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ORIGINAL PAPER

Screening and evaluation of laccase produced by different *Trichoderma* species along with their phylogenetic relationship

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Abstract

Laccases have high biotechnological potential in industries since they catalyze the oxidation of many chemical compounds. The production of laccases by fungi has been extensively studied due to their secretion of enzymes and rapid growth using cheap substrates. *Trichoderma;* the versatile fungal genus includes species of great biotechnological value and considered as a magnificent industrial cell factory of enzymes. In this study, 10 *Trichoderma* species were screened for laccase enzyme production by submerged cultivation. The studied species were identified by internal transcribed spacer (ITS) gene sequences. Guaiacol (0.04%) as an enzyme substrate in plate medium was used for the selection of maximum laccase-enriched *Trichoderma* species by formation of visual color halo intensity. This activity was evaluated by liquid submersion (flask medium) also. The absorbance of laccase contained broth was measured by a spectrophotometer (450 nm). The highest laccase production was obtained by *T. atroviride* (2.62 U/mL). *Trichoderma cremeum* and *T. longipile* showed medium laccase potency, while *T. beinartii* exhibited weak laccase secretion ability. Laccase from *T. atroviride* was purified by SDS-PAGE and the molecular weight was determined (57 kDa). The laccase was confirmed by their respective amino acid sequences, and the phylogenetic tree was constructed for further analysis.

Keywords Guaiacol · Internal transcribed spacer (ITS) · Oxidation · Polymerization · Phylogeny

Abbreviations

ITS	Internal transcribed spacer
U/mL	Enzyme activity unit/mL
E.A	Enzyme activity
IAGS	Institute of agricultural sciences, University of the Punjab, Lahore
CTAB	Cetyl trimethylammonium bromide
BLAST	Basic local alignment search tool
NCBI	National center for biotechnology information
MAFFT	Multiple alignment using fast Fourier transform
MEGA	Molecular evolutionary genetics analysis

Introduction

Trichoderma belongs to *Hypocrea* (Ascomycota) (Savoie et al. 2001) and introduced by Persoon (1974). These fungal species are commonly present in all climatic zones (Błaszczyk et al. 2014) and love to live in soil, root, and foliar environment (Huang et al. 2011). Rapid growth with maximum laccase potential is a characteristic of *Trichoderma* species (Kubicek et al. 2003). These species act as bio control agents (Benitez et al. 2004), biopesticides and fungicides (Howell 2003), releasing antibiotics (Vinale et al. 2008) during plant–pathogenic fungal interaction (Mukherjee et al. 2012), resistive to biotic and abiotic stresses (Mastouri et al. 2012) and play a vital role in plant diversity (Druzhinina et al. 2011; Mathys et al. 2012).

Many kingdoms have been explored for laccase potential, but fungal laccase processed higher activities (Bhamare et al. 2016) as asomycetous laccase sensu stricto exhibit superb qualities than basidiomycetes (Cazares-Garcıa et al. 2013). *Trichoderma* species are called "aggressive biodegraders" due to lytic enzymes important for cell wall components (Strakowska et al. 2014; Ramı´rez-Cavazos et al. 2014),

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periplasmic space, and fungal conidial membrane (Pokorny et al. 2005).

This multicopper enzyme is constitutive and inducible in nature (Kiiskinen et al. 2004; Sharma et al. 2005) and reported molecular weight of laccase ranges from 50 to 97 kDa (Chandra et al. 2015). This is an attractive biocatalyst in industrial applications (Giardina et al. 2015). Oxidative coupling reactions are also catalyzed by fungal laccase (Rodríguez-Couto 2019). This is useful in biofuel production and textiles finishing (Abd El Monssef et al. 2016). This enzyme possess "Janus-faced" range of activities (Giardina et al. 2010) *e.g.,* azodye oxidation (Gomi et al. 2011), biochemical bleaching of pulp (Kalyani et al. 2012), biosensors constructor (Bilal et al. 2019; Gomes and Rebelo 2003), delignification of plant products (Copete-Pertuz et al. 2019), producer of other enzymes (Wang et al. 2002), humification (Hatakka 2001), paper processing (Michael et al. 2005), phenolics, non-phenolics and miscellaneous degradation (Litwińska et al. 2019), pollutants detoxifier (Tortella et al. 2013), polymerization or depolymerization processes (Viswanath et al. 2014), steroid transformation (Priyadarsini et al. 2011), wine and water discoloration (Ikehata et al. 2004), xenobiotic compounds degradation (Gomi et al. 2011), use in nanotechnology (Chhaya and Gupta 2010) and pharmaceutical products (Priyadarsini et al. 2011) as well. The major applications in the field of medicine are anticancer drugs (Sekme et al. 2013), hormone derivatives, preparation of antiviral agents, and antioxidants preparation (Rocasalbas et al. 2013). Furthermore, laccase markers are used in immunoassays (Warsinke et al. 2000), and to design energy transformation systems (Chen et al. 2001) and detoxify organic pollutants (Lynch and Moffat 2005).

Few literatures have been reported on laccase production from *Trichoderma* species (Holker et al. 2002). Although this is an expensive industrial enzyme, a major problem encountered by industrialists is the low production level from its native hosts. This study was designed to screen *Trichoderma* sp. with minimum cost and maximum laccase under optimum conditions. The laccase and strongest candidate were identified by the construction of molecular phylogeny. These samples were packed and stored (4 °C) in germ-free polythene bags from different sites in Lahore, Punjab Pakistan.

Materials and methods

Isolation *Trichoderma* species

Soils of 5–10 cm depth were collected from the Sugar beet rhizosphere. These samples were packed and stored (4 °C) in germ-free polythene bags from different sites of Lahore, Punjab Pakistan.

Malt Extract Agar (MEA) medium was prepared with little modification in g/L by adding Malt Extract 20, Agar 20, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1, Yeast Extract 4, glucose 20, and peptone 6 (Coll et al. 1993) at pH 5.5. Streptomycin (200 mg/L) was added as an antibacterial agent. Complete MEA medium was sterilized in an autoclave for 20 min at 121 ºC. This medium was used to obtain the fungal isolations. The serial dilution made to get the pure *Trichoderma* cultures on Petri plates. These plates were incubated at 30 °C. After 7–10 days, pure mycelium was cultivated and stored at 4 °C. The pure living cultures were deposited in Institute of Agricultural Sciences, University of the Punjab, Lahore (IAGS).

Identification, DNA extraction, sequence alignment, and molecular phylogeny

A modified CTAB procedure was followed to extract the total genomic DNA from the specimens (Doyle and Doyle 1987). Nuclear ribosomal ITS regions were used to study the target species. Sequencing of PCR products (Bidriectional) was done by Macrogen Inc. South Korea. The consensus was generated from both ITS1F and ITS4 in BioEdit version 7.2.5 (Hall 1999) and then homology searches were performed at the National Center for Biotechnology Information (NCBI) Web site using BLAST. Dataset of ITS initial blast (BLAST) and literature accessions were downloaded from GenBank (Benson et al. 2017). Sequences were aligned and edited using ClustalX 2.1 (Larkin et al. 2007) and BioEdit (Hall 1999). Sequences retrieved from GenBank and the newly generated sequences were aligned with MAFFT v. 10 (http://mafft.cbrc.jp/alignment/server/index.html; (Katoh and Standley 2013). The alignment was set at 596 positions. These sequences were used to construct the phylogenetic tree by using the maximum-likelihood method. The phylogeny was performed on MEGA version 10.0 (Tamura et al. 2011). Bootstrap values were adjusted from 1000 replicates. This phylogenetic tree helps to find the exact phylogeny of the filamentous fungi of samples. Branches less than 50% bootstrap replicates were collapsed.

Screening of laccase producing *Trichoderma*

All collected fungal isolates were cultured on Petri plates containing sterilized MEA augmented with 0.04% guaiacol and 0.01% (w/v) chloramphenicol (to avoid bacterial growth) adjusted at pH 5.5. The purpose of this synthetic substrate was to identify those *Trichoderma* species excreted maximum laccase by formation of maroon brown color zone around its mycelia colony. These Petri plates were incubated at 28–30 °C for 72 h and then screened for the formation

of reddish brown zones around the fungal colonies (Kalra et al. 2013).

Laccase production medium and inoculation in submerged culture

After identification of *Trichoderma* species, pure agar culture blocks (5 mm) were inserted into a flask (250 mL) containing the basal nutrient medium without agar. At 28 °C, 150 rpm, the flasks were incubated for 8 days. The fungal liquid culture with mycelia was centrifuged at 10, 000 rpm at 4 °C for 20 min followed by filtration using Whatman filter papers. The obtained extracellular fluid supernatant was used for further investigations containing crude laccase.

Assay of laccase activity

The above-mentioned supernatant comprised of crude laccase used for the determination of enzymatic activity by measuring the oxidation of guaiacol substrate (Gao et al. 2011). Sodium acetate (50 mM) buffer (pH 4.5) and guaiacol (2 mM) were used for quantification. The reaction mixture for quantification of laccase under spectrophotometer comprised 1 mL crude enzyme supernatant, 3 mL sodium acetate buffer and 1 mL guaiacol (Chefetz et al. 1998) vortexed for 30 s, after that, it was incubated for 10 min at 30 °C. After 30 s absorbance was measured at 465 nm ($\varepsilon_{465} = 12{,}100\ M^{-1}\ cm^{-1}$). Formula used for calculation was E.A = (A* V) / (t * €* v), where E.A = Enzyme Activity (U/mL), A = Absorbance at 465 nm, V = Total volume of reaction mixture (mL), v = enzyme volume (mL), t = Incubation time (min) and € = Extinction Coefficient ($M^{-1}\ cm^{-1}$) (Gao et al. 2011). The data of laccase from all *Trichoderma* species were compared to commercial *Trametes versicolor* laccase (Sigma/38429) as a control.

Enzyme purification gel electrophoresis

Laccase purification was conducted by the method of Chefetz et al. (1998). Filtrate was centrifuged at 13,000 rpm for 20 min at 10 °C and supernatant subjected to ammonium sulfate precipitation. Precipitates were dialyzed and loaded to DEAE-Cellulose anion-exchange column, equilibrated by 10 mM sodium acetate buffer (pH 5.5). The fraction containing laccase was pooled, concentrated, and dialyzed overnight. The DEAE-purified sample was loaded on to the column by adding 3 mL fraction. This fraction was dialyzed and purity of the laccase with its molecular weight was determined by SDS-PAGE (Duran et al. 2003) and visualized by the staining gel with Coomassie Brilliant Blue R-250. The relative molecular mass was estimated using standard molecular weight markers [ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa), and bovine serum albumin (67.0 kDa)].

Analysis of amino acid sequence and phylogram

The protein sequencer ABI Procise 491HT was used to sequence the laccase band by the method of Matsudaira (1987).

Statistical analysis

Assays were carried out in triplicate, and the results were presented as mean ± standard deviation.

Results

In this study, 10 species of *Trichoderma* were evaluated for laccase activity. The colony characteristics were also determined (Fig. S1, Table 1). *Trichoderma* fungal colonies were screened for evaluation of laccase potential by guaiacol substrate. Due to the variation in substrates utilization, this extracellular protein gained commercial and industrial importance. Pure cultures of these species were identified on molecular basis using ITS markers. Maximum-likelihood analysis was applied to construct the phylogenetic tree to identify these species (Fig. S2). Purified fungal mycelium made a specific clade with its neighboring species with maximum bootstrap values confirmed robust tree topology. Phylogenetic analysis clearly facilitated in identification of filamentous species of *Trichoderma*.

Out of ten, four species were able to oxidize guaiacol in shake flask than other *Trichoderma* species including *T. cremeum, T. longipile, T. citrinoviride,* and *T. atroviride*. Reddish brown color was produced by oxidation of guaiacol by extracellular laccase secretion in the plate media, but the incubation time and growth rate varied according to the individual colony. Laccase secretion potential in preliminary screening was in this order *T. atroviride* > *T. cremeum* > *T. longipile* and > *T. citrinoviride*. Amongst all species, *Trichoderma atroviride* was more efficient in laccase production confirmed by guaiacol enzyme assay (Fig. S3). This species also showed fast growth and dark brown coloration within 3 days of incubation followed by *T. cremeum, T. longipile,* and *T. citrinoviride*. The mycelium of these species was stored at 4 °C for farther analysis.

In this study, the optimum conditions were applied to find the strongest candidates and compared with commercial laccase as a control of *Trametes versicolor* laccase (5 U/mL). *Trichoderma cremeum, T. longipile,* and *T. citrinoviride* exhibited strong laccase activity in submerge shake flasks less than *T. atroviride*. The laccase activity from *T. atroviride* was 2.62 U/mL (Fig. 1) considered the strongest

Table 1 List of Laccase (U/mL) producing *Trichoderma* Species

Sr no	Species name	Voucher no	GenBank accessions	Conidia shape	Pigmentation	Colony appearance	Laccase activity (U/mL)
1	*T. harzianum*	IAGST21	MW785562	Globose to subglobose	Green	Floccose/cottony white	1.12 ± 0.03
2	*T. viride*	IAGST22	MW898148	Globose	Gray to green	Highly intricate	1.24 ± 0.05
3	*T. pseudokoningii*	IAGST23	MW785566	Oblong ellipsoidal	White	Ringed	1.16 ± 0.01
4	*T. cremeum*	IAGST24	MW785565	Oblong	Creamy	Circular	1.98 ± 0.09
5	*T. longipile*	IAGST25	MW785564	Oblong	Incarnate/green	Circular	1.84 ± 0.02
6	*T. atroviride*	IAGST26	MW325977	Globose to subglobose	Green	Highly intricate	2.62 ± 0.01
7	*T. citrinoviride*	IAGST27	MW785567	Ellipsoidal	Yellowish green	Rough and pigmented	1.65 ± 0.01
8	*T. beinartii*	IAGST28	MW785569	Smooth oblong	Diffusible	Concentric zones	0.42 ± 0.06
9	*T. asperellum*	IAGST29	MW785568	Subglobose/ovoidal	Green to dark green	Concentric rings	1.25 ± 0.08
10	*T. virens*	IAGST30	MW785563	Ellipsoidal to obovoid	Light yellow/green	Floccose	1.03 ± 0.07

candidate in laccase production. Secondary productive species were *T. cremeum* (1.98 U/mL), *T. longipile* (1.84 /mL) and *T. citrinoviride* (1.65 U/mL) (Fig. 1). Literature cited that *T. atroviride, T. harzianum* and *T. reesei* were the strongest and competent laccase secreting candidates amongst *Trichoderma* species by the utilization of augmented media. But the laccase activities related to these species were low in this experimental work. The lowest value of laccase (U/mL) in the shake flask was demonstrated by *T. beinartii* (0.42 U/mL) (Fig. 1). Many of these species have not been explored till yet in literature. This study has been explored many laccase producing *Trichoderma* species (Fig. 1). The strongest candidate, *T. atroviride* was used for farther analysis.

The purification of laccase from *Trichoderma atroviride* was determined by 60% ammonium sulfate precipitation method. After the partial purification, the molecular

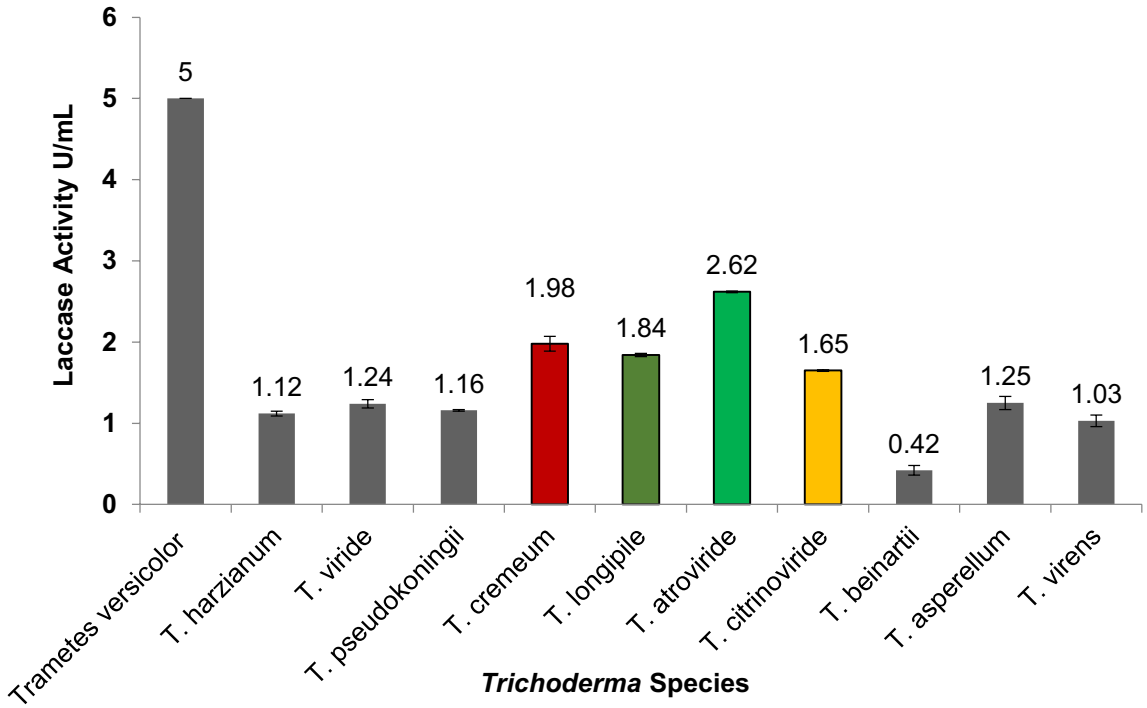

Fig. 1 Comparative evaluation of laccase activity (U/mL) from *Trichoderma* Species compared with commercial laccase (*T. versicolor*)

weight was estimated by SDS-PAGE. Standard protein markers were used to quantify the purified *Ta*Lacc1. The molecular weight was ~ 57 kDa determined by SDS-PAGE (Fig. S4). The band position after staining facilitated in the quantification.

The previously reported literature distinguishes the laccase genes from the other copper blue oxidases in comparative analysis of laccase sequences. The length of amino acid sequences of *T. atroviride* laccase was near to the typical fungal laccases (510 aa). The calculated molecular weight of the protein sequence was nearly ~ 57 kDa.

The purified *Ta*Lacc1 of *Trichoderma atroviride* was identified by amino acids sequences and conducted by Blast analysis of the database in GenBank. A phylogram of *T. atroviride* was constructed to find the exact identity of laccase. The sequences of various laccases were obtained from the NCBI GenBank database used as queries to find the laccase genes of *T. atroviride*, which removed the doubts regarding laccase identity.

For the phylogenetic analysis of the putative sequences of laccase, multiple alignments were performed with CLUSTALW in BioEdit software. The alignments were manually adjusted. Based on the generated alignments, phylogenetic trees were constructed with MEGA version 10.0. Statistical significance was evaluated with a bootstrapping of 1000 repetitions. The phylogeny was confirmed by using the maximum likelihood method, and the alignments were differentially edited to corroborate the topology of the tree (Fig. 2).

Laccase of *T. atroviride* exhibited close phylogenetic relationships with other *Trichoderma* species. The reason behind is the protein included in phylogenetic analysis were chosen according to the same criteria of methodology. The ITS sequences of all *Trichoderma* species were deposited in GenBank (Table 1).

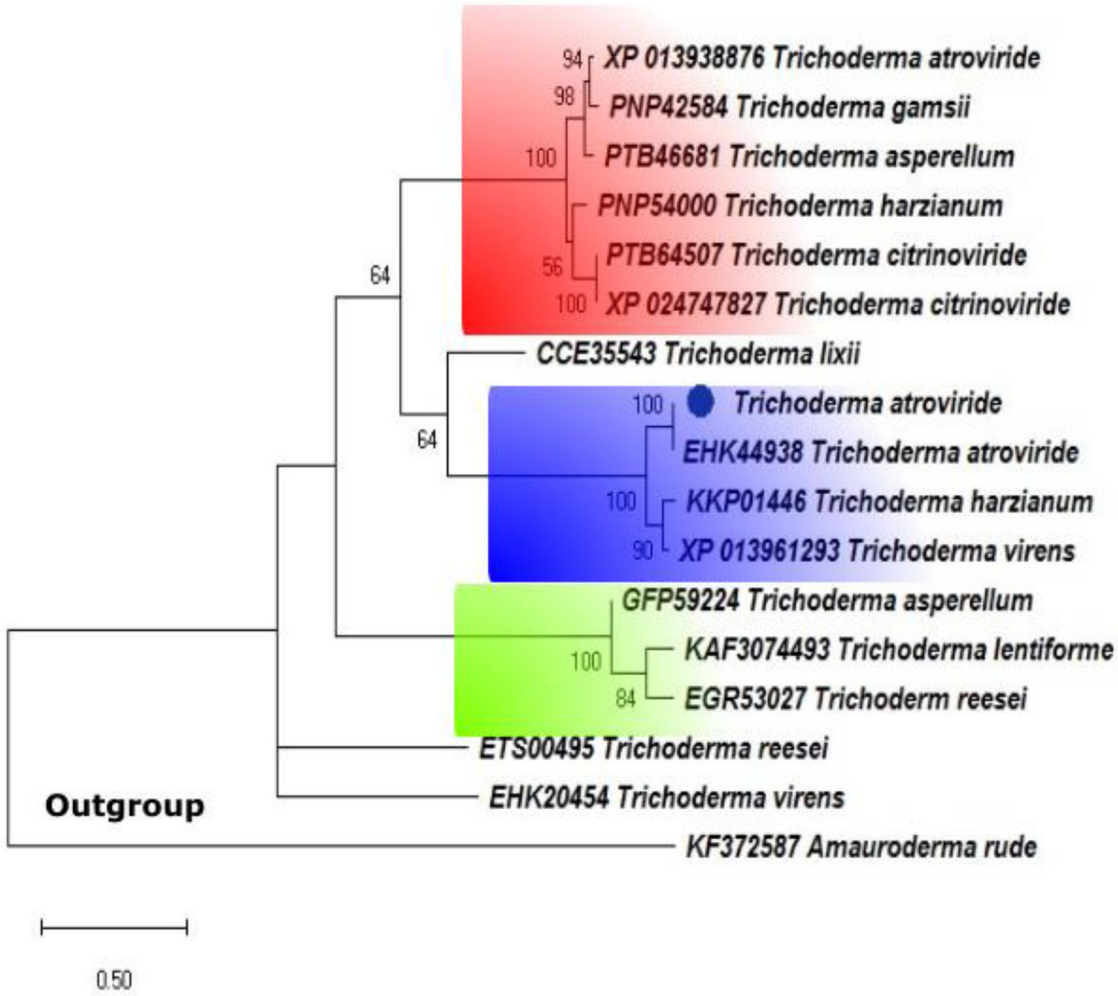

Fig. 2 Phylogram of amino acids sequence of purified laccase of *T. atroviride* indicated by (Colors indicated different clades) (color figure online)

Discussion

Trichoderma–filamentous fungi are the largest group of microorganisms which plays an important role in the environment. These species were selected due to the rapid growth rate in the media rather than other filamentous fungi. Natural substrates (e.g. agricultural residues, wheat bran, and rice straw/husk) mimic the natural conditions under which the particular fungus grows (Brijwani et al. 2010) and prolonged cultivation period is required for production of maximum laccase (Shekher et al. 2011; Rodríguez et al. 2005; Pandey et al. 1999). In this study, preference was given to a synthetic substrate. Substrate spectra of numerous laccases from fungal origin have been reported. Experimental procedure and choice of compound present difficulty in data comparison (Endo et al. 2003; Smirnov et al. 2001; Xu 1996). This enzyme possesses overlapping range of substrate specificity and non-specific towards substrates form one type of laccase to another. Laccase oxidize ortho and paradiphenol, but usually more efficient towards paradiphenol (Wood 1980). Guaiacol is an ortho-substituted compounds typically referred as the best substrates for most laccases (Thurston 1994). *Trichoderma* species collected from Sugar beet rhizosphere, formed a brown-zone around and below colony on the Plate-containing guaiacol substrate, which was a characteristic laccase production (extracellular) on a solid medium of filamentous fungi (Thurston 1994; Madhavi and Lele 2009).

The major issue behind this study was the high cost of laccase with low production level. In this experiment, strong *Trichoderma* candidates were screened with high laccase concentrations by the optimized cultural conditions. Nutrient broth beneath the mycelium in submerge flasks enhanced the extracellular secretion of laccase.

Holker et al. (2002) identified extracellular laccase in *Trichoderma* sp., however, little information is available on intracellular laccases in *Trichoderma* as well as other fungal species (Assavaning et al. 1992). Pokorny et al. (2005) worked on periplasmic laccase (extracellular) activity of *T. harzianum, T. atroviride,* and *T. viride* during maturation of conidia. Laccase in conidia synthesizes pigments and other constituents that safeguard the cells from stress factors (UV light, enzymatic lysis, and temperature, etc.) (Chakroun et al. 2010).

Sadhasivam et al. (2009) worked on *Trichoderma harzianum* by augmented $CuSO_4$ (1 mM) in a liquid medium. This yielded high laccase (4.36 U/mL) after the 4th day. *T. reesei* possessed 1.22 U/mL in liquid medium and worked well in acidic pH (4.5) and 27 °C temperature. Desai and Nityanand (2011) entitled *T. harzianum* the strongest candidate amongst all *Trichoderma* species, whereas, Holker et al. (2002) found maximum laccase activity in spores of *T. atroviride* and *T. harzianum* found in this study.

Trichoderma belongs to those fungi which comprised minimum number of laccase genes (Cázares-García et al. 2013). However, the results in the present study indicated that laccase genes were earlier reported in ascomycetes should be reviewed in the future, as it is not possible for all of them to code the laccases. Molecular weight of *T. atroviride* of this study was in agreement with the previously reported fungal laccases, having between 55 and 70 kDa. In *Trichoderma*, purified laccases from *T. harzianum* WL1 and *T. atroviride* CTM 10,476 presented glycosylated laccase of 79 kDa and 80 kDa, respectively (Chakroun et al. 2010; Sadhasivam et al. 2009) contrary kDa of this purified laccase. In literature during evolution, *T. reesei* lost maximum quantity of the genetic message, while retained in *T. virens* (Kubicek et al. 2011). The generated data during speciation, it is possible that *T. atroviride* and *T. virens* maintained total laccase genes as common ancestor has, while *T. ressei* and *T. harzianum* lost extracellular laccase genes (Cázares-García et al. 2013).

This genus has preserved limited functional laccase amino acids in the course of evolution. Mostly, the whole protein sequence of intracellular laccase is preserved, while the evolutionary patterns of extracellular laccase are variable (Cázares-García et al. 2013). So, the recognition of *Trichoderma* laccase is important to analyze the robust phylogenetic analyses of this enzymatic function.

In this study, many *Trichoderma* species were evaluated for laccase potential to find out the strongest candidate. Only *T. atroviride* a biotechnological workhorse of the genus *Trichoderma* has been reported as a strong laccase producing organism. In addition, phylogenetic and evolutionary analyses were also carried out to identify the *Trichoderma* species as well as laccase enzymes.

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Declarations

Conflict of interest There is no any conflict of interest.

References

Abd El Monssef RA, Hassan EA, Ramadan EM (2016) Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. Annal Agri Sci 61(1):145–154. https://doi.org/10.1016/j.aoas.2015.11.007

Assavaning A, Amornikitticharoen B, Ekpaisal N, Meevootisom V, Flegel TW (1992) Isolation, characterization and function of

laccase from *Trichoderma*. Appl Microbiol Biotechnol 38(2):198–202. https://doi.org/10.1007/BF00174468
Benitez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int J Microb 7:249–260
Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J et al (2017) GenBank. Nuc Acids Res 45(D1):D37–D42
Bhamare HM, Sayyed RZ (2016) Microbial Laccase: production and their potential applications In book: advances in bio and medico. Sciences p.173–190
Bilal M, Rasheed T, Nabeel F, Iqbal HMN (2019) Hazardous contaminants in the environment and their laccase assisted degradation, a review. J Environ Manag 234:253–264. https://doi.org/10.1016/j.jenvman.2019.01.001
Błaszczyk L, Marek S, Krzysztof S, Jolanta L, Małgorzata J (2014) *Trichoderma* spp. application and prospects for use in organic farming and industry. J Plant Prot Res 54:4
Brijwani K, Oberoi HS, Vadlani PV (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochem 45(1):120–128. https://doi.org/10.1016/j.procbio.2009.08.015
Cazares-Garcıa SV, Vazquez-Garciduenas MS, Vazquez-Marrufo G (2013) Structural and phylogenetic analysis of laccases from *Trichoderma*: a bioinformatic approach. PLoS ONE 8(1):e55295. https://doi.org/10.1371/journal.pone.0055295
Chakroun H, Mechichi T, Martinez MJ, Dhouib A, Sayadi S (2010) Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds. Process Biochem 45:507–513. https://doi.org/10.1007/s00253-002-1012-x
Chandra R, Chowdhary P (2015) Properties of bacterial laccases and their application in bioremediation of industrial wastes. Environ Sci Process Imp 17:326–342. https://doi.org/10.1039/C4EM00627E
Chefetz B, Chen Y, Hadar Y (1998) Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl Environ Microbiol 64(9):3175–3179. https://doi.org/10.1128/AEM.64.9.3175-3179.1998
Chen T, Barton SC, Binyamin G, Gao Z, Zhang Y, Kim HH (2001) A miniature biofuel cell. J Am Chem Soc 123:8630–8631. https://doi.org/10.1021/ja0163164
Chhaya U, Gupte A (2010) Optimization of media components for laccase production by litter-dwelling fungal isolate *Fusarium incarnatum* LD-3. J Basic Microb 50(1):43–51. https://doi.org/10.1002/jobm.200900203
Coll PM, Abalos JMF, Villanueva JR, Santamaria R, Perez P (1993) Purification and characterization of phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59:2607–2613
Copete-Pertuz LS, Alandete-Novoa F, Jersson P, Correa-Londoño GA, Mora-Martínez AL (2019) Enhancement of ligninolytic enzymes production and decolourising activity in Leptosphaerulina sp. by co–cultivation with *Trichoderma viride* and *Aspergillus terreus*. Sci Total Environ 646:1536–1545. https://doi.org/10.1016/j.scitotenv.2018.07.387
Cordoba Canero D, Roncero MIG (2008) Influence of the chloride channel of *Fusarium oxysporum* on extracellular laccase activity and virulence on tomato plants. Microbiol 154:1474–1481
Couto SR, Sanromán MA (2005) Application of solid-state fermentation to ligninolytic enzyme production. Biochem Eng J 22(3):211–219. https://doi.org/10.1016/j.bej.2004.09.013
Desai SS, Nityanand C (2011) Microbial laccases and their applications: a review. Asian J Biotech 3:98–124. https://doi.org/10.3923/ajbkr.2011.98.124
Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15
Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA et al (2011) *Trichoderma*, the genomics of opportunistic success. Nat Rev 9:749–759. https://doi.org/10.1038/nrmicro2637
Duran N, Rosa M, Annibale A, Gianfreda L (2003) Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. Enzyme Microb Technol 31:907–931. https://doi.org/10.1016/S0141-0229(02)00214-4
Endo K, Hayashi Y, Hibi T, Hosono K, Beppu T et al (2003) Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. J Biochem 133:671–677. https://doi.org/10.1093/jb/mvg086
Gao HJ, Wang YW, Zhang WT, Wang WL, Mu ZM (2011) Isolation, identification and application in lignin degradation of an ascomycete GHJ-4. African J Biotech 10(20):4166–4174
Giardina P, Sannia G (2015) Laccases: Old enzymes with a promising future. Cell Mol Life Sci 72:855–856. https://doi.org/10.1007/s00018-014-1821-y
Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G (2010) Laccases: a never-ending story. Cell Mol Life Sci 67:369–385. https://doi.org/10.1007/s00018-009-0169-1
Gomes SA, Rebelo MJ (2003) A new laccase biosensor for polyphenols determination. Sensors 3:166–175. https://doi.org/10.3390/s30600166
Gomi N, Yoshida S, Matsumato K, Okudomi M, Konno H, Hisabori T, Sugano Y (2011) Degradation of the synthetic dye amaranth by the fungus *Bjerkendera adusta* inference of the degradation pathway from an analysis of decolorized products. Biodegradation 22:1239–1245. https://doi.org/10.1007/s10532-011-9478-9
Hall TA (1999) BioEdit: a user–friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nuc Acids Sym Series 41:95–98
Hatakka A (2001) Biodegradation of lignin. In: Hofrichter M, Steinbüchel A (eds) Lignin, humic substances, and coal. Wiley-VCH, Weinheim, pp 129–179
Holker H, Dohse J, Ho¨fer M, (2002) Extracellular laccases in ascomycetes *Trichoderma atroviride* and *Trichoderma harzianum*. Folia Microbiol 47:423–427. https://doi.org/10.1007/BF02818702
Howell CR (2003) Mechanisms employed by Trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis 87:4–10. https://doi.org/10.1094/PDIS.2003.87.1.4
Huang X, Chen L, Ran W, Shen Q, Yang X (2011) *Trichoderma harzianum* strain SQR-T37 and its bio-organic fertilizer could control *Rhizoctonia solani* damping-off disease in cucumber seedlings mainly by the mycoparasitism. Appl Microbiol Biotech 91:741–755. https://doi.org/10.1007/s00253-011-3259-6
Ikehata K, Buchanan ID, Smith DW (2004) Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. J Environ Eng Sci 3:1–19. https://doi.org/10.1139/s03-077
Kalra K, Chauhan R, Shavez M, Sachdeva S (2013) Isolation of laccase producing *Trichoderma* spp. and effect of pH and temperature on its activity. Int J Chem Tech 5(5):2229–2235
Kalyani D, Dhiman SS, Kim H, Jeya M, Kim I, Lee J (2012) Characterization of a novel laccase from the isolated *Coltricia perennis* and its application to detoxification of biomass. Proc Biochem 47:671–678. https://doi.org/10.1016/j.procbio.2012.01.013
Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Boil Evol 30(4):772–780. https://doi.org/10.1093/molbev/mst010
Kiiskinen LL, Kristiina K, Michael B, Erkko Y, Matti S, Markku S (2004) Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiol 150:3065–3074

Kubicek CP, Bissett J, Druzhinina I, Kullnig-Gradinger C, Szakacs G (2003) Genetic and metabolic diversity of *Trichoderma*: a case study on South-East Asian isolates. Fung Genet Biol 38:310–319. https://doi.org/10.1016/S1087-1845(02)00583-2

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V et al (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style for *Trichoderma*. Genome Biol 12:R40. https://doi.org/10.1186/gb-2011-12-4-r40

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H et al (2007) ClustalW and ClustalX version 2.0. Bioinformatics 23(21):2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Litwińska K, Felix B, Falko M, Rüdiger B, Twan R, Gotthard K (2019) Characterization of recombinant laccase from *Trametes versicolor* synthesized by *Arxula adeninivorans* and its application in the degradation of pharmaceuticals. AMB Express 9:102. https://doi.org/10.1186/s13568-019-0832-3

Lynch JM, Moffat AJ (2005) Bioremediation–prospects for the future application of innovative applied biological research. Ann Appl Biol 146(2):217–221. https://doi.org/10.1111/j.1744-7348.2005.040115.x

Madhavi V, Lele SS (2009) Laccase: properties and applications. BioResources 4(4):1694–1717

Mastouri F, Björkman T, Harman GE (2012) *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. Mol Plant-Microb Int 25:1264–1271. https://doi.org/10.1094/MPMI-09-11-0240

Mathys J, De Cremer K, Timmermans P, Van Kerckhove S, Lievens B, Vanhaecke M et al (2012) Genome-wide characterization of ISR induced in Arabidopsis thaliana by *Trichoderma hamatum* T382 against Botrytis cinerea infection. Front Plant Sci 3:108. https://doi.org/10.3389/fpls.2012.00108

Matsudaira P (1987) Sequence from picomole quantities of proteins electro blotted onto poly vinylidene difluoride membranes. J Bio Chem 262(21):10035–10038. https://doi.org/10.1016/S0021-9258(18)61070-1

Michael MT, Georg MG, Astrid R (2005) Degradation of Azo dyes by laccase and ultrasound treatment. Applv Environ Microbiol 71:2600–2607. https://doi.org/10.1128/AEM.71.5.2600-2607.2005

Mukherjee PK, Buensanteai N, Moran-Diez ME, Druzhinina IS, Kenerley CM (2012) Functional analysis of the non- ribosomal peptide synthetases (NRPSs) in *Trichoderma virens* reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. Microbiology 158:155–165

Pandey A, Selvakumar P, Soccol CR, Nigam P (1999) Solid state fermentation for the production of industrial enzymes. Current Sci 10:149–162

Persoon CH (1794) Disposita methodica fungorum. Römer's Neues Mag Bot 1:81–128

Pokorny R, Vargovic P, Holker U, Janssen M, Bend J, Hudecová D et al (2005) Developmental changes in *Trichoderma viride* enzymes abundant in conidia and the light-induced condition signalling pathway. J Basic Microbiol 45:219–229. https://doi.org/10.1002/jobm.200410354

Priyadarsini RI, Bhuvaneswari V, Kumar KS (2011) Isolation, identification and phylogenetic analysis of White rot fungus and heterologous expression of gene encoding laccase. J Appl Sci Environ Sanit 6(1):69–83

Ramırez-Cavazos L, Junghanns C, Ornelas-Soto N, Cardenas-Chavez D, Hernandez- Luna C, Demarche P et al (2014) Purification and characterization of two thermostable laccases from *Pycnoporus sanguineus* and potential role in degradation of endocrine disrupting chemicals. J Mol Catal b: Enzym 108:32–42. https://doi.org/10.1016/j.molcatb.2014.06.006

Rocasalbas G, Francesko A, Touriño S, Fernández-Francos X, Guebitz GM, Tzanov T (2013) Laccase-assisted formation of bioactive chitosan/gelatin hydrogel stabilized with plant polyphenols. Carbohydr Polym 92(2):989–996. https://doi.org/10.1016/j.carbpol.2012.10.045

Rodríguez RD, Gabriela H, José AS, Miguel J, Mario CNS, Inmaculada GR, Inmaculada S (2019) Enhancing laccase production by white-rot fungus *Funalia floccosa* LPSC 232 in co-culture with *Penicillium commune* GHAIE86. Folia Microbiol 64:91–99. https://doi.org/10.1007/s12223-018-0635-y

Sadhasivam S, Savitha S, Swwaminathan K (2009) Redox-mediated decolorization of recalcitrant textile dyes by *Trichoderma harzianum* WL1 laccase. World J Microbiol Biotechnol 25:1733–1741. https://doi.org/10.1007/s11274-009-0069-4

Savoie JM, Gerardo M, Michèle M (2001) Variability in brown line formation and extracellular laccase production during interaction between white rot basidiomycetes and *Trichoderma harzianum* biotype Th2. Mycologia 93(2):243–248.https://doi.org/10.1080/00275514.2001.12063154

Sekme S, Nese A, Inci A (2013) Studies on laccase activity in the filamentous fungus *Trichoderma reesei*. IUFS J Bio Res Art 72(2):37–42

Sharma KK, Kapoor M, Kuhad RC (2005) In vivo enzymatic digestion, in vitro xylanase digestion, metabolic analogues, surfactants and polyethylene glycol ameliorate laccase production from *Ganoderma* sp. kk-02. Lett Appl Microbiol 41:24–31. https://doi.org/10.1111/j.1472-765X.2005.01721.x

Shekher R, Sehgal S, Kamthania M, Kumar A (2011) Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzyme Res 2011:217861. https://doi.org/10.4061/2011/217861

Smirnov SA, Koroleva OV, Gavrilova VP, Belova AB, Klyachko NL (2001) Laccases from basidiomycetes: physicochemical characteristics and substrate specificity towards methoxyphenolic compounds. Biochemistry 66:774–779. https://doi.org/10.1023/A:1010216829856

Strakowska J, Błaszczyk L, Chełkowski J (2014) The significance of cellulolytic enzymes produced by *Trichoderma* in opportunistic lifestyle of this fungus. J Basic Microbiol 54(Suppl. 1):S2-13. https://doi.org/10.1002/jobm.201300821

Tamura K, Daniel P, Nicholas P, Glen S, Masatoshi N, Sudhir K (2011) Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Bio Evo 28(10):2731–2739. https://doi.org/10.1093/molbev/msr121

Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140(1):19–26

Tortella G, Durán N, Rubilar O, Parada M, Diez MC (2013) Are white-rot fungi a real biotechnological option for the improvement of environmental health? Critical Rev Biotech 85(51):1–8. https://doi.org/10.3109/07388551.2013.823597

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*–plant–pathogen interactions. Soil Biol Biochem 40:1–10. https://doi.org/10.1016/j.soilbio.2007.07.002

Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimha G (2014) Fungal laccases and their applications in bioremediation. Enzyme Res 163242:1–21. https://doi.org/10.1155/2014/163242

Wang CJ, Thiele S, Bollag JM (2002) Interaction of 2,4,6-trinitrotoluene (TNT) and 4-amino-2,6-dinitrotoluene with humic monomers in the presence of oxidative enzymes. Arch Environ Contam Toxicoly 42:1–8. https://doi.org/10.1007/s002440010284

Warsinke A, Benkert A, Scheller FW (2000) Electrochemical immunoassays. Fresenius J Anal Chem 366:622–634. https://doi.org/10.1007/s002160051557

Wood DA (1980) Production, purification and properties of extracellular laccase of *Agaricus bisporus*. J Gen Microbiol 117:327–338
Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35:7608–7614. https://doi.org/10.1021/bi952971a