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Functional and transcriptomic investigation of laccase activity in the presence of PCB29 identifies two novel enzymes and the multicopper oxidase repertoire of a marine-derived fungus

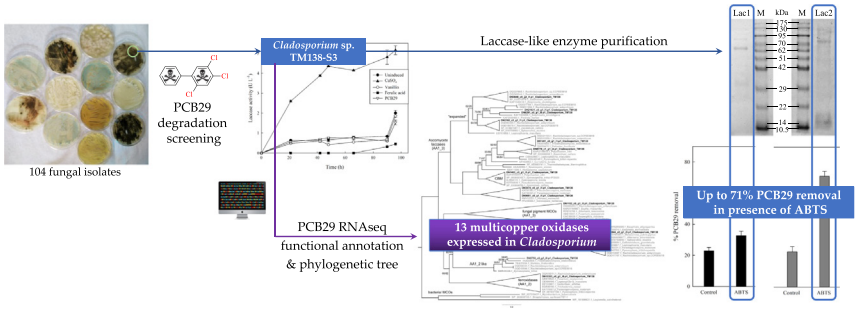
Efstratios Nikolaivits [a], Romanos Siaperas [a], Andreas Agrafiotis [a], Jamal Ouazzani [b], Antonios Magoulas [c], Anastasia Gioti [c], Evangelos Topakas [a,*]

[a] Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece
[b] Institut de Chimie des Substances Naturelles, ICSN, CNRS, Gif sur Yvette, France
[c] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Heraklion, Greece

HIGHLIGHTS

- 104 fungal invertebrate symbionts were screened for PCB29-transforming ability.
- Three most competent strains expressed laccase activity in the presence of PCB29.
- Two enzymes with laccase activity were isolated from *Cladosporium* sp. TM-138-S3.
- Isolated enzymes were able to remove up to 71% of PCB29 in presence of mediators.
- RNAseq identified the multicopper oxidase repertoire of *Cladosporium* sp. TM-138-S3.

GRAPHICAL ABSTRACT



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ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs), that can be detected in a variety of environments including the human body, adversely affecting global health. Bioremediation is an emerging field for the detoxification and removal of environmental pollutants, with novel biocatalysts appropriate for this task being in high demand. In this study, a biobank of novel fungal strains isolated as symbionts of marine invertebrates was screened for their ability to remove 2,4,5-trichlorobiphenyl (PCB29). The most efficient strains were studied further for their ability to express laccase activity, the most commonly associated extracellular activity involved in the removal of aromatic pollutants and encoded in fungi by the enzymatic class of multicopper oxidases (MCOs). The strain expressing the highest laccase activity, *Cladosporium* sp. TM138-S3, was cultivated in the presence of copper ions in a 12 L bioreactor and two enzymes exhibiting laccase activity were isolated from the culture broth through ion-exchange chromatography. The two enzymes, Lac1 and Lac2, were biochemically characterized and showed similar characteristics, although an improved ability to remove PCB29 (up to 71.2%) was observed for Lac2 in the presence of mediators. In parallel, we performed RNAseq of the strain growing in presence and absence of PCB29 and reconstructed its transcriptome assembly. Functional annotation allowed identifying the MCO repertoire of the fungus, consisting of 13 enzymes. Phylogenetic analysis of Ascomycete MCOs further allowed classifying these enzymes, revealing the diversity of laccase activities in *Cladosporium* sp. TM138-S3.

* Corresponding author at: Iroon Polytechniou 9, Zografou Campus, 15780 Athens, Greece.
E-mail address: vtopakas@chemeng.ntua.gr (E. Topakas).

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1. Introduction

Polychlorinated biphenyls (PCBs) are a class of halogenated compounds, the members of which consist of a biphenyl substituted by 1–10 chlorine atoms. There are 209 PCB congeners which, despite differences in their physicochemical properties, show low water solubility, high lipophilicity, low vapor pressure, high chemical stability and the tendency to persist in the environment and the human body (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2015). Even though PCB production has been banned since the 1980s, 1.5 tons of PCBs had been manufactured and a large amount released in the environment, leading to leaching of PCBs from the Antarctic to the Arctic (Sharma et al., 2018). Chronic exposure to PCBs is proved to have toxic effects on nerves, skin, immune and reproductive systems, while it may also cause teratogenicity, endocrine disruption, and a predisposition to cancer (Loganathan and Masunaga, 2020). Other diseases that have been associated with high concentrations of PCBs in the body are cardiovascular, hypertension and diabetes (Carpenter, 2015).

A number of conventional methods for removal of PCBs have been tested so far, including incineration, landfilling remediation and dredging, or their combination. These methods are however expensive, time-consuming and may release toxic compounds or produce secondary contaminants (Nair and Abraham, 2019). Bioremediation has been proposed as a cost-effective and eco-friendly alternative, based on the use of living organisms for the removal, detoxification or assimilation of pollutants (Patel and Kumar, 2017; Ren et al., 2016). Organisms that have already been successfully used in this context are bacteria, fungi, algae and plants. Microorganisms in particular are ubiquitous on our planet and participate in important physicochemical processes, such as element cycling, geochemical processes, and degradation of pollutants (Pushpanathan et al., 2014).

Marine environment covers two thirds of the earth's surface and represents >95% of the biodiversity of the total environment (Dalmaso et al., 2015). This huge biodiversity arises from the wide variety of marine habitats, where different conditions of temperature, pressure, dissolved oxygen, salinity and light are present (Nikolaivits et al., 2017). Marine-derived fungi have proven valuable catalysts that can be applied in various biotechnological processes, due to their interesting properties acquired through years of adaptation in extreme environmental conditions (Nicoletti and Andolfi, 2018; Nikolaivits et al., 2017). So far, marine-derived fungi have been used in bioremediation of oil constituents, such as polycyclic aromatic hydrocarbons (PAHs) and aliphatic alkenes, 2,4,6-trinitrotoluene (TNT) or hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), deriving from unexploded ordnance (Birolli et al., 2018; Bovio et al., 2017; Nicoletti and Andolfi, 2018). There are also reports regarding the degradation of chlorinated pesticides, such as 1,1-dichloro-2,2-bis-(4-chlorophenyl) ethane, esfenvalerate and dieldrin (Nicoletti and Andolfi, 2018).

Multicopper oxidases (MCOs) catalyze the four-electron reduction of dioxygen to water with concurrent oxidation of a variety of substrates. MCOs contain at least four coppers divided into three copper centers (Type 1, Type 2 and binuclear Type 3) (Jones and Solomon, 2015). Among MCOs, laccase enzymes have been heavily studied for the removal of a wide range of phenolic and non-phenolic pollutants, chlorinated or not (Bilal et al., 2019; Chandra and Chowdhary, 2015; Mandic et al., 2019; Wang et al., 2018). Laccases from marine sources in particular have been isolated from various organisms and used for the bioremediation of synthetic dyes, pesticides and other xenobiotics (Theerachat et al., 2019). Additionally, laccase-mediator systems (LMS) have been also utilized in cases where laccase redox potential was inadequate for the oxidation of a certain pollutant or when the pollutant could not reach the laccase active site due to steric hindrance. In these systems, a synthetic or natural compound that acts as a laccase substrate (mediator) is added for oxidation by the enzyme, generating highly reactive stable organic radicals that act as a catalyst oxidizing the target pollutant by mechanisms different from the enzymatic one (Ashe et al., 2016; Cañas and Camarero, 2010).

The present work describes the screening of 104 fungal strains isolated as symbionts of mesophotic and shallow-reef invertebrates (Nikolaivits et al., 2019, 2020) for their potential in the bioremediation of the pollutant 2,4,5-trichlorobiphenyl (PCB29). The most potent strains in terms of PCB29 bioconversion yield were chosen for further investigation by screening for laccase activity in respective culture supernatants. Induced expression and purification of the responsible enzymes, as well as PCB29 removal assays in the presence of mediators were performed on the fungal strain exhibiting the highest activity, *Cladosporium* sp. TM138-S3. This strain was previously subjected to whole-genome sequencing, and its assembly is publicly available (Gioti et al., 2020). Aiming to enhance -omic data for this fungus and in order to identify MCOs expressed during bioconversion, we performed massive RNA sequencing (RNAseq) of the strain growing in marine broth in presence and absence of PCB29. Functional annotation of the assembled transcriptome and phylogenetic tree reconstruction allowed the identification and classification of the MCO repertoire of *Cladosporium* sp. TM138-S3.

2. Materials and methods

2.1. Fungal biobanks

Microorganisms utilized in the present study were fungal isolates deriving from marine invertebrates (e.g. sponges, soft corals, anemones, echinoderms), collected from different parts of the globe, namely the eastern and western Mediterranean Sea, the Red Sea and the Andaman Sea in the east Indian Ocean. Invertebrate collection was carried out by iMare Natural S.L. (Granada, Spain) for the east Mediterranean Sea, by the Tel Aviv University (Israel) for the east Mediterranean and Red Sea and by Chulalongkorn University (Bangkok, Thailand) for the Andaman Sea. The isolation of symbiotic fungi from these invertebrates was performed by the Natural Products and Medicinal Chemistry Department (ICSN) at the French National Center for Scientific Research (CNRS). The collection and isolation took place in the framework of the European research program TASCMAR (Horizon 2020, grant agreement no. 634674).

Two fungal biobanks were constructed from the above samples: one called TARMIC consisting of 60 fungal strains (mainly ascomycetes), which were exclusively isolated from invertebrates of the mesophotic marine zone, while the second biobank, called MICLIB, consists of 44 fungal strains, 20 of which were isolated from the upper mesophotic zone, and the remaining were from shallow reefs. A detailed list of the fungal strains identified is presented in Table S1, where isolates with code names TM derive from TARMIC biobank, while isolates with code names ML derive from MICLIB biobank.

2.2. Culture conditions and resting cell reactions

Fungal strains were grown on Difco™ Marine Agar 2216 (BD Biosciences, NJ, USA) plates at 27 °C for 5 days. These plates were used to inoculate submerged cultures with Difco™ marine broth (MB) 2216 (pH 7.6) at 27 °C and 150 rpm shaking. After 5 days, the biomass was filtered using 0.2 μm-pore filters and used as a biocatalyst (10% w/v) in 15-mL reactions with ultrapure water saturated with PCB29 (Sigma-Aldrich, USA). Reactions were left under mild shaking at 27 °C for 10 days. On the final day, the remaining reaction including fungal biomass was extracted with an equal volume of ethyl acetate and was analyzed by gas chromatography (GC), as described below, in order to determine the PCB amount absorbed by the isolates and thus not bio-converted. Control reactions with no addition of fungal biomass were also performed, extracted in the same manner and taken into account in PCB degradation removal yield calculations.

2.3. Detection and quantification of PCB29

Reaction samples were analyzed for the quantification of the remaining PCB29 using a SHIMADZU GC-17A apparatus incorporating a 30-m Equity®-5 capillary GC column, following a modified methodology (Cossu et al., 2013). One-microliter samples were injected in splitless mode in the injector held at 280 °C. The oven temperature program was initially set to 90 °C for 3 min, then raised to 220 °C at a rate of 30 °C min^{-1} and held for 1 min and finally raised to 280 °C at 25 °C min^{-1} and held for 2 min. Detection was performed by an ECD-17 electron capture detector of 10 mCi activity, held at 300 °C. The retention time of PCB 29 was approximately 10.35 min and the calibration curve ($y = 2.54 \cdot 10^{-8}x$) had an $R^2 = 0.999$. Each sample was analyzed in triplicate.

2.4. Induction and measurement of laccase activity

Fungal cultures inoculated from 5-day-grown plates were incubated in MB for 3 days at 27 °C. Grown biomass (5% v/v) was used to inoculate 50-mL cultures and left to incubate at the same conditions for 3 days, prior to the addition of inducers. Laccase activity was measured in the culture medium of selected fungal strains with high PCB-bioconversion ability, induced either with 0.02 mM PCB or other typical laccase inducers (0.5 mM $CuSO_4$, 2 mM vanillin and 2 mM ferulic acid).

A typical laccase assay was performed in 250 μL 50 mM phosphate-citrate buffer pH 4.5 containing 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 25 μL of enzyme preparation. Monitoring of ABTS oxidation was performed for 10 min on a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMaxPro software (version 1.1, Molecular Devices, Sunnyvale, CA, USA) set at 35 °C. One unit of laccase activity was determined as the amount of enzyme that produces 1 μmol of oxidized ABTS per minute. The concentration of oxidized ABTS was calculated using $\varepsilon_{420nm} = 36{,}000\ M^{-1}\ cm^{-1}$.

2.5. Purification of laccase-like enzymes

Cladosporium sp. TM138-S3 was cultivated in a 15-L bioreactor (MBR, Switzerland) with a 12-L working volume where laccase activity was induced with the addition of 0.5 mM $CuSO_4$. Culture broth was centrifuged (10,000 ×*g*, 15 min, 4 °C) and the supernatant was filtered through a 0.45-μm pore size filter and then concentrated using a Pellicon® Cassette Acrylic Holder and a Pump system equipped with a 30 kDa cassette, all provided by Millipore (USA). The resulting concentrate was further concentrated using an Amicon device with a 10 kDa cut-off membrane. This concentrate was dialyzed in a 20-mM piperazine-HCl buffer at pH 5.5 and loaded on a pre-equilibrated with the same buffer Q-sepharose column (2.5 × 25 cm). Proteins were eluted by a linear gradient increase of NaCl concentration 0–0.5 M (4 mL/min flow rate). Fractions of 8 mL were collected, and their protein content and laccase activity were measured. The homogeneity of the fractions was checked by SDS-PAGE. Two fractions showing laccase activity were further purified using DEAE-Cellulose resin equilibrated in piperazine-HCl buffer pH 5.5 and Q-sepharose resin equilibrated in Bis-Tris buffer pH 7, respectively.

2.6. Biochemical characterization of isolated laccase-like enzymes

The effect of temperature and pH on enzymatic activity was tested by performing the laccase assay (10 min) at different temperatures (30–65 °C) or pH buffers (citrate/phosphate pH 2–6, sodium phosphate pH 6–7 and Tris-HCl pH 8–9), respectively. Substrate specificity for the two enzymes was tested by incubation with various phenolic compounds (2 mM catechol, pyrogallol, 2,6-dimethoxyphenol, L-DOPA, gallic acid, guaiacol, hydroquinone and caffeic acid) at 35 °C for 18 h, based on a method described previously (Nikolaivits et al., 2018). The enzyme amount (units) used was the same for both enzymes.

The ability of purified laccase-like enzymes to bioconvert 1 μM PCB29 was tested by incubation at 35 °C for 20 h in an Eppendorf Thermomixer Comfort operating at 900 rpm. Reactions were extracted with 3 volumes of ethyl acetate and residual PCB29 was measured on GC, as described above.

2.7. RNA extraction and sequencing

Total RNA was extracted from two, 5-day submerged cultures of *Cladosporium* sp. TM138-S3 in MB and in MB supplemented with 0.02 mM PCB29 as described in Section 2.1. The polyA+ fraction of total RNA was purified, and strand-specific libraries were constructed with the Thermo Fisher Ion Total RNA-seq kit v.2 (Cat. 4475936) and sequenced in single-end mode by Genomnia (Milan, Italy) on an Ion Torrent S5 instrument. The raw sequencing data from each sample were deposited at the NCBI SRA database (SRR12633902: 28,139,398 reads, SRR12633901: 28,280,510 reads).

2.8. Bioinformatic analysis

Reads from Ion Torrent sequencing were processed to remove adapters, short reads and low quality bases with Trimmomatic (Bolger et al., 2014) and contaminant reads with Kraken2 (Wood et al., 2019). Prior to *de novo* assembly with Trinity v.2.8.5 (Grabherr et al., 2011), error correction was performed with Pollux (Marinier et al., 2015) and rRNA reads were removed using sortmeRNA v.4 (Kopylova et al., 2012). Transcriptome completeness was assessed using BUSCO v.3.1.0 against the Pezizomycotina database (Simão et al., 2015). The assembly is accessible in GenBank (accession no.: GIVB00000000).

Candidate coding regions were predicted from the transcriptome assembly with TransDecoder (Haas et al., 2013). The predicted peptides were searched for sequence homology using BLASTp (Camacho et al., 2009) against the SwissProt database (UniProt Consortium, 2019) and were annotated for domain content using Pfam queries (El-Gebali et al., 2019), for transmembrane helices using TMHMM v.2 (Sonnhammer et al., 1998), for signal peptides (SP) using SignalP v.4.1 (Petersen et al., 2011) and for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the respective database. They were further classified into Carbohydrate Active Enzymes (CAZy) families with Run_dbcan v.2 using hmmscan, Diamond and Hotpep tools, or dbCAN tools (Lombard et al., 2014; Zhang et al., 2018) and into distinct Gene Ontology (GO) terms. Based on reports of fungal MCOs with two Cu-oxidase domains (Nakamura and Go, 2005; Rivera-Hoyos et al., 2013), we searched for proteins that contain at least two of the Cu-oxidase, Cu-oxidase_2 and Cu-oxidase_3 Pfam domains to identify an exhaustive set of putative MCOs in *Cladosporium*. These proteins and their corresponding transcripts were aligned to the genome of *Cladosporium* sp. TM138-S3 (Gioti et al., 2020). From this initial extensive dataset of 32 putative MCOs (Data file S1), three-domain (3d)MCOs as defined in Gräff et al. (2020) were identified by choosing those that contain all three Cu-oxidase Pfam domains and that were classified as members of the Auxiliary Activity Family 1 (AA1) of the CAZy database by at least two dbCAN tools. The MW and isoelectric point of putative MCOs were calculated with the EMBOSS package (Rice et al., 2000) following removal of predicted signal peptides, where present. Extracellular 3dMCOs were defined as those that possess SPs and have no predicted transmembrane helices in the mature protein.

For the phylogenetic analysis, we included most representative taxa from each of the clades of the tree published from Copete and colleagues, along with fungal MCOs retrieved from GenBank for under-represented clades, a *Cladosporium* sp. TM138-S3 protein not present in the set of 32 putative MCOs but identified as homologous to an ascorbate oxidase (AO) in Copete et al. (2015), and bacterial MCOs serving as an outgroup. Protein alignments were performed with MUSCLE within

Seaview (Gouy et al., 2010), and informative sites were selected with Gblocks (Castresana, 2000). Maximum-Likelihood (ML) trees were estimated using IQ-TREE (Nguyen et al., 2015). Node support was calculated using 5000 ultrafast bootstraps (UFBoot) (Minh et al., 2013) and 5000 iterations of the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010).

Detailed bioinformatic methods are available at Supplementary Material.

3. Results and discussion

3.1. Potential of marine-derived fungi for PCB29 removal

In our previous studies, we investigated the bioremediation potential of two fungal biobanks comprising strains isolated from marine invertebrates, collected from different parts of the world and different depths (shallow and mesophotic reefs) (Nikolaivits et al., 2019, 2020). Although these microorganisms come from pristine environments, some showed promising potential for the degradation of the chlorinated pollutant 2,4-dichlorophenol (2,4-DCP). In the present study, we investigated the ability of strains from the same biobanks to remove PCB29, a comparatively more toxic and complex pollutant, bearing two benzene rings and three chlorine atoms. Screening of strains took place at a PCB29 concentration equal to its solubility in water at 27 °C, which was estimated to be approximately 1 μM.

Out of the 104 strains tested, 72 were able to biotransform PCB29 at some level (Table S1). The average removal percentage for these strains is 72%, with almost half of the isolates (47%) transforming PCB29 at percentages over 90%. These isolates belong to the genera *Penicillium* (35%), *Aspergillus* (29%), *Cladosporium* (18%), *Purpureocillium* (6%) and 12% to other species of Ascomycota. From these, 8 isolates showed pollutant bioconversion percentages exceeding 98.5%. These were *Aspergillus* sp. TM122-S2, *Penicillium* sp. TM125-S3, *Cladosporium* sp. TM138-S3, *Purpureocillium lilacinum* TM138-S4, *Alternaria* sp. TM141-S1, *Aspergillus* sp. ML136-S2, *Aspergillus fumigatus* ML138-S2 and *Penicillium steckii* MLm53-S3.

There is only a handful of studies reporting PCB removal from fungi. For example, Mouhamadou and colleagues used fungi isolated from heavily PCB-contaminated soil to remove a mixture of PCBs at percentages 29–85% after 7 days (Mouhamadou et al., 2013). The most competent strains (>70% removal) belonged to the genera *Doratomyces*, *Myceliophthora*, *Phoma* and *Thermoascus*. *Phanerochaete chrysosporium*, one of the most studied fungi for bioremediation purposes, has been also reported to biotransform the PCB mixtures Aroclor 1242, 1254 and 1260, with low-substituted PCB congeners being removed more efficiently (Sharma et al., 2018). Marine-derived fungi have been utilized so far for the degradation of synthetic dyes and PAHs (Birolli et al., 2018; Bonugli-Santos et al., 2016; D'Souza et al., 2006; Verma et al., 2010), but not of chlorinated pollutants. Marine-derived fungi able to degrade PCB have enormous potential for the development of biocatalysts for remediation of polluted saline environments due to their natural tolerance to high salinity.

The exact mechanism of fungal degradation of PCBs remains unknown. Products of 4,4′-dichlorobiphenyl (PCB15) degradation by *Phanerochaete* species were previously determined by GC/MS, revealing that the initial hydroxylation of the benzene rings may constitute a crucial step (Kamei et al., 2006). In the present work, we also attempted to identify the metabolites of PCB29 degradation by *Cladosporium* sp. TM18-S3 using GC–MS/MS analysis. Unfortunately, the detection of metabolites in the reaction extracts was impossible with the employed methodology, probably due to the fact that metabolite concentrations were lower than the detection limit of the method. Based on the literature, fungi tend to use their extracellular oxidative enzyme system as defense against various xenobiotics, such as dioxins, PAHs, trinitrotoluene, dyes, pesticides, and PCBs (Marco-Urrea et al., 2015; Sivaperumal et al., 2017). This system consists of lignin-degrading enzymes, which include the peroxidase and laccase families, both non-specific with regards to their target substrates (Passatore et al., 2014). In many cases, activation of PCB degradation mechanisms requires restriction of carbon and/or nitrogen sources (Sharma et al., 2018).

3.2. PCB29 removal could be associated with laccase activity

In order to get a better understanding of the PCB29 biotransformation mechanism, we attempted to measure enzymatic activities in the extracellular medium that are potentially involved in biodegradation of this pollutant, namely biphenyl dioxygenase, catechol dioxygenase and laccase. Laccase was the only activity that could be detected in three out of the eight strains tested. As one can see in Fig. 1, *Cladosporium* sp. TM138-S3 shows the highest laccase activity at day 3, almost 20 times higher than *Alternaria* sp. TM141-S1. Screening of marine-derived microorganisms for the expression and isolation of novel enzymes with properties desirable for bioremediation processes is a widespread method inspired by the biochemical diversity of these microorganisms (Batista-García et al., 2017; Lima and Porto, 2016; Nikolaivits et al., 2017; Theerachat et al., 2019). This approach has been mostly adopted in screening of fungi for their ability to decolorize and biodegrade synthetic dyes (Ben Ali et al., 2020b; Dhouib et al., 2005; Kiiskinen et al., 2004). Here, the same approach allowed identifying a laccase-like activity associated with bioconversion of a complex chlorinated organic pollutant. We cannot however exclude that other enzymatic activities may be able to degrade PCB in *Cladosporium* sp. TM138-S3 or other fungi. A sensible and exhaustive metabolite analysis during PCB bioconversion may resolve this issue in future experiments.

3.3. Large-scale production of laccase activity in Cladosporium sp. TM138-S3

Aiming to express the laccase activity from *Cladosporium* sp. TM138-S3 at large scale, we investigated other typical laccase inducers, namely $CuSO_4$, vanillin and ferulic acid, along with PCB29. Ferulic acid inhibited laccase expression in the tested conditions, while similar expression levels were observed in presence and in absence of vanillin and PCB29. On the contrary, addition of $CuSO_4$ increased the expressed laccase activity by 2.6-fold (Fig. 2).

Our results are in agreement with numerous cases throughout literature, where copper ions were reported to be the most effective laccase inducer (Ben Ali et al., 2020b; Gomaa and Momtaz, 2015; Theerachat et al., 2019). The addition of copper ions can increase laccase expression levels 13- and 20-fold in liquid cultures of marine-derived fungi *Pestalotiopsis* sp. J63 (Chen et al., 2011) and *Polyporus brumalis*

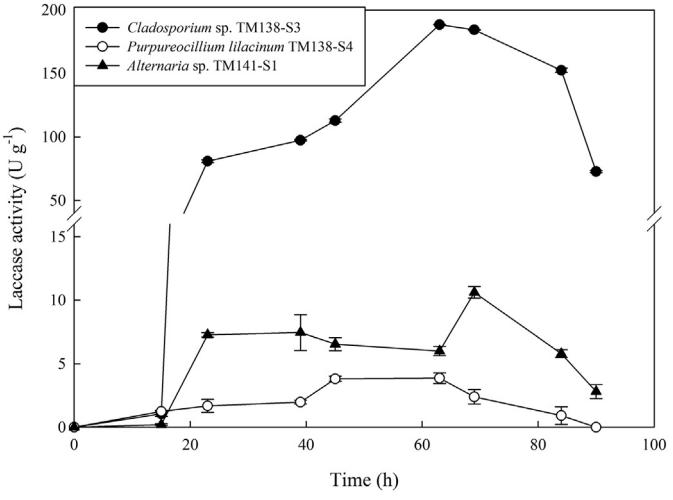

Fig. 1. Extracellular laccase activity during removal of PCB29 by selected isolates: *Cladosporium* sp. TM138-S3 (●), *Purpureocillium lilacinum* TM138-S4 (○) and *Alternaria* sp. TM141-S1 (▲).

ibrc05015 (Theerachat et al., 2019), respectively. Even though, in our work, ferulic acid showed an inhibitory effect on laccase activity, there are many reports demonstrating its inducing activity in fungal cultures (Bertrand et al., 2013; Subramanian et al., 2014; Nakade et al., 2012). Regarding vanillin, this is a substrate (Zeng et al., 2017) and a mediator (Rivera-Hoyos et al., 2013) for laccase activity, but it has also been shown to act as an inducer (Cavallazzi et al., 2005). Lastly, even though the addition of PCBs in *Pleurotus ostreatus* cultures can increase laccase activity up to 20 times (Gayosso-Canales et al., 2012), this was not observed with the *Cladosporium* sp. TM138-S3 isolate.

We then tested different concentrations of the most potent laccase inducer, $CuSO_4$, in the range between 0.1 and 1.5 mM. Overall, Cu^{2+} concentration in the tested range did not affect laccase expression dramatically. Specifically, considering as 100% the maximum activity recorded, activity was higher than 68% at all copper concentrations tested. Copper concentrations with the highest induction effect were 0.25 and 0.5 mM with negligible differences between them, while the lowest expression levels were observed at concentrations 0.1 and 1.5 mM, which were the lowest and highest tested (Fig. S1).

Even though copper ions act as a laccase inducer in fungi, they can also have antifungal activity at high concentrations, since they react with proteins, nucleic acids and other metabolites related to important cellular functions (Subramanian et al., 2014; Passarini et al., 2015). Indicatively, the presence of copper at concentrations up to 0.5 mM increased laccase expression in the marine fungus *Pestalotioptis* sp. up to 13 times, while a sharp decrease was observed at higher concentrations (Chen et al., 2011). The low activity observed at 0.1 mM concentration may be attributed to the small amount of available Cu^{2+} ions, not sufficient for all active sites of the expressed protein molecules so that the post-translational modification of the enzyme is not supported (Jaber et al., 2017; Theerachat et al., 2019). Additionally, in plant cell cultures supplemented with copper at concentrations lower than 100 μg L^{-1}, production of inactive copper-free laccases or production of active laccases until copper depletion was observed (Gianfreda et al., 1999).

3.4. Isolation of enzymes with laccase activity

The *Cladosporium* sp. TM138-S3 extracellular medium derived from a 12-L bioreactor expressing laccase activity was processed as described in Section 2.4. For the isolation of enzymes with laccase activity, the crude extracellular medium was loaded onto a Q-sepharose ion exchange column equilibrated in pH 5.5. Elution was performed with increasing NaCl concentrations, resulting in two fractions with laccase activity. In order to purify these further, the first fraction, containing an enzyme hereafter termed Lac1, was passed through a second Q-sepharose resin equilibrated at pH 6.5, while the second fraction, containing an enzyme hereafter termed Lac2, was passed through DEAE-cellulose resin at pH 5.5. The final activity recovery was 17% for Lac1 and 23% for Lac2. The purity of the fractions, as well as the MW of the proteins were determined by SDS-PAGE (Fig. 3). The MW of Lac1 appears to be ca. 65 kDa with some higher MW impurities, while that of Lac2 ca. 75 kDa. Lac2 was rather unstable under these storage conditions (piperazine-HCl buffer, pH 5.5), since protein lysates are observed on SDS-PAGE.

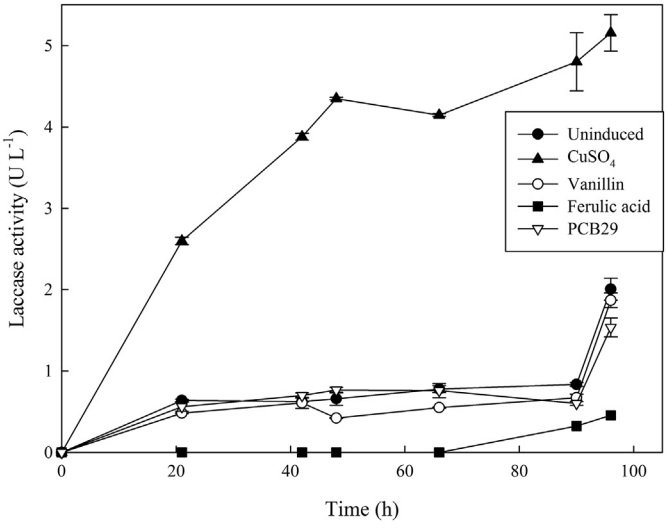

Fig. 2. Effect of different inducers, namely $CuSO_4$ (▲), vanillin (○), ferulic acid (■) and PCB29 (▽) on the expression levels of laccase activity measured in liquid cultures, compared to the uninduced cultures (●).

Expression of marine-derived enzymes under saline conditions has not been widely studied, and especially at a large-scale. Examples include the large-scale expression of a copper-induced laccase by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 (Mainardi et al., 2018) and that of a lab-scale expression of a laccase from *Cerrena unicolor*, both isolated through ion-exchange chromatography (D'Souza-Ticlo et al., 2009; Mainardi et al., 2018). These laccases showed a MW of approximately 60 kDa, within the range of reported MWs for fungal laccases (Baldrian, 2006; Rivera-Hoyos et al., 2013). The MW of the isolated enzymes of *Cladosporium* sp. TM138-S3 are also in agreement with the above range, while Lac2 in particular shows a similar MW of 75 kDa to a laccase isolated from the closely related species *Cladosporium cladosporioides* (Halaburgi et al., 2011) and from another marine-derived fungus, *Stemphylium lucomagnoense* (Ben Ali et al., 2020a). The MW of Lac1 is similar to the one measured for the heterologously expressed laccases of the marine-derived *Pestalotiopsis* sp. (Wikee et al., 2019).

3.5. Biochemical characterization of purified enzymes

The effect of temperature on the activity of isolated enzymes was studied in a temperature range of 30–65 °C. The optimum temperature was observed at 50 °C for both enzymes, while Lac2 appears to maintain higher activity at lower temperatures (Fig. 4a). Overall, however, both enzymes retained >65% of their maximum activity at all tested temperatures.

Regarding optimal pH, both enzymes presented their maximum activity at pH 3 and had almost identical profiles (Fig. 4b). Both laccases

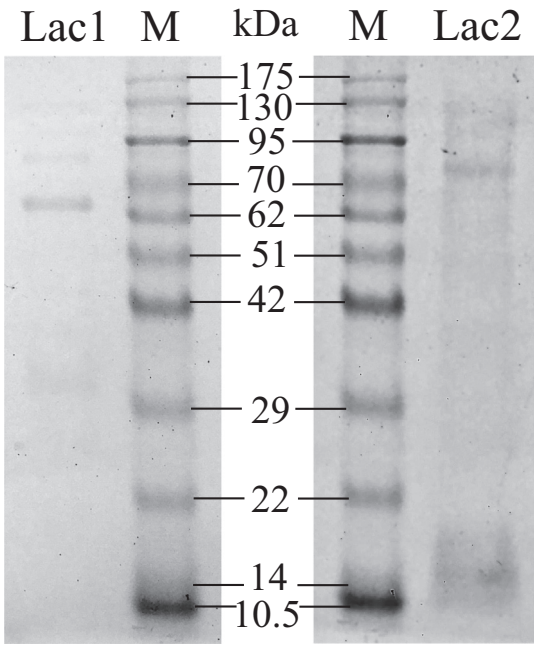

Fig. 3. SDS-PAGE gel of the resulting laccase preparations after two steps of ion-exchange chromatography.

lose 70% of their activity at pH 2, but retain activity over 50% at the range of pH 3–5, while for pH over 6, activity is almost completely lost. The activity of both Lac1 and Lac2 shows a significant decrease at pH values > 6. Specifically, for neutral pH, Lac1 and Lac2 show activity of 4% and 2% respectively, while for alkaline pH values they show no activity at all. The laccase isolated from the cyanobacterium *Spirulina platensis* CFTRI also exhibited a similar pH-profile (Afreen et al., 2017). Marine-derived fungal laccase-like enzymes, seem to be thermostable and exhibit their optimum temperature above 50 °C, while the optimum pH is highly dependent on the substrate used. For instance, they show an optimum pH of 2–3 when tested on ABTS and 5 when tested on syringaldazine (D'Souza-Ticlo et al., 2009; Mainardi et al., 2018; Wikee et al., 2019).

In order to test the substrate specificity of the two isolated enzymes, we used various phenolic compounds, adding the same amount of Lac1 and Lac2, measured under standard assay conditions. The qualitative results presented in Table 1 show that Lac1 has a broader substrate scope than Lac2. However, none could oxidize L-DOPA. Lac2 was also unable to oxidize hydroquinone and caffeic acid, and generally showed a preference for benzene rings with three substitutions. The highest activity of Lac1 was observed against compounds with 3 ring substitutions, which are the most common laccase substrates (Afreen et al., 2017; Baldrian, 2006; Dwivedi et al., 2011; Kiiskinen et al., 2004). Generally, *para*-substituted phenols, such as hydroquinone and caffeic acid, oxidize in a lower rate (Blaich and Esser, 1975; Senthivelan et al., 2016).

Table 1
Substrate specificity of purified laccases. Symbols according to absorbance: (−) < 0.1; 0.1 ≤ (+) < 0.2; 0.2 ≤ (++) < 0.3; 0.3 ≤ (+++).

Substrate	Lac1	Lac2
Catechol	++	+
Pyrogallol	+++	++
2,6-Dimethoxyphenol	+++	++
L-DOPA	−	−
Gallic acid	+++	+++
Guaiacol	++	++
Hydroquinone	++	−
Caffeic acid	+	−

3.6. Identification of MCOs in Cladosporium sp. TM138-S3

The laccase activity is encoded by the large family of MCOs or Auxiliary Activity family 1 (AA1) proteins. These show a broad substrate range, and many are not functionally characterized (Baldrian, 2006; Kües and Rühl, 2011). Massive DNA or RNA sequencing approaches have been releasing a wealth of putative MCO sequences, all of which have in theory the potential to oxidize ABTS. Since the genome of *Cladosporium* sp. TM138-S3 is available but not yet structurally annotated (Gioti et al., 2020), we performed RNA extraction and Ion Torrent sequencing (RNAseq) from two samples grown in saline conditions (marine broth) in absence and presence of PCB29. The goal was to provide transcriptome data for future genome annotation and identify *Cladosporium* sp. TM138-S3 MCOs that may be responsible for PCB29 removal. The *de novo* assembly of the resulting RNAseq reads (pooled from both conditions) with Trinity provided 29,246 isoforms corresponding to 13,797 unique transcripts. Based on BUSCO analysis, the assembly is 71.9% complete, which indicates that combining transcripts from these two growth conditions allows recovering the majority of expressed genes in *Cladosporium sp.* TM138-S3. However, as expected in RNAseq data, some transcripts were incomplete or missing, with fragmented and missing Pezizomycotina single-copy orthologs estimated at 20.8% and 7.3% respectively.

The transcriptome assembly of *Cladosporium* sp. TM138-S3 (Table S2) was structurally annotated using TransDecoder to identify open reading frames, providing 26,228 predicted proteins, that we functionally annotated combining searches against Swissprot, Pfam, dbCAN, CAZy, TMHMM, SignalP and Panther (Mi et al., 2005) tools and databases. Proteins that contained the three Cu-oxidase Pfam domains and were classified as members of the MCO family of the CAZy database by at least two dbCAN tools, were selected as *Cladosporium* sp. TM138-S3 three-domain (3d)MCOs. There are reports of fungal MCOs with two domains (Rivera-Hoyos et al., 2013), but we chose to focus on typical fungal three-domain MCOs (Gräff et al., 2020; Nakamura and Go, 2005). Twelve proteins were identified as 3dMCOs with these criteria; functional annotation data on these can be found in Data file S1. Similar numbers have been reported in other Ascomycetes, with 13 MCOs reported in the *Aspergillus niger* genome (Tamayo Ramos et al., 2011) and 13–14 in the genomes of *Fonsecaea* species (Moreno et al., 2017). Studies on the repertoire of MCOs, or AA1 proteins, in marine-derived fungi are scarce, and thus inconclusive with regards to numbers: 18 AA1 proteins were predicted in the genome of the Basidiomycete *Peniophora* sp. CBMAI 1063 (Brenelli et al., 2019), while in marine Ascomycetes, AA1 numbers seem reduced: Only 3 were detected in the genome of *Scopulariopsis brevicaulis* (Kumar et al., 2015), while the combination of transcriptomics with secretome analysis failed to identify AA1 proteins in *Stemphylium lucomagnoense*, despite laccase activity being detected (Ben Ali et al., 2020c). Using a similar approach, 5 AA1 proteins were identified in the mangrove fungus *Pestalotiopsis* sp. (Arfi et al., 2013).

Four of the identified *Cladosporium* sp. TM138-S3 3dMCOs are predicted to be extracellular; three of these (DN1152_c0_g1_i5.p1, DN2162_c0_g1_i1.p1 and DN6291_c0_g1_i8.p1) have predicted MW 64–79 kDa and isoelectric points below pH 6.5, that is, within the range of the two proteins isolated here through ion-exchange

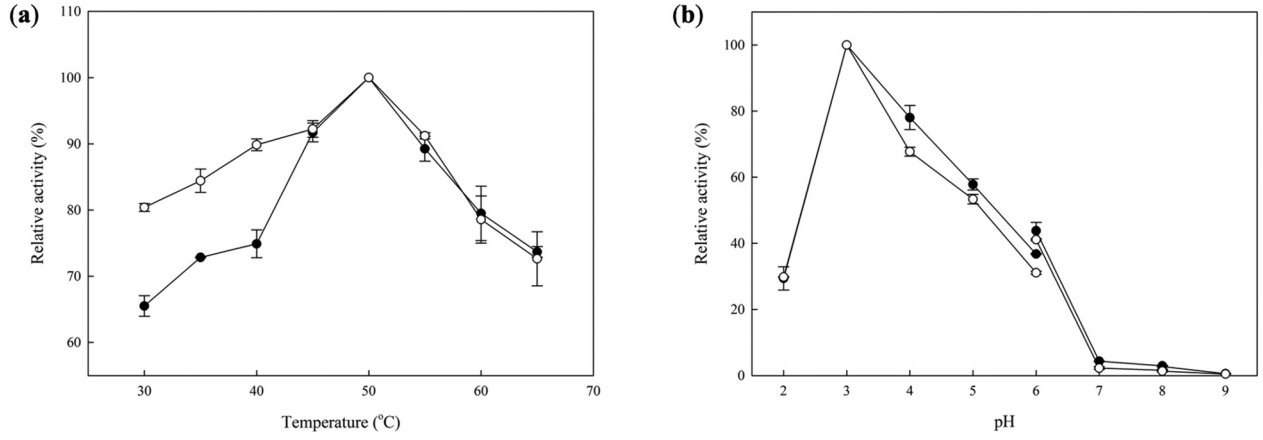

Fig. 4. Effect of temperature (a) and pH (b) on the activity of Lac1 (●) and Lac2 (○).

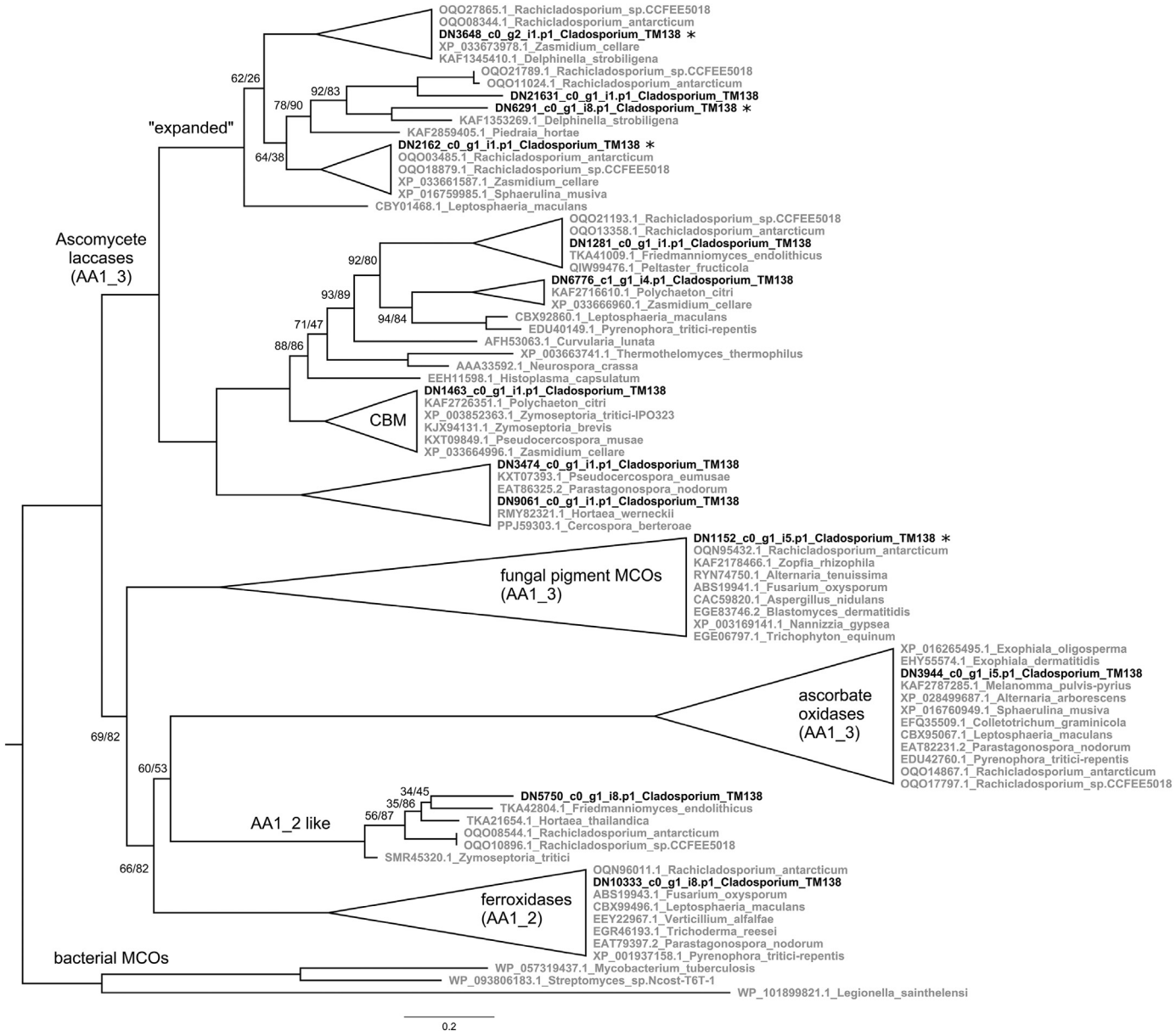

Fig. 5. Maximum Likelihood tree (LogL = −17,905.3456) from the amino-acid alignment of fungal MCOs. Numbers on nodes correspond to ultrafast bootstrap and SH-like approximate likelihood ratio test values, and are not shown for well-supported clades (i.e. where they are >95 and 80, respectively). Clades that have been collapsed for simplicity are well supported in terms of both tests. The tree was rooted with bacterial MCOs. Stars denote proteins predicted as extracellular.

chromatography. One should note, however, that the number of putative and extracellular MCOs identified in the transcriptome assembly is probably underestimated. Although a search for 3dMCOs in preliminary annotations of the *Cladosporium* sp. TM138-S3 genome indicated that all are present in the transcriptome assembly, one third of these was revealed to be partial (data not shown), which affects the detection of Cu-oxidase Pfam domains and SPs in the N-terminus. Structural and functional annotation of the *Cladosporium* sp. TM138-S3 genome is expected to reveal the full repertoire of MCO enzymes in this fungus.

Availability of genomic and transcriptomic sequences for fungi with potential in bioremediation represents a fundamental resource in research, aiming to unlock their metabolic capacities for the industry. However, the field is still at its infancy with regards to marine-derived fungi. In this context, the transcriptome assembly provided here complements the recently-published full genome sequence of *Cladosporium sp.* TM138-S3 (Gioti et al., 2020). The transcriptome data are a prerequisite for the future genome annotation of this isolate that shows promising enzymatic capacities.

3.7. Phylogenetic classification of Cladosporium MCOs

There is currently a lack of consensus with regards to MCO protein naming and classification, the latter being based on different criteria in different studies/phyla, such as domain or motif presence, intron structure, similarity or phylogenies. One of the most widely used databases for carbohydrate active enzymes, CAZy, has classified fungal MCOs or Auxiliary Activity family 1 (AA1) proteins on the basis of sequence similarity into three classes; laccases (AA1_1) only present in Basidiomycetes, ferroxidases (AA1_2) and laccase-like MCOs (AA1_3). Previous phylogenetic studies have allowed distinguishing AA1_3 class fungal MCOs into Basidiomycete laccases, Ascomycete laccases, fungal pigment MCOs and ascorbate oxidases (AO), albeit with limited tree support or

using simplified phylogenetic reconstruction methodologies (Copete et al., 2015; Haddad Momeni et al., 2019; Hoegger et al., 2006; Moreno et al., 2017; Tamayo Ramos et al., 2011). Since phylogenetic analysis of gene families holds great potential in evolutionary-based classification of novel/uncharacterized proteins, we used a Maximum Likelihood approach incorporating a total of 81 taxa in order to place the *Cladosporium* sp. TM138-S3 3dMCOs identified in this study in a phylogenetic tree (Fig. 5).

Based on this tree, the *Cladosporium* sp. TM138-S3 transcriptome encodes representatives from all the previously described classes, except for the Basidiomycete group laccases, as expected. Most *Cladosporium* sp. TM138-S3 3dMCOs, classified by dbCAN tools as AA1_3, were part of the well-supported Ascomycete laccase group, while the remaining two AA1_3 class proteins grouped together with fungal pigment MCOs (DN1152_c0_g1_i5.p1, predicted as secreted) and AO (DN3944_c0_g1_i5.p1), respectively. The *Cladosporium* sp. TM138-S3 AO representative was manually retrieved from predicted proteins with BLASTp, since the protein contains 1/3 Cu-oxidase domains and was thus not identified as a 3dMCO based on our criteria. The number of *Cladosporium* sp. TM138-S3 ferroxidases is yet unclear. While one of the two 3dMCOs that were classified as AA1_2 grouped together with other ferroxidases, the second (DN5750_c0_g1_i8.p1) formed a well-supported separate group (AA1_2-like) along with uncharacterized proteins retrieved as its most similar homologs in this study. Whether these proteins are ferroxidases or AO remains unclear, since clade support remains low in this part of the tree, most probably due to low sampling of taxa.

Inclusion of the *Cladosporium* AA1_3 laccases along with their homologs in the phylogeny revealed the presence of several well-supported clades within the biggest clade, which contains Ascomycete laccases. One of these clades is the "CBM" clade, containing MCOs that possess the starch-binding-domain CBM20. These modular extracellular laccases were recently characterized in *Zymoseptoria* species and formed a separate group named as "Septoria group" based on a Neighbor Joining tree (Haddad Momeni et al., 2019). The fungal MCO tree presented here confirmed with an independent and highly reliable tree reconstruction approach (ML) monophyly of this group, and further showed that CBM-containing MCOs are also present in the *Cladosporiaceae* (DN1463_c0_g1_i1.p1) and the *Capnodiaceae* (*Polychaeton citri* homolog) families.

Interestingly, the phylogenetic tree revealed the presence of a new monophyletic group ("expanded"), which is distinct from all previously characterized MCOs. This group was built with homologs of a *Leptosphaeria maculans* protein (CBY01468.1) of previously unresolved phylogenetic placement (Copete et al., 2015). The expanded clade comprises four *Cladosporium* sp. TM138-S3 proteins, three of which are predicted to be secreted, along with homologs from the closely related *Rachicladosporium* and other species from the *Capnodiales* and *Dothideales* families.

Overall, the phylogenetic analysis revealed the diversity of *Cladosporium* sp. TM138-S3 3dMCOs and some potentially new phylogenetic clades; whether these represent genuine gene family expansions is worth further investigating once more genomic/transcriptomic data from marine-derived fungi become available. Sequence data on marine-derived laccase activity enzymes are lacking either because they come from biochemical studies or are not publicly available. Future enrichment of the MCO phylogeny with sequences from marine or salt-tolerant taxa (such as the halotolerant *Hortaea werneckii* in the phylogenetic tree) is expected to further elucidate the evolutionary history of MCOs in fungi.

3.8. Lac1 and Lac2 effectively remove PCB29

Laccase-like enzymes, especially from Ascomycetes, commonly display low redox potentials that cannot oxidize recalcitrant bulky pollutants or non-phenolic compounds. The discovery of LMS systems enabled the expansion of the substrate scope of laccase-like enzymes to these compounds. LMS systems mimic the fungal biodegradation mechanism of lignin that produces reactive oxygen species (e.g., hydroxyl, peroxyl and hydroperoxyl radicals) along with ligninases (Ashe et al., 2016).

Typically, the removal of PCBs by fungi has been associated with their extracellular oxidases, such as laccase-like enzymes (Takagi et al., 2007). Independently of the enzyme identity, however, the ultimate goal of this work was to investigate whether the enzymes isolated here as induced by copper ions can effectively remove PCB29. When Lac1 and Lac2 were tested on their own, removal of PCB29 was found to be approximately 23%. When ABTS was added at a low concentration to act as mediator, Lac1 enhanced its removal ability by 1.4-fold, while Lac2 activity was enhanced 3-fold, reaching a satisfactory removal yield of 71.2% (Fig. 6). Gallic acid and pyrogallol were also tested as mediators for Lac2, but they did not prove as effective as ABTS. This result may be attributed to the enhanced activity of the enzymes on ABTS and the possible instability of gallic acid and pyrogallol radicals (Chandra and Chowdhary, 2015). ABTS-mediated oxidation proceeds via an electron transfer mechanism, where stable ABTS dication ($ABTS^{2+}$) resulting from ABTS cationic radical ($ABTS^{\bullet+}$) oxidizes the target compound (Chandra and Chowdhary, 2015). On the other hand, when a phenolic compound is used as a mediator, phenoxyl radicals (PhO•) are produced by the enzyme, which oxidize the target compound through the hydrogen atom transfer (HAT) mechanism (Cañas and Camarero, 2010). These different oxidation mechanisms may also result in different products from the same pollutant, as for instance in the case of anthracene and benzo[*a*]pyrene when oxidized by *Pycnoporus cinnabarinus* laccase and different mediators (ABTS, 1-hydroxybenzotriazole and *p*-coumaric acid; Cañas et al., 2007).

ABTS has been previously reported to mediate the biodegradation of tetra- and pentachloro hydroxy PCBs by a laccase from *T. versicolor* (Keum and Li, 2004). In addition, ABTS proved to be the most efficient mediator tested in the case of olsalazine degradation by the crude extracellular laccase preparation of a marine-derived fungus, *Aspergillus aculeatus* (Bankole et al., 2021). In other marine-derived fungi studied in the context of decolorization of synthetic dyes, 1-hydroxybenzotriazole (HBT) has also been shown to be an efficient mediator, for instance when applied to a copper-induced extracellular laccase preparation from *Trichoderma asperellum* (Ben Ali et al., 2020a, b, c), or to the only case of purified laccase from marine sources, isolated from *Pestalotiopsis* sp. (Wikee et al., 2019).

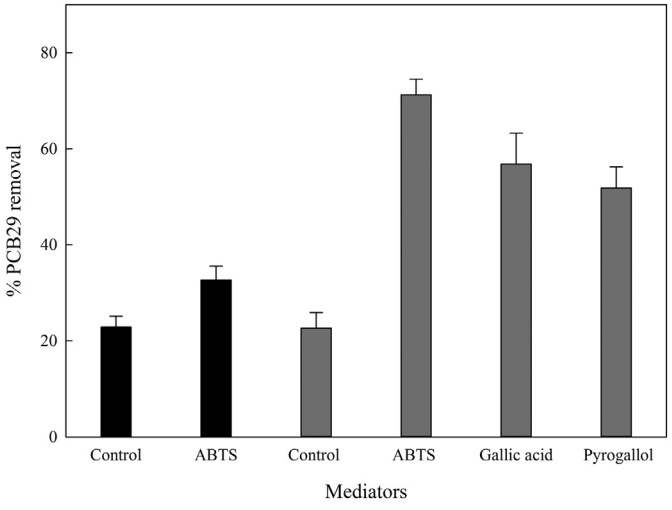

Fig. 6. Percentage removal of PCB 29 in reactions containing either Lac1 (black) or Lac2 (grey) with ABTS as mediator, or Lac2 and other mediators (gallic acid and pyrogallol), compared with reactions with no addition of mediator (Control).

4. Conclusions

PCBs, although banned for decades, still pose a serious threat to human health and wildlife. Representing one of the most complex classes of polychlorinated compounds, these pollutants are recalcitrant and thus persist in the environment. Our study identified fungal isolates, which originated from invertebrates of pristine marine regions, with the ability to remove PCB29. One of the laccase-like enzymes isolated from a *Cladosporium* species was able to remove up to 71% of PCB29. Combining transcriptomic and biochemical analysis on this *Cladosporium* isolate proved a successful strategy for enriching the still-restricted arsenal of marine-derived enzymes suitable for bioremediation applications. Further investigation of other *Cladosporium* enzymes identified in the transcriptome assembly is expected to elucidate the mechanisms of oxidative degradation of xenobiotics in saline environments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145818.

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CRediT authorship contribution statement

Efstratios Nikolaivits: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Romanos Siaperas:** Software, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Andreas Agrafiotis:** Investigation, Writing – review & editing. **Jamal Ouazzani:** Resources, Funding acquisition. **Antonios Magoulas:** Resources, Writing – review & editing, Funding acquisition. **Anastasia Gioti:** Conceptualization, Methodology, Software, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Evangelos Topakas:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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