR and Poly R-478. The other 24 fungal strains decolorized RBBR within ten days. Of these strains, *I. lacteus*, *P. incarnata*, *P. subacida*, *P. crassa*, *P. acerina*, and *T. betulina* decolorize several dyes such as RBBR, Orange II, Reactive Blue, and/or Poly R-478 [40, 52, 53]. The 28 WRF strains capable of decolorizing RBBR within 10 days were selected for Step 3, the PAH tolerance assays. RBBR is a hydrophobic anthraquinonic dye related to an intermediate product of the degradation of anthracene [54, 55]. The RBBR decolorization by fungi and bacteria is correlated to lignocellulose-degrading activity [54, 56, 57]. Subsequently, the selected WRF demonstrated their ability to degrade RBBR dye beyond the phenolic compounds which are similar structure with PAHs. The genera *Gymnopus*, *Hymenochaete*, and *Hyphoderma* fell into category C (11 to 15 days) were not selected for Step 3.

Table 2 White rot fungal species and their ranked oxidative activities for gallic and tannic acid expressed by the color intensities of Bavendamm's reaction in Step 1 of the proposed screening protocol (*DB* dark brown, *BR* brown, *YB* yellowish brown, *YE* yellow, and *N* not reaction)

No.	WRF	Step 1		Selected	No.	WRF	Step 1		Selected
		G	T				G	T	
1	*Abortiporus biennis* KUC10346	YE	YB		61	*Perenniporia fraxinea* KUC10499	YE	YE	
2	*Agaricus* sp. KUC10131	YE	YE		62	*Perenniporia maackiae* KUC10246	YB	YB	
3	*Agrocybe pediades* KUC10068	YE	N		63	*Perenniporia narymica* KUC10095	YB	YB	
4	*Athelia epiphylla* KUC10396	N	YB		64	*Perenniporia subacida* KUC10195	YB	BR	●
5	*Bjerkandera adusta* KUC10279	YE	N		65	*Phanerochaete crassa* KUC10106	N	YB	
6	*Bjerkandera adusta* KUC10437	YE	N		66	*Phanerochaete crassa* KUC10319	DB	BR	●
7	*Bjerkandera adusta* KUC10481	YE	N		67	*Phanerochaete crassa* KUC10456	YB	YB	
8	*Bjerkandera adusta* KUC10300	YE	N		68	*Phanerochaete sordida* KUC10088	BR	YB	●
9	*Bjerkandera adusta* KUC10323	YE	N		69	*Phanerochaete sordida* KUC10119	YB	N	
10	*Ceriporia lacerata* KUC10108	YE	YE		70	*Phanerochaete sordida* KUC10123	DB	YB	●
11	*Ceriporia lacerata* KUC10193	YE	YE		71	*Phanerochaete sordida* KUC10147	YB	YB	
12	*Ceriporia lacerata* KUC10171	YE	YE		72	*Phanerochaete sordida* KUC10252	YB	YB	
13	*Ceriporia lacerata* KUC10449	YE	YE		73	*Phanerochaete sordida* KUC10351	BR	YB	●
14	*Cerrena consors* KUC10202	YE	YE		74	*Phanerochaete sordida* KUC10423	BR	YB	●
15	*Cerrena consors* KUC10265	YE	YE		75	*Phanerochaete sordida* KUC10403	BR	YB	●
16	*Cerrena consors* KUC10524	YE	YE		76	*Phanerochaete* sp. KUC10353	YB	YB	
17	*Cerrena unicolor* KUC10210	YE	YE		77	*Phanerochaete stereoides* KUC10101	YE	YE	
18	*Clitopilus* sp. KUC10107	YE	YB		78	*Phanerochaete stereoides* KUC10190	YB	YB	
19	*Cylindrobasidium laeve* KUC10062	N	N		79	*Phanerochaete velutina* KUC10400	YB	YB	
20	*Dentipellis dissita* KUC10112	YE	YB		80	*Phlebia acerina* KUC10443	YB	BR	●
21	*Fuscoporia gilva* KUC10234	YB	BR	●	81	*Phlebia acerina* KUC10479	YB	BR	●
22	*Ganoderma applanatum* KUC10185	YE	YE		82	*Phlebia radiata* KUC10076	YB	BR	●
23	*Ganoderma* sp. KUC10204	YE	YB		83	*Phlebia radiata* KUC10216	YB	YB	
24	*Gymnopus* sp. KUC10139	YB	BR	●	84	*Pleurotus pulmonarius* KUC10517	N	N	
25	*Hydnochaete tabacina* KUC10522	YB	YB		85	*Pleurotus salmoneostramineus* KUC10173	YE	N	
26	*Hymenochaete innexa* KUC10512	YB	YB		86	*Plicaturopsis crispa* KUC10184	YE	YB	
27	*Hymenochaete intricata* KUC10293	BR	BR	●	87	*Polyporales* sp. KUC10105	N	N	
28	*Hymenochaete yasudai* KUC10066	YB	YB		88	*Polyporales* sp. KUC10299	N	N	
29	*Hyphoderma praetermissum* KUC10272	YB	YB		89	*Polyporales* sp. KUC10398	N	N	
30	*Hyphoderma* sp. KUC10057	YE	N		90	*Porostereum crassum* KUC10134	BR	BR	●
31	*Hyphoderma* sp. KUC10305	YB	BR	●	91	*Porostereum crassum* KUC10226	DB	BR	●
32	*Hyphodermella corrugata* KUC10254	YE	YE		92	*Porostereum crassum* KUC10390	DB	BR	●
33	*Irpex lacteus* KUC10150	YE	YB		93	*Porostereum spadiceum* KUC10094	N	N	
34	*Irpex lacteus* KUC10097	YE	YB		94	*Porostereum spadiceum* KUC10120	N	N	
35	*Irpex lacteus* KUC10200	YE	YB		95	*Porostereum spadiceum* KUC10129	YE	N	
36	*Irpex lacteus* KUC10336	YE	YB		96	*Porostereum spadiceum* KUC10385	YE	N	
37	*Irpex lacteus* KUC10357	BR	YB	●	97	*Porostereum spadiceum* KUC10416	YE	N	
38	*Irpex lacteus* KUC10344	N	YE		98	*Porostereum spadiceum* KUC10502	YE	N	
39	*Lycoperdon perlatum* KUC10211	YE	N		99	*Psathyrella* sp. KUC10343	N	N	
40	*Marasmiellus sp.* KUC10451	YB	YB		100	*Pycnoporus sanguineus* KUC10245	YB	YB	
41	*Microporus vernicipes* KUC10194	YB	BR	●	101	*Pycnoporus sanguineus* KUC10175	YB	YB	
42	*Microporus vernicipes* KUC10250	BR	BR	●	102	*Radulomyces confluens* KUC10127	YE	YE	
43	*Mycoaciella* sp. KUC10124	YE	YB		103	*Scytinostroma caudisporum* KUC10411	YB	YB	
44	*Oudemansiella canarii* KUC10263	BR	BR	●	104	*Scytinostroma* sp. KUC10314	YB	YB	
45	*Oudemansiella canarii* KUC10280	BR	BR	●	105	*Scytinostroma* sp. KUC10186	YB	YB	
46	*Panaeolus subbalteatus* KUC10069	YE	N		106	*Steccherinum murashkinskyi* KUC10317	YB	N	
47	*Panellus stipticus* KUC10289	YB	YB		107	*Steccherinum ochraceum* KUC10420	YB	YE	

Table 2 (continued)

No.	WRF	Step 1		Selected	No.	WRF	Step 1		Selected
		G	T				G	T	
48	*Panellus stipticus* KUC10401	YB	YB		108	*Trametes betulina* KUC10282	YB	BR	●
49	*Peniophora incarnata* KUC10078	BR	DB	●	109	*Trametes elegans* KUC10176	YB	YB	
50	*Peniophora incarnata* KUC10113	BR	DB	●	110	*Trametes hirsuta* KUC10439	YB	YB	
51	*Peniophora incarnata* KUC10217	DB	BR	●	111	*Trametes hirsuta* KUC10132	YB	YE	
52	*Peniophora incarnata* KUC10218	DB	DB	●	112	*Trametes trogii* KUC10258	YE	YE	
53	*Peniophora incarnata* KUC10222	BR	BR	●	113	*Trametes versicolor* KUC10137	YB	YB	
54	*Peniophora incarnata* KUC10395	BR	BR	●	114	*Trametes versicolor* KUC10166	YB	YB	
55	*Peniophora incarnata* KUC10431	BR	BR	●	115	*Trametes versicolor* KUC10290	YB	YB	
56	*Peniophora incarnata* KUC10503	DB	BR	●	116	*Trametes versicolor* KUC10404	YB	YB	
57	*Peniophora nuda* KUC10320	YE	YB		117	*Trametes versicolor* KUC10510	YB	YB	
58	*Peniophora* sp. KUC10325	YE	YB		118	*Trametes versicolor* KUC10373	YB	YB	
59	*Perenniporia fraxinea* KUC10180	YE	YE		119	*Trechispora* sp. KUC10442	YB	YE	
60	*Perenniporia fraxinea* KUC10262	YE	YE		120	*Vuilleminia cystidiata* KUC10330	YB	YB	

Table 3 also shows the results of the PAH tolerance tests, through which 14 WRF strains were screened out of the 28 previously selected in Step 2. In general, PAHs in contaminated fields are not present as individual PAH compounds, but rather, they are found in mixtures [58]. Hence, the degradation of PAHs was focused on mixtures, and on the major contaminant PAHs with 3 or 4 rings. The top 10 WRF strains exhibited over 50% tolerance to the PAH mixture and these strains belong to the five different fungal species: *M. vernicipes*, *P. incarnata*, *P. subacida*, *P. acerina*, and *P. radiata*. The other 4 WRF strains, which all belong to *P. sordida*, were additionally selected based on their radial growth rates (> 5 mm/day) on the MEA plates in the presence of the PAH mixture. These final 14 WRF strains exhibited a sufficient capability for water treatment contaminated with textile dyes and phenolic compounds. Also, they might be applied to terrestrial remediation of environment contaminated with mixture of hydrocarbons.

PAH biodegradation assessment in liquid cultures

Most of the 14 WRF strains screened by the proposed protocol showed significant enhancement in PAH biodegradation activity following their inoculation into the PAH-spiked microcosms, as shown in Fig. 2. The averaged overall degradation percentages varied from > 30 to 90% for phenanthrene (PHE), > 10–80% for anthracene (ANT), > 10–90% for fluoranthene (FLT) and > 20–95% for pyrene (PYR). Fungal activity for PAH degradation varied depending on the PAH type and WRF strain (Fig. 2). This study indicates that the proposed screening protocol specifically narrowed down the 120 WRF strains to those 14 strains with PAH degradative capabilities, but at the same time, it should be noted that their PAH degradative activities largely varied by strain. The screened WRF strains that exhibited higher overall degradation percentages for all 4 representative PAHs were concentrated only in the genera *Peniophora* (5 strains, > 40–90% overall degradation percentages). The rest of the selected fugal species, *Phanerochaete* (4 strains), *Phlebia* (2 strains), *Microporus* (2 strains) and *Perenniporia* (1 strain) generally showed larger variations in their overall degradation percentages for PAHs (from 10 to > 80% of overall degradation percentages). For example, the PAH-spiked microcosms inoculated with *M. vernicipes*, *P. subacida*, *P. acerina*, or *P. radiata* strains had generally lower overall degradation percentages, near or below 50%, compared to *P. incarnata*.

The PAH-spiked microcosms inoculated with each of the 5 *P. incarnata* strains (KUC10078, KUC10113, KUC10218, KUC10395, and KUC10503) exhibited the highest degradation percentages of over 70% for 3 of the PAHs (phenanthrene, fluoranthene and pyrene), as shown in Fig. 2. The very high initial total PAH concentrations of 120 mg L^{-1} decreased to approximately 10–20 mg L^{-1} at the end of the 14-day experiment in the microcosms inoculated with each of the 5 *P. incarnata* strains. However, all *P. incarnata* strains exhibited relatively lower biodegradative activity for anthracene (~ 40–70%), which is likely related to the low dissolution rate of anthracene in aqueous culture. The aqueous solubility of anthracene (0.015 mg/L) is much lower than those of phenanthrene (1–2 mg/L), fluoranthene (0.25 mg/L) and pyrene (0.12–0.18 mg/L). The dissolution rates of non-aqueous liquid PAHs phase may influence the biodegradative activity of the WRF [59]. Interestingly, anthracene was more significantly degraded (up to about 80%) in the microcosms inoculated with the two *P. sordida* strains, KUC10088 and KUC10351. In the PAH tolerance tests (Step 3), high growth rates (> 5 mm/day) were also observed for the same two strains of *P. sordida*. However, the other two *P. sordida*

Table 3 The results of the RBBR decolorization tests (Step 2) and PAH tolerance assays (Step 3)

No.	WRF	Step 2		Step 3			
		RBBR test	Selected	PAHs TR	Selected	Radial growth rate (mm/day)	Selected
1	*Phanerochaete sordida KUC10403*	A	●	+ +		**5.87**	●
2	*Phanerochaete sordida KUC10423*	A	●	+ +		**5.53**	●
3	*Phanerochaete sordida KUC10123*	A	●	+ +		4.88	
4	*Porostereum crassum KUC10226*	A	●	+ +		3.19	
5	*Phlebia radiata KUC10076*	B	●	+ + +	●	4.32	
6	*Phlebia acerina KUC10479*	B	●	+ + +	●	4.15	
7	*Peniophora incarnata KUC10113*	B	●	+ + +	●	3.59	
8	*Peniophora incarnata KUC10078*	B	●	+ + +	●	3.29	
9	*Peniophora incarnata KUC10395*	B	●	+ + +	●	3.14	
10	*Peniophora incarnata KUC10218*	B	●	+ + +	●	3.11	
11	*Perenniporia subacida KUC10195*	B	●	+ + +	●	2.82	
12	*Peniophora incarnata KUC10503*	B	●	+ + +	●	2.81	
13	*Microporus vernicipes KUC10250*	B	●	+ + +	●	2.78	
14	*Microporus vernicipes KUC10194*	B	●	+ + +	●	2.61	
15	*Phanerochaete sordida KUC10088*	B	●	+ +		**6.00**	●
16	*Phanerochaete sordida KUC10351*	B	●	+ +		**5.66**	●
17	*Porostereum crassum KUC10134*	B	●	+ +		3.92	
18	*Irpex lacteus KUC10357*	B	●	+ +		3.78	
19	*Phlebia acerina KUC10443*	B	●	+ +		3.56	
20	*Peniophora incarnata KUC10431*	B	●	+ +		3.16	
21	*Oudemansiella canarii KUC10263*	B	●	+ +		2.99	
22	*Peniophora incarnata KUC10217*	B	●	+ +		2.87	
23	*Peniophora incarnata KUC10222*	B	●	+ +		2.81	
24	*Phanerochaete crassa KUC10319*	B	●	+ +		2.76	
25	*Porostereum crassum KUC10390*	B	●	+ +		2.33	
26	*Oudemansiella canarii KUC10280*	B	●	+ +		2.22	
27	*Trametes betulina KUC10282*	B	●	+ +		2.02	
28	*Fuscoporia gilva KUC10234*	B	●	+ +		1.12	
29	*Gymnopus sp. KUC10139*	C					
30	*Hymenochaete intricata KUC10293*	C					
31	*Hyphoderma sp. KUC10305*	C					

The RBBR results show the time needed for full plate decolorization: 5 days (A), 6–10 days (B) or 11–15 days (C). PAH tolerance was estimated by the percent tolerance rate (%) on MEA media plates amended with the mixture of PAHs, expressed as + (0–30%), + + (30–50%), and + + + (50–70%)

strains selected by the screening protocol, KUC10423 and KUC10403, showed lower bulk degradation percentages for anthracene, below 40–50%, which indicates strain-specific behavior for PAH biodegradation.

It appears that the relevance to PAH biodegradation of some of the 14 WRF strains screened through the proposed protocol has been shown in the literature. For example, the ligninolytic enzyme of the genus *Phlebia* has been previously reported (Hildén et al. [60]; Lee et al. [19]). The significant PAH biodegradation potentials of genera *Peniophora*, *Phanerochaete*, and *Phlebia* have also been previously reported [19, 29, 60]. The screened *M. vernicipes*, *P. subacida*, *P. acerina*, and *P. radiata*, exhibited more moderately increased PAH degradation activities and have not been extensively documented as PAH-degrading WRF in the literature. The PAH biodegradation potential by *M. vernicipes* for pyrene was only indirectly assessed previously with a respiratory experimental technique [61]. The screening protocol, in combination with the PAH-spiked microcosm experiments for biodegradation, workably screened 14 WRF and was generally in good agreement with the known PAH-degrading WRF in the literature. This suggests a strategic protocol that may enable us to focus more effectively on the most promising fungal species (e.g., *P. incarnata* in this

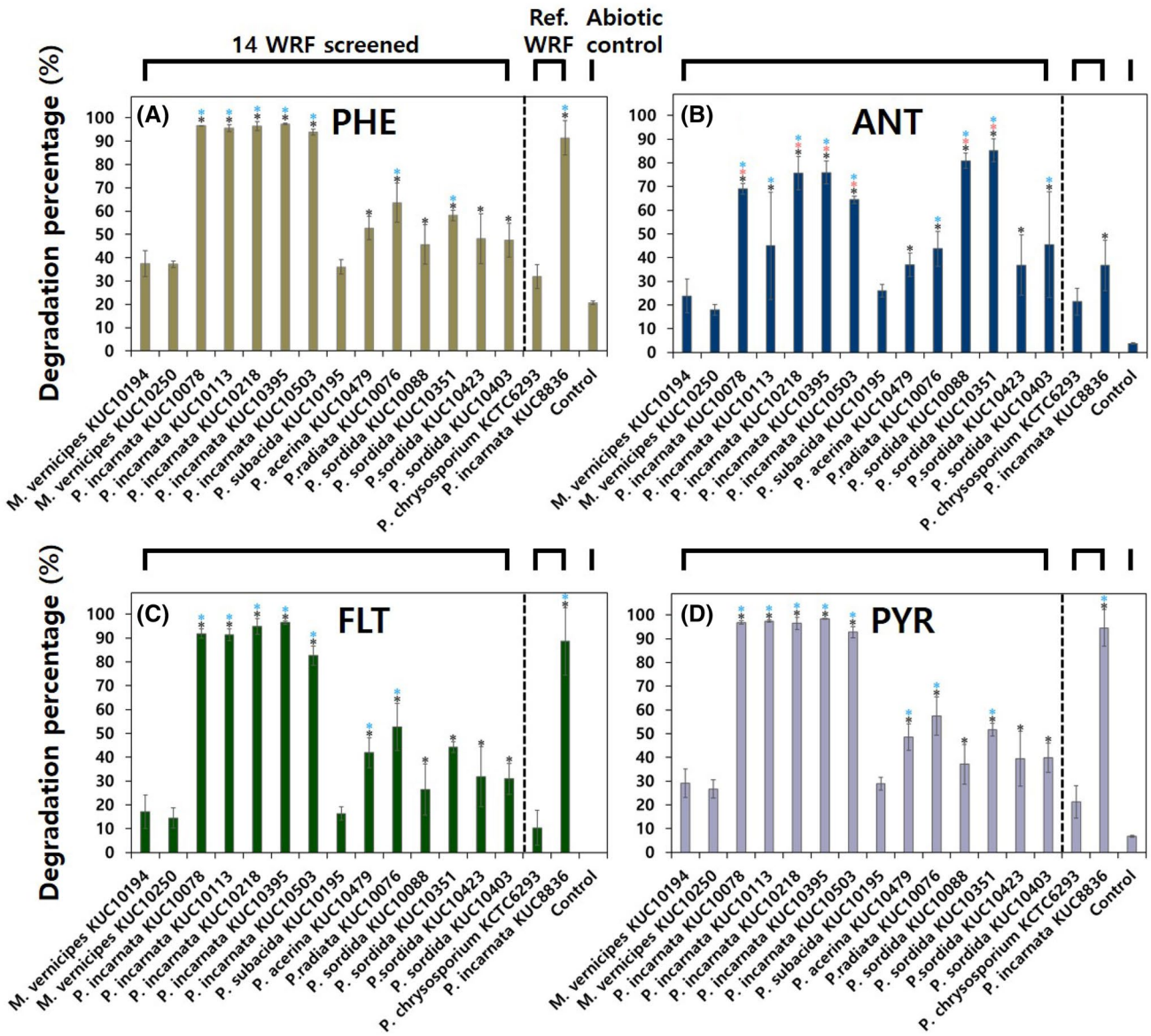

Fig. 2 The PAH degradation percentage of the 14 WRF strains selected by the stepwise screening protocol proposed in this study, along with those of reference PAH-degraders. The statistically significant differences ($p < 0.001$) in the degradation percentages of the selected strains and three controls are represented by asterisks: *P. chrysosporium* KCTC6293 (blue), *P. incarnata* KUC8836 (light red), and the abiotic control (black)

study) with a greater potential for PAH biodegradative activity. *P. incarnata* is also known as rosy crust, a wood rotting fungus. *P. incarnata* is related to *P. piceae* and *P. crassitunicata* based on its internal transcribed spacer (ITS) region in the phylogenetic cluster (Fig. 3). The five strains of *P. incarnata*, KUC10078, KC10113, KUC10218, KUC10395 and KUC10503, are slightly different with the three clusters (Cluster 1, *P. incarnata* KUC10395 and KUC10078; Cluster 2, *P. incarnata* KUC1218 and KUC10113; and Cluster 3, *P. incarnata* KUC10503). However, they are very closely matched to each other. *P. incarnata* has only relatively recently received attention as a potential PAH degrader [41, 58].

Tracked ligninolytic enzyme activity during PAH biodegradation

During PAH degradation, most of the screened WRF strains produced the three principal ligninolytic enzymes (laccase, MnP and LiP) in the PAH-spiked microcosms. As shown in Fig. 4, greater production of the three principal ligninolytic enzymes results in higher total PAH degradation

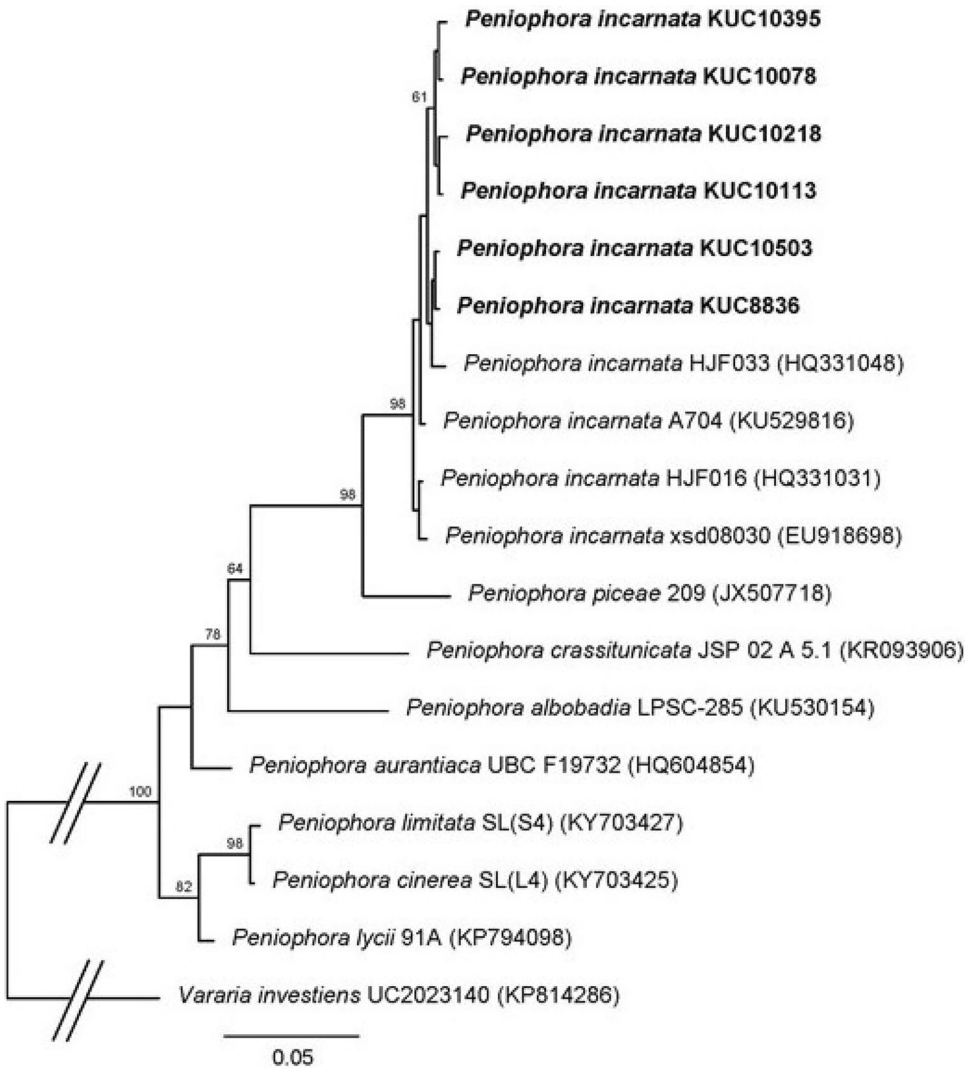

Fig. 3 Neighbor-joining tree for the five *Peniophora incarnata* strains (1000 bootstrap replicates)

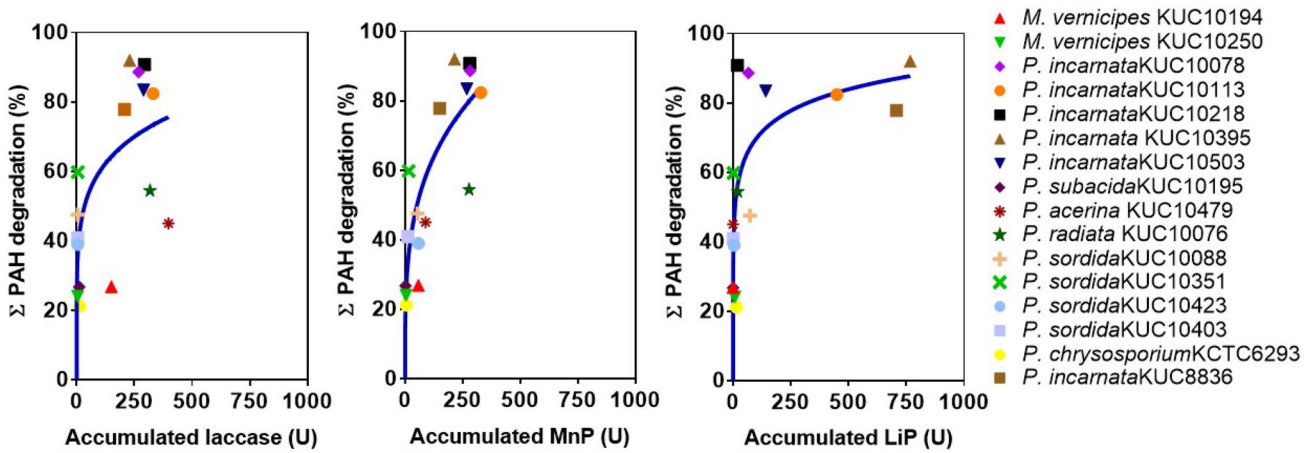

Fig. 4 Relationship between ligninolytic enzyme production and total PAH degradation (phenanthrene, anthracene, fluoranthene, and pyrene) by the selected PAH-degrading WRF

efficiency (for the 4 PAHs), indicating enzyme-induced PAH degradation.

With a focus on the *P. incarnata* group, a shift in ligninolytic enzyme production was observed during PAH degradation in the 14-day microcosm experiment. As shown in Fig. 5, the majority of *P. incarnata* strains produced laccase (EC 1.10.3.2) and MnP (EC 1.11.1.13) enzymes in notably greater quantities in the early days, whereas the onsets of the LiP (EC 1.11.1.14) production intensified only in the later days of PAH degradation. In the microcosms inoculated with *P. incarnata* KUC 10,113, for example, laccase and MnP enzyme productions were highest at 92 U mL^{-1} and 93 U mL^{-1}, respectively, on Day 4 of the experiment (Fig. 5). However, LiP production by strain KUC 10,113 reached a peak of over 200 U mL^{-1} on Day 14 of the experiment. It should be noted that these observations are only based on the enzyme secretion patterns the *P. incarnata* group. The secretion patterns of ligninolytic enzymes appeared to be highly strain-specific, even within the *P. incarnata* group exhibiting the strongest PAH degradative activity. For instance, *P. incarnata* KUC 10,503 produced the greatest secretions of laccase (~70 U mL^{-1}) and MnP (~60 U mL-1) around Day 10 of the experiment, which was considerably delayed compared to other *P. incarnata* strains (< 40 U mL^{-1}).

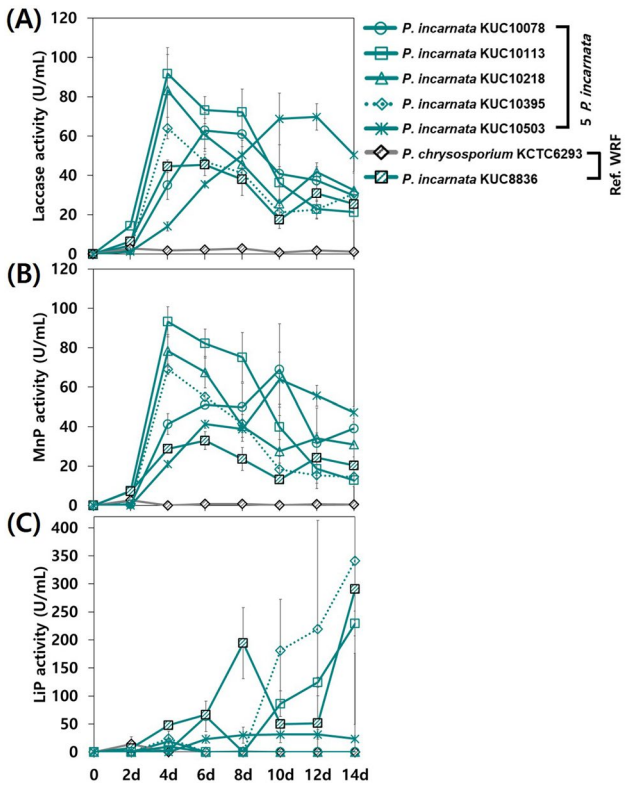

Fig. 5 Ligninolytic enzyme production of the five *Peniophora incarnata* strains during PAH degradation

In this study, the shifts in enzyme secretion in the *P. incarnata* group may be related to variations in the physicochemical characteristics (e.g., dissolution rates) of each carbon substrate (PHE, ANT, FLT, and PYR), as well as to potential intermediate compounds during PAH biodegradation. The physicochemical characteristics of the PAHs may influence their bioavailability (e.g., there is a large variation in the aqueous solubilities of the four PAHs: 0.015–2 mg L^{-1}). As discussed above, the degradation of anthracene in the *P. incarnata*-inoculated microcosms tended to be distinctly lower than for the other PAHs. It is speculated that the time-variable ligninolytic enzyme activity may be due to changes in the abundance of bioavailable dissolved substrates in the PAH-water system. The earlier laccase and MnP enzyme secretions by the majority of *P. incarnata* strains may be related to the presence of readily bioavailable carbon and nutrient (nitrogen) sources, and metal inducers (e.g., Mn^{2+}), in the ME media and acetone (miscible solvent), as well as to dissolved PAHs in the early stage of biodegradation. The delayed production of LiP may be related to slowly dissolving PAHs and intermediate compounds formed by the early enzymatic activity. Laccase is a phenoloxidase with a broad substrate specificity that includes phenols, aromatic or aliphatic amines, and the corresponding reactive radicals [13].

The early production of laccase by WRF associated with phenolic compounds and aromatic amines has been reported [12]. MnP oxidizes Mn(II) to Mn(III), which then oxidizes phenol rings to phenoxy radicals, and MnP has been shown to mediate the initial steps in the degradation of high-molecular-mass lignin [12, 13]. It is known that LiP, which is secreted for secondary metabolic activity associated with nutrient limitation (e.g., nitrogen), is a strong oxidizer capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers and PAHs [1]. The clades of both MnP and LiP belong to Class II (fungal secretory peroxidase), which play crucial roles in the degradation of lignin [12, 62]. This study suggests the possibility of employing the sequential productions of the major ligninolytic extracellular enzymes associated with variable substrate bioavailability during PAH biodegradation in a PAH-water system. However, the individual roles and specific interactive relationships of extracellular laccase, MnP and LiP secreted by *P. incarnata* exposed to multiple PAHs have not been extensively understood. Further research is needed to understand the in-depth mechanisms of peroxidase-catalyzed biodegradation of multiple PAHs, and the respective PAH quinones, by the *P. incarnata* group.

Laccase and MnP gene expression during pyrene biodegradation

The RT-qPCR-based gene expression analyses were performed using the pyrene-spiked liquid media inoculated

with the five screened *P. incarnata* strains, by targeting the genes *pilc1* and *pimp1*. At the same time, corresponding ligninolytic enzyme activity was measured using the fungal samples obtained from the same pyrene-spiked liquid media. Figure 6 presents the *pilc1* and *pimp1* gene expressions relative to the *act1* gene (reference), and the concurrent ligninolytic enzyme production in the pyrene-spiked liquid media after 7 days of incubation. The co-production of major ligninolytic enzymes (laccase, MnP and LiP) during pyrene biodegradation coincided with the expression of the laccase- and MnP-encoding genes (*pilc1* and *pimp1*), although the estimated expressions varied largely across the fungal strains (Fig. 6 and Table S1). The expression of laccase and MnP genes for ligninolytic enzyme production by *Peniophora incarnata* has recently been reported [41]. These genes with GenBank accession numbers KJ622360 and KJ622361 for *pilp1* and *pimp1*, respectively, have been unveiled by a full-length cDNA sequence.

Early on, laccase was more readily produced in the pyrene-spiked microcosms than MnP and LiP, except by *P. incarnata* KUC10218. In later stages, MnP production was generally greater than LiP production. For example, laccase, MnP and LiP productions in the pyrene-spiked liquid media inoculated with *P. incarnata* KCU10078 were estimated at 165, 107, and < 20 U mL^{-1} at the end of the 7-day experiment. This preferential pattern of ligninolytic enzyme secretions, which is presumably due to changes in substrate bioavailability associated with the transient dissolution

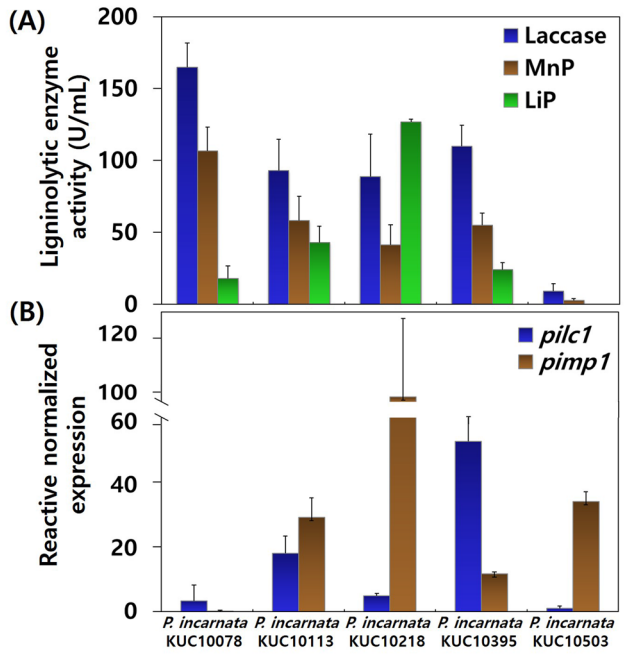

Fig. 6 Ligninolytic enzyme production (laccase and MnP) and gene expression for the target genes *pilc1* and *pimp1* in pyrene-spiked liquid media inoculated with the five screened *P. incarnata* strains

of hydrophobic PAHs as a carbon source, was similarly observed in the microcosms spiked with the 4 PAHs, as shown in Fig. 5. This indicates that the sequential production of important ligninolytic enzymes may be reproducible in pyrene-spiked liquid media. It is known that the regulation of laccase and peroxidase genes is affected by environmental signals such as the concentrations of carbon, nitrogen, and metal ions, as well as by xenobiotic compounds, temperature, daylight hours, and WRF growth conditions [13]. The lines of evidence gathered from gene expression analyses coupled with assessments of ligninolytic enzyme production and PAH biodegradation suggest a potential upregulation of laccase- and MnP-related genes for the secretion of the ligninolytic enzymes in the presence of pyrene by at least some of the *P. incarnata* strains (KUC10218, KUC10395, and KUC10503, $p < 0.05$).

Implications for bioremediation

The results of the screening protocol were in good agreement with the previously reported WRF related to the degradation of phenolic and non-phenolic aromatic compounds (Table 4). Most of the fungal species screened at each step of the proposed screening protocol have been previously reported in the literature as significant to biotechnology, because they utilize gallic acid, lignin, dioxin, polychlorinated biphenyl (PCB), chlorobenzene, pentachlorophenol (PCP), trichloroethylene (TCE), dyes (RBBR, Orange II, Reactive Blue, Poly R-478), and PAHs. This affirms that the screening protocol broadly covers WRF capable of degrading organic contaminants, including PAHs. Of the fungal strains screened in this study, the five *P. incarnata* strains capable of actively producing ligninolytic enzymes and degrading the 4 PAHs are considered highly promising biological agents for the bioremediation of PAH-contaminated soils and water.

However, the effects of the mass transfer rate of non-aqueous phase liquids (NAPLs) through environmental matrices (soil and water) to fugal biomass may still significantly affect the applicability of the screened PAH-degrading WRF for bioremediation. The potential implications for bioremediation are only indirectly suggested by the sequential production of ligninolytic enzymes associated with substrate bioavailability. For example, limited anthracene biodegradation was observed in the five *P. incarnata* group in this study (Fig. 2). However, Lee et al. [41] reported a significant increase in the degradation rates of anthracene due to the addition of Tween 80 in the same malt extract nutrient media amended with *Peniophora incarnata* KUC8836. Tween 80 is a well-known nonionic surfactant that increases the aqueous solubility of hydrophobic organic carbon (HOC) in NAPL-water systems, which increases bioavailability and improves degradation efficiency for anthracene, which has a

Table 4 The screened fungal species, as found in the literature

Fungal species	Step 1: Gallic and tannic acids			Step 2: Dye decolorization (RBBR)			Step 3: PAH tolerance		
	Selection	Literature information		Selection	Literature information		Selection	Literature information	
		Reference	Chemical		Reference	Chemical		Reference	Chemical
Peniophora incarnata	●	[40]	Gallic acid	●	[40]	RBBR	●	[41]	PAHs
Microporus vernicipes	●	[51]	Lignin	●			●		
Perenniporia subacida	●			●	[52]	RBBR	●		
Phlebia acerina	●	[47]	Dioxin	●	[52]	RBBR	●		
Phlebia radiata	●	[47]	PCB, Dioxin	●	[53]	OrangeII, Reactive Blue	●		
Phanerochaete sordida	●	[47]	Dioxin, Chlorobenzene	●	[52]	RBBR, Poly R-478	●	[29]	PAHs
Porostereum crassum	●			●	[52]	RBBR, Poly R-478			
Phanerochaete crassa	●	[48]	PCP	●	[52]	RBBR, Poly R-478			
Oudemansiella canarii	●			●					
Irpex lacteus	●	[47]	TCE	●	[52]	RBBR, Poly R-478			
Fuscoporia gilva	●			●					
Trametes betulina	●			●	[52]	RBBR, Poly R-478			
Hymenochaete intricata	●				[52]	RBBR			
Gymnopus sp.	●				[52]	RBBR			
Hyphoderma sp.	●								

lower aqueous solubility than the other 3 PAHs considered in this study [63]. At the same time, Tween 80 might act as a co-substrate, causing increased ligninolytic enzyme production under nutrient- and substrate-limited conditions [41].

On the other hand, ligninolytic enzymes are extracellular and can theoretically diffuse into less accessible pore spaces where PAHs are trapped in soil and sediment matrices. Prior to implementing bioremediation techniques with PAH-degrading WRF screened and verified through the proposed protocol, mass transfer effects associated with the characteristics of NAPLs (e.g., dissolution rates) and environmental matrices (e.g., sorbed HOCs) should be carefully characterized, along with fungal growth conditions (e.g., temperature, pH, oxygen supply, and nutrient accessibility), system scales, biodegradation kinetics, and so on. In addition, potential competition with indigenous microbial communities, including predation effects and fungi-bacteria interactions and mobilization, needs to be considered in future comprehensive feasibility studies for bioremediation with the selected WRF [24, 64].

Beginning with 120 WRF strains that were isolated previously, the proposed, simple screening protocol selected 14 PAH-degrading WRF. Screening took approximately 40 days, including the incubation periods required at each screening step (visualizations of lignin degradation activity, decolorization, and tolerance to target PAHs) and the following PAH biodegradation microcosm experiment. The suggested stepwise screening procedure provides a practical, strictly culture-dependent framework capable of screening a relatively large number of WRF isolates (120), compared to the previous WRF screening procedures for smaller numbers of isolates suggested in the literature [10, 11, 27–30]. Prior to comprehensive PAH bioremediation feasibility studies and field implementation, the screening procedure should be further optimized depending on the screening purpose, scale of the problem, and available laboratory equipment and infrastructure.

Conclusions

This study screened 14 PAH-degrading white rot fungal strains out of 120 strains randomly collected from various forests in South Korea. A simple, stepwise screening protocol, which combines Bavendamm's reaction (Step 1), RBBR decolorization assays (Step 2), and PAH tolerance assays (Step 3), was subsequently verified by a series of the assessments of PAH biodegradation, ligninolytic enzyme

secretion, and laccase- and peroxidase-encoding genes. It appeared that the screening protocol identified the 14 WRF exhibiting low to high degradation activity for the 4 PAHs considered (phenanthrene, anthracene, fluoranthene, and pyrene). Most of the WRF strains screened through the proposed protocol have been previously reported as the environmentally significant fungal species capable of degrading phenolic and non-phenolic compounds including lignin, dioxin, dyes, PCB, TCE, and PAHs. Of the isolated strains, *Peniophora incarnata* exhibited the strongest PAH-biodegradation activity in this study. The secretion of the three principal ligninolytic enzymes (laccase, MnP and LiP) by the selected WRF was monitored during PAH biodegradation, and the expression of genes encoding the ligninolytic enzymes (laccase and MnP) confirmed the occurrence of ligninolytic-enzyme-induced PAH biodegradation. Laccase and MnP secretions by *Peniophora incarnata* were involved in the early stages of PAH biodegradation, whereas LiP production was generally involved later. It is speculated that the sequential ligninolytic enzyme secretions were presumably due to changes in substrate bioavailability associated with the varying solubilities of the 4 PAHs in the PAH-water system. The proposed screening and verification framework can be adapted for different screening purposes and scopes. This study suggests a simple screening protocol and transcript-level verification for PAH-degrading white rot fungi with potential applications as biological resources for the bioremediation of PAH-contaminated soils and water.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest to declare.

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