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Screening white-rot fungi for bioremediation potential of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

Ligninolytic fungi contain a number of representative strains consisting of mainly white-rot fungi (WRF) that produce lignin-modifying enzymes (LME) such as laccases and manganese peroxidases. Lignin-modifying enzymes are multipurpose enzymes which have potential for application in various fields such as, for example, bioremediation and biomass conversion. Because of the non-specific nature of these enzymes, they are also capable of biodegradation and removal of xenobiotic pollutants. In this study we used a tiered screening process where we screened over 70 Vietnamese WRF fungal isolates for LME activity and subsequently for the ability to breakdown the dioxin TCDD. After the initial screening we selected four fungal strains, which belong to the order of Polyporales, which excreted high laccase enzyme levels. The most active fungus being isolate FMD21, a species of *Rigidoporus*, which was isolated from a forest in the South of Vietnam and which produced both laccase and manganese peroxidase. In the optimized PDSRb medium, FMD21 laccase levels reaced activities of 238800 U/L after 10 days while MnP activity showed the highest activity at day 4 of aproximately 40 U/L. 2,3,7,8-TCDD, which is the most toxic dioxin congener, is a persistent organic pollutant of which few organisms are known that break it down. After the final screening, FMD21 was the only fungus capable of degrading TCDD and was able to reach a breakdown percentage of 73% after 28 days culture with a start concentration of 0.5 pg TEQ/μL TCDD. Co-cultivation experiments of up to three fungi were performed to test for a synergistic breakdown effect of TCDD but such an effect was not observed. FMD21 is a fungus that shows a potential to be used as a bioremediation agent to clean up dioxin contamination in the environment.

1. Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), more commonly known as dioxins (PCDD/Fs), are persistent organic and toxic environmental pollutants formed from various sources which include industrial processes, such as the paper pulping industry and the manufacturing of organochlorine-based herbicides (Chang, 2008). Also, incomplete combustion and the incineration of municipal and industrial waste can be sources of dioxin emissions into the environment. Due to their high chemical and thermostability, dioxins are difficult to remove from the environment by conventional remediation techniques. Bioremediation, in which (micro)organisms degrade xenobiotic pollutants, may be an alternative and feasible method to remove dioxins from the environment (Chang, 2008). Here we focus on mycoremediation, where fungi or fungal enzymes are used for xenobiotic degradation. Fungi show a wide range in degradation potential for many types of pollutants, from wrapping paper to hazardous compounds in soil and sediment (Singh, 2006). Wood-degrading fungi, such as white-rot fungi (WRF) have been studied previously and have shown the ability to breakdown not only complex natural polymers such as lignin and chitin, but also wide range xenobiotic compounds such as phenols and chlorinated phenols. They are ubiquitous in the environment and during growth they construct a wide hyphal network of spreading mycelia which gives them good access to less bioavailable soil pollutants and low soluble compounds. The actual breakdown of lignin and xenobiotics is done by the secretion of lignin-modifying enzymes (LMEs). LMEs, such as laccase, manganese peroxidase and lignin peroxidase, are key enzymes made by fungi

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which, in nature, are involved in the wood delignification processes in which lignocellulose is broken down in order to reach nutrient resources such as cellulose. Different WRF species produce a different repertoire of LME enzymes at different yields making not all WRF species suitable for mycoremediation. There have been several studies focusing on the degradation of persistent organic pollutant by means of wood-degrading fungi (Singh, 2006; Strong and Claus, 2011; Viswanath, 2014). Several strains of white-rot fungi are used as model organic pollutant degraders, such as *Phanerochaete chrysosporium*, *Phlebia lindtneri* and *Pleaurotus ostreatus*. However, most studies focus on polychlorinated biphenyls (PCBs); polycyclic aromatic hydrocarbons (PAH) or pesticides, while there is limited information on degradation of dioxins, in particular 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Of the dioxin and dioxin-like compounds TCDD is the most toxic congener to humans, as well as being the most lipophilic and chemically stable of the different dioxin congeners (EPA, 2010). Takeda et al. (1996) has shown that TCDD was degraded by *Phanerochaete sordida* YK-624. *Phanerochaete* spp. are known for their metabolic versatility and their effectivity in the degradation of many recalcitrant pollutants. In the study presented here, isolated fungi from tropical forests in Vietnam were sampled and screened for the presence of lignin-modifying enzymes which may be a key factor for the removal of chlorinated phenolic compounds. Subsequently, they were screened for their bioremediation potential by degradation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most toxic and recalcitrant dioxin, within the condition in which the fungi can secrete fairly LMEs.

2. Materials and methods

2.1. Fungal strains

Seventy-six basidiomycetes fungi were collected and isolated from different forests located in Vietnam (for details of the isolates see supplemental Table 1). Fungal strains that showed laccase activity in the form of a reddish colour on potato dextrose agar (PDA) plates containing 0.01% of guaiacol as an indicator were selected. These strains were maintained under paraffin oil for long-term preservation at room temperature. After reactivation and sub-culturing on Potato Dextrose Agar (PDA) plates with 0.01% guaiacol, the fungi were used as inoculant for further study.

2.2. Chemicals

Laccase substrate 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), buffer constituents such as sodium acetate, acetic acid, sodium malonate and malonic acid for enzyme assays in this study were purchased from Sigma-Aldrich (St. Louis, USA). Organic solvents such as n-hexane, iso-propanol (HPLC grade, suitable for Calux) used for TCDD extraction and subsequent DR-CALUX® analysis were purchased from Biosolve Chimie (Dieuze, France) except dimethyl sulfoxide (DMSO) which was purchased from Acros Organics (New Jersey, USA). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was bought from the Cambridge Isotope Laboratories (Tewksburry, USA). Ingredients for fungal cultivation, which included potato dextrose broth was obtained from Sigma-Aldrich (St. Louis, USA) and soy meal, rice bran were purchased locally, and tap water was supplied by Waternet (Amsterdam, The Netherlands). Water used in biochemical reactions was super-demineralized water purified by Elga-purelab FLEX.

Table 1
Effect of cultivation conditions on laccase production by *Cerrena* sp. FBV41.

Factors	Effect on Laccase production
Type of water	Positive effect
Tap water	Negative effect
Distilled water	
$CuSO_4$	Negative effect
Supplementation (0.25 mM)	Positive effect
No supplementation	
Light	Ineffective
Natural day-night	Ineffective
In dark	
Component sources	Ineffective
Inoculation method	Negative effect
With agar plug	Positive effect
With 3.3 % of fungal liquid culture	

2.3. Fungal enzyme assays

Fungal cultural medium was filtrated through a 0.22 µm membrane to obtain crude extracellular enzymes of which enzyme activities were determined spectrophotometrically. All assays were performed in triplicate in flat bottom 96 well microplates and the absorbance was measured by a microplate reader Spark 10 M (TECAN, Männedorf, Switzerland). One unit of activity was defined as the amount of enzyme that catalysed 1 µmol of substrate per minute at 30 °C. Enzyme activity was expressed in units per liter (U L^{-1}).

For laccase activity, the assay mixture contained 0.5 mM ABTS as a substrate; 20 mM sodium acetate buffer pH 3 and 35 µl of extracellular enzyme to a final volume of 175 µl. The oxidation of ABTS was monitored by determining the increase of blue-green colour of the radical cation ABTS ($ABTS^{\bullet+}$) within two minutes at $\lambda = 420$ nm ($\varepsilon = 36\,000$ $M^{-1}\,cm^{-1}$), 30 °C (Srinivasan et al., 1995).

The manganese peroxidase (MnP) assay was modified based on Wariishi et al. (1992). MnP oxidizes Mn^{2+} to Mn^{3+}, this product combined with malonate to form a stable complex with an absorbance at $\lambda = 270$ nm ($\varepsilon = 11\,590$ $M^{-1}\,cm^{-1}$), 30 °C (Wariishi et al., 1992). The reaction mixture contained 5 mM $MnSO_4$; 0.1 mM H_2O_2; 50 mM sodium malonate buffer pH 4.5; and 35 µl extracellular enzyme in a total volume of 175 µl.

2.4. LMEs screening and laccase production

Lignolytic enzyme producing fungi were initially screened on PDA plates containing 0.01% guaiacol (Kiiskinen et al., 2004). Fungal strains which were active showed a dark reddish-brown colour and these were selected to the second step of screening. Subsequently, three agar plugs of fully grown fungi on PDA were used to inoculate 50 ml potato dextrose broth (PDB) medium in a 250 ml Erlenmeyer flask and cultivated at 30 °C and 200 rpm for screening. Laccase activity served as a proxy for LME activity and was measured daily. Strains that showed the highest activities were selected for LME production optimization.

LME production of selected fungi was improved by a three-step cultivation procedure. Firstly, fungal subcultures between the second and fifth passage on 0.01% guaiacol PDA plates were used as inoculant and grown for 5–7 days at 30 °C to ensure the highest laccase enzyme activity and maintain a stable enzyme production. Secondly, fungal mycelium from the subculture was used for submerged fermentation: three 1 cm agar plugs were transferred into 150 ml PDB medium in a 250 ml Erlenmeyer flask. The third stage was also conducted in 250 ml Erlenmeyer flasks with 150 ml of PDSRb medium containing potato dextrose powder 24 g; soy meal 5 g; and rice bran 1 g per liter, pH 7, 3.3% v/v of inoculant from the previous step. Cultural conditions were at 200 rpm and 30 °C for five days for the second step and ten days for the third step. Tap water (provided by Waternet, Amsterdam, The Netherlands) was used to carry out every cultivation without the supplementation of copper. Laccase and MnP activity were measured every day. All experiments were performed in triplicate. Crude extracellular enzymes were harvested and filtered through a 0,25 µm membrane prior to its use in TCDD degradation studies.

Fig. 1. Morphology of *Rigidoporus* sp. FMD21. **A**: Fungus on decaying tree branch in Dong Nai biosphere reserve, Vietnam; **B**: Aerial hyphae on PDA plate; **C**: Vegetative hyphae on PDA plate with guaiacol, red-brown colour as an indicator for lignin-modifying enzymes.

2.5. Fungal classification and molecular typing

Fungal DNA was extracted using the DNeasy plant mini kit (Qiagen, Hilden, Germany). For phylogenetic analysis, both the ITS1-5.8S-ITS2 region and 18S rDNA genes were amplified by PCR using fusion DNA polymerase with primers ITS1/ITS4 (White et al., 1990) and EF4/EF3 (Smit et al., 1999) respectively.

Because of the similarity in phenotype and internal transcribed spacer (ITS) sequences of some selected fungi, we used an additional fingerprinting technique in order to distinguish between selected fungi at the genomic level. In this study, **re**trotransposon **m**icrosatellite **a**mplified **p**olymorphism (REMAP) was applied as a molecular typing method. Three different combinations out of three primers, including UBC807 for single sequence repeat (SSR) specific primer; marY1-LTR-L and marY1-LTR-R for outward-facing long terminal repeat (LTR) primer, were conducted in PCR reaction. PCR fragments were separated by long agarose gel electrophoresis and we adapted image analysis by GelCompar II according to the description of Le et al.(2008).

2.6. TCDD analysis by DR CALUX®

TCDD was quantified using the DR-CALUX® assay (**D**ioxin **R**esponsive-**C**hemical **a**ctivated **lu**ciferase gene e**x**pression) (Besselink et al., 2004; Hoogenboom et al., 2006; van Vugt-Lussenburg et al., 2013). This assay is based on activation of the Ah-receptor pathway. AhR is activated by ligands such as dioxin or dioxin-like compounds, specifically, 2,3,7,8-TCDD used in this investigation (Denison and Heath-Pagliuso, 1998; Giesy et al., 2002; van Vugt-Lussenburg et al., 2013). Total residual TCDD in samples was extracted by shaken-solvent extraction using n-hexane/iso-propanol. Fungal cultivation including fluid and biomass were transferred to glass extraction bottles, followed by iso-propanol and n-hexane with the 1:1:2 ratio of volume. The Erlenmeyer itself was rinsed 3 times with n-hexane which was then incorporated into the cultural fluid and biomass to go into extraction procedure. After shaking at 250 rpm for 30 min, top layer was transferred into new glass tubes, new n-hexane was added into extraction bottle and repeated the same for three times. The combined extracts were concentrated by evaporation to a volume of approximately 1 ml before cleaned up by sulphuric acid silica column which contains three layers: the top layer of the column is sodium sulphate, second layer of 20% sulphuric acid silica and the final layer of 33% sulphuric acid silica. TCDD was eluted by mixture of n-Hexane/DEE (100/3) solution, the solvent after that was evaporated and the sample was transferred to DMSO for exposure in the DR CALUX® bioassay.

2.7. Experimental setup for TCDD degradation

TCDD degradation studies were performed using four selected fungi FMD21; FBV41; FBV311 and FBD154 and two combinations: Co-FMD21 consisted of FMD21, FBV311, and FBD154; and Co-FBV41 consisted of FBV41, FBV311, and FBD154. Laccase and MnP activity were measured in the liquid medium at several time points during the whole 28-day cultivation process (Fig. 5). TCDD was spiked at the start of the 28-day cultivation with 0.5 pgTEQ/μL. TCDD was analyzed in fungal cultures after 28 days of cultivation. The percentage of TCDD degradation was calculated based on the difference between TCDD following fungal cultivation for 28 days and the control. Two controls were used, i.e., control 1: which contained all components, without fungal inoculants and under the similar conditions, but was analysed at the time zero (0 h incubation), the starting point of the experiment. In addition, another control, Control_2 was the same as Control_1 but underwent similar cultivation as the fungal samples and was then analysed at 28 days of incubation. Fungi were cultivated in 50 ml PDSRb medium in triplicate. Moreover, q-PCR with strain-specific primers based on the ITS sequence was carried out to detect the proportion of each strain present in the combination culture. q-PCR was performed using the iQ SYBR green from Biorad (Hercules, USA) according to the manufacturer's instructions. Laccase and MnP were measure daily.

3. Results and discussion

3.1. Fungal screening for LME production

Fungal fruit body and fungal mycelia were collected from decaying wood and leaf litter in the forest. Specimens were stored in sterilized containers and transported to the laboratory at 10 °C within three days after sampling (Fig. 1A). Collected specimens were used as material for purification, fungal mycelia on solid PDA were sub-cultured to get pure strains (Fig. 1B; C). The majority of the isolates (67 out of 76 isolates) showed the oxidation of guaiacol (Supplemental Table 1). We selected the eight strains that showed the highest activity based on the size and shade of the halo of red-brown oxidised guaiacol (Table 2). We selected two strains from Ba Vi National Park in the North of Vietnam (FBV41 and FBV311); two from A Luoi in the Middle of Vietnam (FAL3 and FAL11) and FBD154 and FMD21 were sampled from Bidoup National Park and Dong Nai Culture and Nature Reserve respectively (South of Vietnam). The overall laccase activity of the selected isolates was not correlated to a specific region of sampling (Fig. 2). FBV40 and FBV41 were sampled from the litter layer whilst the others were sampled from rotting branches.

Subsequently, the eight selected fungi underwent a secondary screening under submerged conditions. The medium used in this step was potato dextrose broth which is an ubiquitous basic medium used in laccase production studies (da Silva Coelho-Moreira et al., 2013; Yang et al., 2014). Laccase activities were measured daily till the activity started to decline. The highest measured laccase activity of each isolate is depicted in Fig. 2. The timepoint at which the highest laccase activity was observed was different for the different strains. FAL3, FAL11 and

Table 2
Classification of selected fungi based on ITS sequences.

Fungus	Origin	Order	Family	Genus	Best score
FBV40	Ba Vi national park	Polyporales	Polyporaceae	*Cerrena*	*Cerrena* sp. HYB07
FBV41	Ba Vi national park	Polyporales	Polyporaceae	*Cerrena*	*Cerrena* sp. isolate Lyc23
FBV311	Ba Vi national park	Polyporales	Steccherinaceae	*Junghuhnia*	*Junghuhnia crustacea* X1127
FTQ8	Tuyen Quang forest	Polyporales	Meripilaceae	*Rigidoporus*	*Rigidoporus vinctus* ICA F02
FAL3	A Luoi forest	Polyporales	Polyporaceae	*Pycnoporus*	*Pycnoporus sanguineus* CBS 358.63
FAL11	A Luoi forest	Hymenochaetales	Hymenochaetaceae	*Inonotus*	*Inonotus pachyphloeus* CBS 193.37
FBD154	Bi-doup Nui Ba national park	Polyporales	Polyporaceae	*Polyporus*	*Polyporus arcularius* DSH92132
FMD21	Dong Nai culture and nature reserve	Polyporales	Meripilaceae	*Rigidoporus*	*Rigidoporus vinctus* NZD-mf190

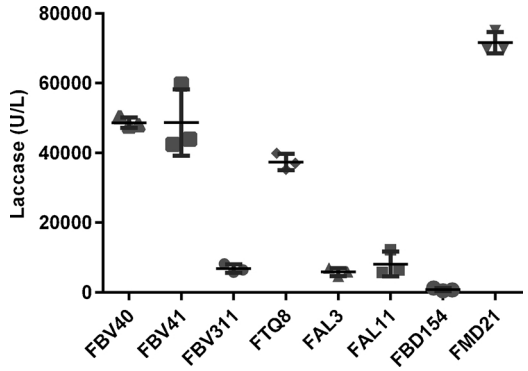

Fig. 2. Screening of laccase activity excreted in the medium from selected fungal strains in potato dextrose broth. The laccase activity represents mean and standard deviation, n = 3 per fungal strain.

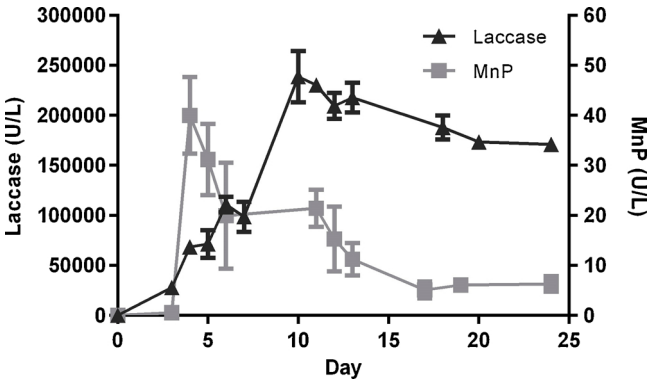

Fig. 3. Laccase and MnP activity excreted by *Rigidoporus* sp. FMD21 in PDSRb medium. Value of each point represents the mean of a triplicate analysis and the error bar represents the standard derivation.

FBV311 showed the highest laccase activity after twelve, seven and eight days of cultivation, respectively. For the five other fungi activities were at their peak after ten days of cultivation. Although high laccase activity was found on the PDA screening plate of FBD154, this strain secreted the lowest laccase activity level in the liquid medium e.g., 794 U/L. In contrast, the highest laccase level was observed in the medium for FMD21 with 71,600 U/L, followed by FBV40, FBV41 and FTQ8. FBV41 and FMD21 were selected as model species to further investigate the culture conditions in which fungi secreted the highest enzyme concentration and activity.

3.2. Optimization of LME production

LMEs are considered as the main group of enzymes which are involved in the transformation of a variety of pollutants therefore in order to get sufficient enzyme activity optimization attempts have been made. Other studies have identified many fungal strains as laccase producers and these strains have been studied in order to improve their enzyme secretion efficiency (Brijwani et al., 2010; Yang et al., 2017). Optimization for laccase production shows that some ingredients can enhance the laccase yield. Additions such as grain bran, soy meal, lignocellulosic wastes or even tomato juice can all enhance laccase production and secretion (Lorenzo et al., 2002; Ramírez-Cavazos et al., 2014; Yang et al., 2017). In our study a similar effect was observed in which soy meal and rice bran enhanced laccase production when added to the medium in which potato dextrose powder was the main carbon source. Using PDSRb medium, several additional factors were tested (Table 1). There was no difference in laccase activity between samples that were cultivated entirely in the dark and samples that were cultured in a natural day-night cycle and also with the type of soy meal and rice bran. We observed, however a significant difference in laccase levels between tap water (provided by Waternet, Amsterdam, The Netherlands) and distilled water. Interestingly, when tap water was used, laccase activity surpassed the distilled water by 8.5 times. The reason for this may be that some trace elements available in tap water act like inducers for laccase production (Hendricks et al., 1995; Schmidt et al., 2005).

Several previous studies have demonstrated that the Cu^{2+} concentration can enhance fungal laccase production (Ramírez-Cavazos et al., 2014; Yang et al., 2017). In this study, laccase activities were compared with to without 0.25 mM of Cu^{2+}. Data shows however that additional copper did not enhance the production but even slightly decreased the activity.

Using the optimized PDSRb medium, the highest laccase activity observed in FBV41 was 65,518 U/L after nine days of cultivation. However, no manganase peroxidase (MnP) or ligning peroxidase (LiP) were detected during the whole cultivation period (15 days). By contrast, FMD21 secreted both laccases and MnP, but not LiP, although MnP activity was much lower than laccase (Fig. 3). Laccase activity in the optimized PDSRb medium increased during the first 10 days and leveled off at 238 800 U/L, which was 3.3 times higher than found when cultivating in basal PDB medium. After 10 days of culturing the activity leveled off and showed a slight decrease in laccase activity in the subsequent days until day 25. MnP activity showed the highest activity at an earlier time point, i.e. at day 4 the peak in MnP activity of about 40 U/L was observed (Fig. 3). The difference in optimal time point of MnP and laccase activity in the FMD21 culture have to be taken into account when selecting an optimal time period for mycoremediation. This is important because both laccase and MnP activities are involved in the degradation of pollutants (Christian et al., 2005; da Silva Coelho-Moreira et al., 2013).

LME producing fungi can be classified according to the types of enzymes they produce and secrete in the extracellular medium. In our collection, laccase and MnP were detectable, but LiP was not detectable in the extracellular medium (data not shown). However, It is uncertain if our collection of fungal strains do have the ability, or not to produce LiP as studies have shown that WRF secrete LIP only during solid-state fermentation or in carbon-limited medium (Hatakka, 2013; Linko and Haapala, 1993). It is evident that there is apparently no universal growth medium in which all types of LME enzymes are produced at a high rate simultaneously by fungus.

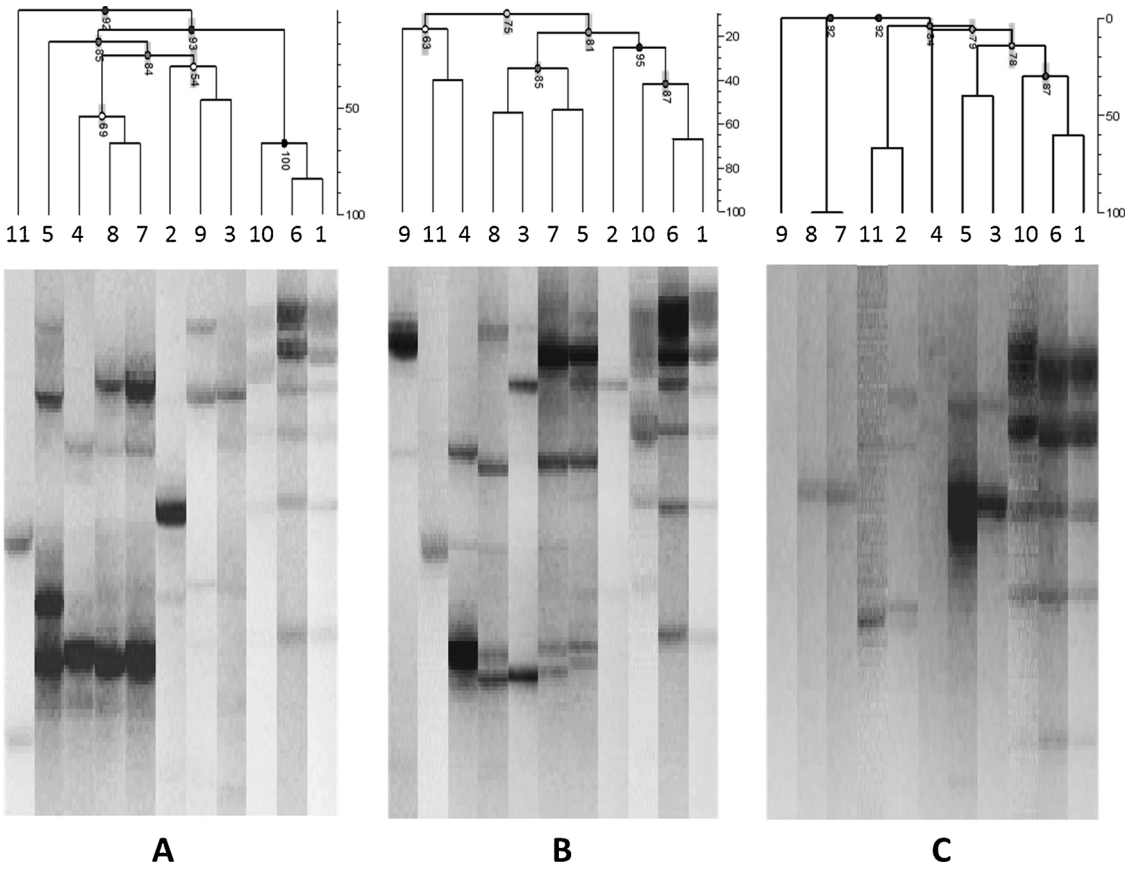

Fig. 4. REMAP analysis on eight potential fungi: Electrophoresis band patterns and dendrogram (by GelCompar II). A, combination of primer UBC807 and marY1-LTR-L; B, combination of primer UBC807 and marY1-LTR-R; C, combination of primer marY1-LTR-L and marY1-LTR-R. Lane 1, 6 and 10, Marker; lane 2, FAL3; lane 3, FTQ8; lane 4, FAL11; lane 5, FMD21; lane 7, FBV40; lane 8, FBV41; lane 9, FBV311; lane 11, FBD154. Gray bar at branches: error flag; number at branches: branch quality; right bar: similarity value.

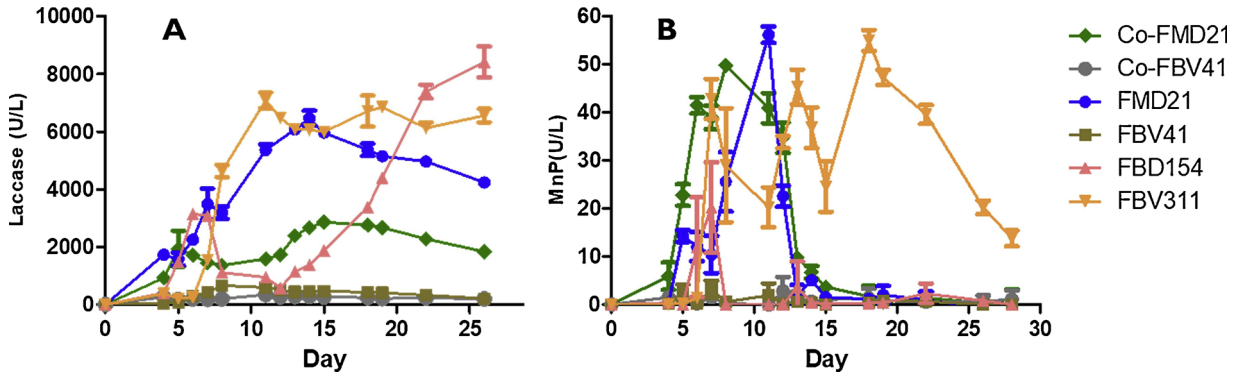

Fig. 5. Lignin-modifying enzymes secreted during cultivation with TCDD in the PDSRb medium. A: Laccase; B: Manganese peroxidase; Co-FMD21: Co-cultivation of *Rigidoporus* sp. FMD21, *Polyporus* sp. FBD154 and *Junghuhnia* sp. FBV311; Co-FBV41: Co-cultivation of *Cerrena* sp. FBV41, *Polyporus* sp. FBD154 and *Junghuhnia* sp. FBV311; FMD21: *Rigidoporus* sp. FMD21; FBV41: *Cerrena* sp. FBV41; FBD154: *Polyporus* sp. FBD154; FBV311: *Junghuhnia* sp. FBV311.

3.3. Fungal classification

Fungal classification and nomenclature based on DNA differences as well as on morphology are still controversial (Jeewon and Kd, 2016). DNA based phylogenies should be based on strongly phylogenetic signal genes such as the internal transcribed spacer (ITS) regions of the rRNA complex and combined with rather conserved genes such as 18S rDNA. Also, when classification is based on morphology it should refer to the origin of the material (culture or specimens, fruit body or vegetative hyphae). Phenotypic characteristics as different life stages of the same fungus have been classified as different species in the past (Jeewon and Kd, 2016). Therefore, both DNA and morphological characteristics have to be determined for a successful fungal classification. ITS regions consisting of ITS1-5.8S-ITS2, and 18S rDNA sequences of the eight selected fungi in the current study were sequenced for classification. Sequencing results show that four fungal isolates: FBV40, FBV41, FTQ8 and FMD21 had a high similarity towards each other and showed 99–100% similarity over the entire length of both the ITS region and 18S rDNA sequences. These four were clustered in the same branch in the phylogenetic tree (Appendix A Figs. A1 and A2). Classification of eight fungi based on the alignment of ITS region sequences with Mycobank database is illustrated in Table 2. Seven out of eight isolates belonged to the order of the Polyporales with only FAL11 being classified in the order of the Hymenochaetales. Within the Polyporales, the

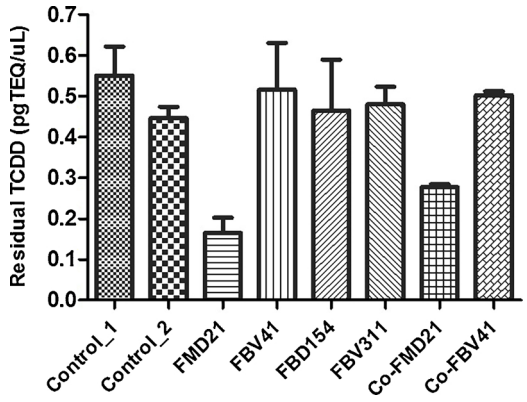

Fig. 6. 2,3,7,8-tetrachlorodibenzo-p-dioxin fungal degradation. Control_1: control at the start (day 0); Control_2: control at the end (day 28); Co-FMD21: Co-cultivation of *Rigidoporus* sp. FMD21, *Polyporus* sp. FBD154 and *Junghuhnia* sp. FBV311; Co-FBV41: Co-cultivation of *Cerrena* sp. FBV41, *Polyporus* sp. FBD154 and *Junghuhnia* sp. FBV311; FMD21: *Rigidoporus* sp. FMD21; FBV41: *Cerrena* sp. FBV41; FBD154: *Polyporus* sp. FBD154; FBV311: *Junghuhnia* sp. FBV311. Data illustrate mean and standard variation, n = 3, $p < 0.05$.

Polyporaceae family is one of the families that has numerous wood-degrading fungal strains which have been thoroughly investigated for LME production and their potential for industrial application (Goltapeh et al., 2013; Riley et al., 2014). Of the seven strains that belonged to the Polyporales FBV40, FBV41, FBV311, FAL3 and FBD154 were Polyporaceae whilst FTQ8 and FMD21 were identified as Meripilaceae due to their morphologies in natural habitat (Fig. 1A). In literature, *Cerrena*, *Polyporus*, *Rigidoporus* and *Pycnoporus* are representative of fungal genera that are efficient LME-producing strains and for which it was shown that they have applicability in biodegradation. For instance, laccase from *Cerrena* sp. HYB07 can decolorize dye used in the textile industry and degrade pharmaceutical waste (Yang et al., 2017). *Rigidoporus* has been studied less intensively but there are studies that have shown that *Rigidoporus lignosus* and its laccase combined with mediators can degrade Poly Aromatic Hydrocarbons (PAHs) (Cambria et al., 2008). Although the fungi in this study were derived from different geographical locations, all isolates that belong to the Polyporales order showed LME activity albeit with a wide variation in activity levels.

Morphologically, FBV40 and FBV41 are different, but DNA sequence results show that they are synanamorphs of a singular strain. To prevent similar identification issues we increased resolution by applying an additional technique called REMAP which is a DNA-fingerprinting technique to distinguish between fungal isolates genotypically (Le et al., 2008). Three combinations of primers were used: combination A yielded the best results with the most abundant in terms of DNA band patterns whilst combination C generated the lowest number of bands (Fig. 4). The dendrogram showed an identical pattern of DNA-bands generated from FBV40 and FBV41 in combination C which was less abundant, in the same combination FTQ8 and FMD21 are also close together. Besides, in combination B, all those four strains shared the same branch whilst in combination A in which the most abundance was observed exhibited the same result for a pair of FBV40 and FBV41 but the completely different pattern for the others. Data that were acquired from REMAP strongly supported the classification of eight fungi using DNA phylogenetic markers. Thus, FBV41, FMD21, FBV311 and FBD154 were selected for TCDD degradation experiments.

3.4. Mycodegradation of 2,3,7,8-tetrachlorodibenzo-p-dioxin

Fungi have the potential to be efficient dioxin degraders, especially the ligninolytic fungi. Several studies have been conducted which show fungal ability to biodegrade dioxin and many other pollutants (Singh, 2006). However, the number of studies focusing on degrading our target substrate, 2,3,7,8-TCDD is limited. Moreover, the possible biodegradation mechanism is not understood. Only a few basidiomycetes species with regard to TCDD degradation have been published. In our current study, fungi were screened for their potential to degrade TCDD (Figs. 5 and 6).

Based on the previous LME experiment, FMD21 and FBV41 were selected to be studied for TCDD degradation as they showed the highest LME activity. We chose to focus on single and combination cultures as a combination of fungi may show a synergistic effect in TCDD breakdown. For practical reasons we chose the following combinations: FMD21, FBV311 and FBD154 (Co-FMD21) and the combination FBV41, FBV311 and FBD154 (Co-FBV41) as these could be distinguished from each other on the DNA level so fungal biomass per species could be determined. In single culture FMD21 and FBV311 showed a stable trend in enzyme production (Fig. 5) in both laccase and MnP, followed by FBV311, however the activity here was much lower than in the optimization, this difference may be explained by the effect of the present of 2,3,7,8-TCDD in the medium or the difference in the volume of cultivation between two experiments (100 ml for optimization and 50 ml for TCDD degradation). By contrast, low laccase and no MnP were detected in FBV41 culture. FBD154 showed highest laccase activity at the end of the experiment and MnP was not detected during 28 days. Regarding fungal combinations, there is a noticeable difference between two combinations in both enzymatic secretion and growth proportion. In Co-FMD21, laccase activity was almost half of the individual cultivations and MnP activity was equal to FMD21 and FBV311 single culture (Fig. 5). MnP activity in the Co-FMD21 culture however did reach higher levels sooner than in the individual cultures. When looking at the fungal abundancies in the co-cultures the largest proportion of biomass in the Co-FMD21 mixture was FMD21 (81%), followed by FBD154 (15%) and FBV311 (4%). Also, Co-FBV41 showed the same pattern but FBV41 dominated this co-culture alone because of 99.7% was contributed by FBV41 whereas the other two had negligible quantities.

Analysis of 2,3,7,8-TCDD in cultivation flasks were performed by DR-CALUXx® after 28 days, and for control 1 at day 0. FMD21 degraded TCDD with the largest amount (73% as compared to control 1 and 65% as compared to control 2). The second largest TCDD degradation was observed in Co-FMD21 which reached 45% of control 1 and 40% of control 2. Although Co-FMD21 contained three fungi which produced laccase and MnP at considerable levels, degradation occurred less efficiently compared to when there was only FMD21 in the culture. Moreover, no degradation was observed for the other fungi when cultivated individually or with the mixture of FBV41; FBV311 and FBD154 (Fig. 6). This indicates that the ability to breakdown pollutants such as 2,3,7,8-TCDD is not a ubiquitous characteristic of white-rot fungi. Co-cultivation of different white-rot fungi did not advance TCDD degradation, and therefore does not seem to be a useful strategy for enhancement of the mycoremediation potential.

FMD21 alone showed TCDD degrading potential among the fungal isolates. Two important ligninolytic enzymes were detected in the culture fluid, of which laccase seems to be the most likely candidate to be involved, because the highest degradation was observed with FMD21 alone as compared to co-FMD21 incubation. Similarly, the laccase activity in FMD21 alone is at least twice as high as compared to the co-FMD21 situation. Further research is needed to investigate the breakdown pathway of 2,3,7,8-TCDD. The involvement of white-rot fungi along with the presence of its lignin-modifying enzymes in the breakdown of PCDD/Fs have been shown before in several different studies (Kamei et al., 2009; Mori and Kondo, 2002; Takada et al., 1996; Valentín et al., 2013). Moreover, 2,7-dichlorodibenzo-p-dioxin as model substrate was intensively investigated by Valli et al. (1999) who suggested a degradation pathway of 2,7-dichlorodibenzo-p-dioxin involving LiP and MnP of *Phanerochaete chrysosporium*. However, there are some possible pathways through which fungi degrade TCDD. Besides using LMEs, it is true that cytochrome P450 systems or other enzymes may contribute to the capability for TCDD degradation of FMD21, but we cannot conclude or identify which one is the major pathway in this work. We expect that TCDD is degraded by a mixture of

enzymes which include LMEs. Here we present a screening study to investigate the potential of Vietnamese WRF to degrade TCDD which remains a risk pollutant and contaminant in agriculture and animal feedstocks. The Polyporale fungus FMD21 showed a high potential for TCDD breakdown, the unique characteristics of FMD21 over other fungi in this study, which may be a potential species used for bioremediation. We carried out TCDD degradation experiments at mild conditions and with the basic medium for LME production in order to achieve the potential fungus which only needs minimal and simple requirements to express its degradation capacities with the outlook for bioremediation application. Further study needs to be undertaken to investigate the practical implications for this species as a bioremediation agent.

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Appendix A

1. **Phylogenetic tree based on 18S rDNA sequences**

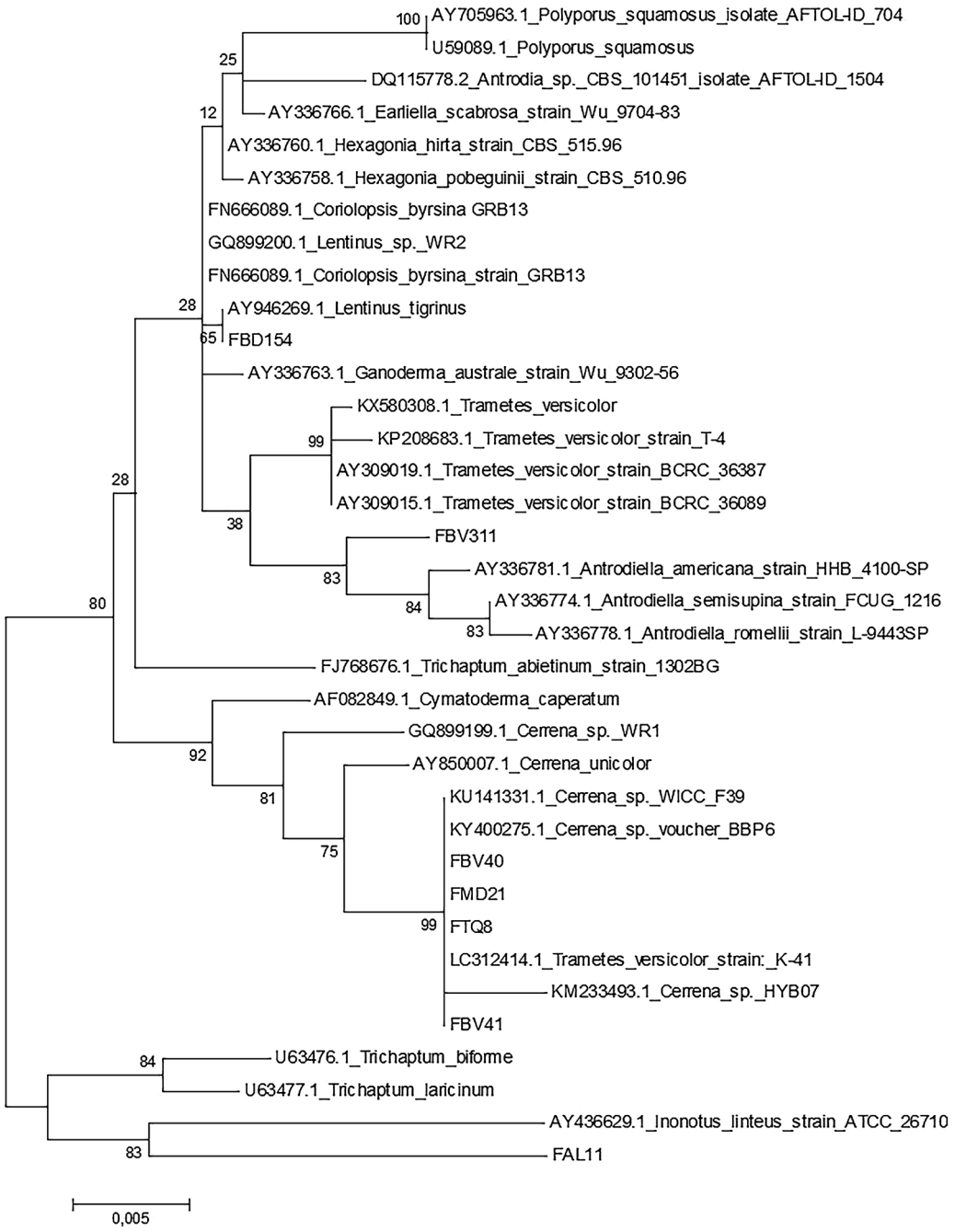

Fig. A1. Phylogenetic tree based on 18S rDNA sequences.

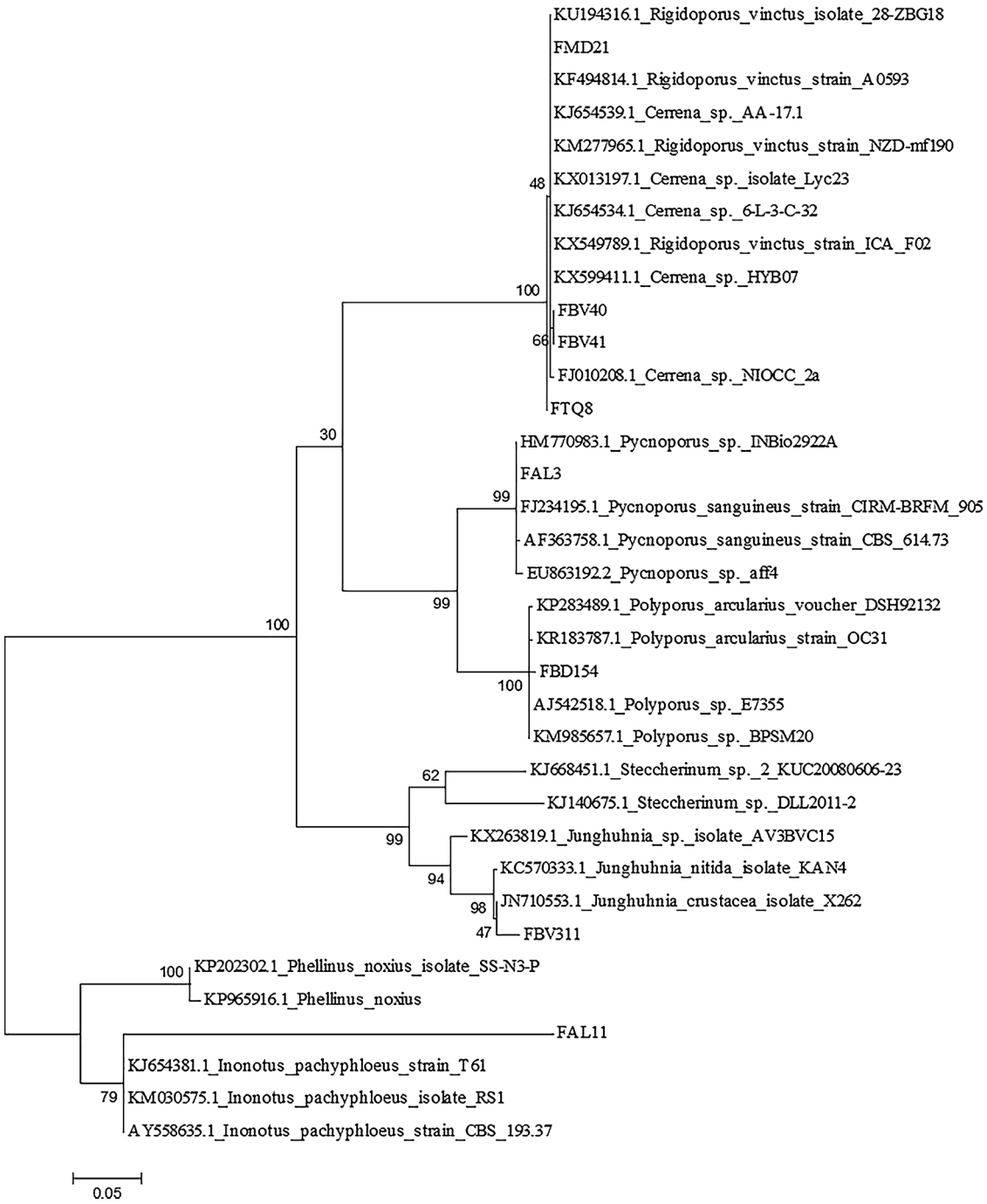

Fig. A2. Phylogenetic tree based on ITS1-5.8S-ITS2 sequences.

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2018.10.059.

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