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Enhancement of ligninolytic enzyme activities in a *Trametes maxima–Paecilomyces carneus* co-culture: Key factors revealed after screening using a Plackett–Burman experimental design

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ABSTRACT

Background: In the industrial biotechnology, ligninolytic enzymes are produced by single fungal strains. Experimental evidence suggests that co-culture of ligninolytic fungi and filamentous microfungi results in an increase laccase activity. In this topic, only the ascomycete *Trichoderma* spp. has been studied broadly. However, fungal ligninolytic-filamentous microfungi biodiversity interaction in nature is abundant and poorly studied. The enhancement of laccase and manganese peroxidase (MnP) activities of *Trametes maxima* as a function of time inoculation of *Paecilomyces carneus* and under several culture conditions using Plackett–Burman experimental design (PBED) were investigated.
Results: The highest increases of laccase (12,382.5 U/mg protein) and MnP (564.1 U/mg protein) activities were seen in co-cultures I3 and I5, respectively, both at 10 d after inoculation. This level of activity was significantly different from the enzyme activity in non-inoculated *T. maxima* (4881.0 U/mg protein and 291.8 U/mg protein for laccase and MnP, respectively). PBED results showed that laccase was increased ($P < 0.05$) by high levels of glucose, $(NH_4)_2SO_4$ and $MnSO_4$ and low levels of KH_2PO_4, $FeSO_4$ and inoculum ($P < 0.05$). In addition, MnP activity was increased ($P < 0.05$) by high yeast extract, $MgSO_4$, $CaCl_2$ and $MnSO_4$ concentrations.
Conclusions: Interaction between indigenous fungi: *T. maxima–P. carneus* improves laccase and MnP activities. The inoculation time of *P. carneus* on *T. maxima* plays an important role in the laccase and MnP enhancement. The nutritional requirements for enzyme improvement in a co-culture system are different from those required for a monoculture system.

1. Introduction

White-rot fungi can offer a biological and environmentally friendly means of reducing environmental pollution by ligninolytic enzymes, such as laccase (EC 1.10.3.2), manganese peroxidase (MnP) (EC 1.11.1.14) and lignin peroxidase (EC 1.11.1.13), which degrade a wide range of environmental pollutants [1,2,3]. Most of the fungal enzymes in industrial biotechnology today are produced by processes involving single microbial strains [4]. However, constitutive extracelullar enzymes from white-rot fungi are only produced in small amounts, so their use in industrial applications has been limited due to low productivity and high economic cost [5,6]. Chemical induction of laccase and MnP by the addition of compounds related to lignin or lignin derivates, phenolic and aromatic compounds, copper, ethanol and contaminated water has been studied [7,8]. However, chemical inducers are expensive and, in some cases, toxic. Therefore, the search for economical and safe laccase and MnP production methods has been one of the main enzyme research topics in the past few decades [5]. Using co-cultures appears to be more successful than using single microorganism cultures, because of the potential to utilize synergisms between the metabolic pathways of the strains involved in the co-culture [4,9]. Previous studies have shown that co-cultivation of some white-rot fungi, such as *Lentinula edodes*, *Pleutotus ostretus*, *Trametes versicolor* and *Phanerochaete chrysosporium*, with the filamentous fungi,

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Trichoderma spp., resulted in high laccase production [5,10,11,12,13,14]. In these cases, only the interactions with the microfungi *Trichoderma* spp., were studied, which is considered an antagonic fungus against edible mushrooms [13] and a fungus with a biological control potential [15].

The mechanism used to enhance laccase activity of white-rot fungi during co-culture with *Trichoderma* spp. is not known [4,12]. Some authors have suggested that the increased laccase activity of white-rot fungi could be a response against *Trichoderma* spp. attack [5,13], due to the synthesis of lytic enzymes, such as chitinase and β-1,3-glucanases, in the mycoparasitism process [16,17]. This hypothesis has arisen because the increased production of laccase occurred when cultures of white-rot fungi were infected with *Trichoderma* spp. mycelia, which seemed to lead to an increase in the production of laccase and lytic enzymes by *Trichoderma* spp. [13,18]. This occurred during antagonism with white-rot fungi strains, and is mainly associated with host cell degradation. Some authors have suggested that laccase production under *Trichoderma* spp. attack is a defense response by white-rot fungi [17,19].

Co-culture of white-rot fungi with soil microfungi has been little studied. Moreover, the effect of the fungi inoculation time on the development of co-cultures and the optimum culture conditions needed to increase enzyme activities have also not been studied to any great extent. Maximum culture condition can be determinated through experimental designs, one of this is the Placket–Burman experimental design (PBED), which is focused in identifying main effects on efficiency, and it uses few experimental trials for large number of factors [20]. This design has been used to study the influence of laccase activity mainly in monocutures of white-rot fungi [21,22]. Therefore, this study was undertaken in order to evaluate: a) the production of laccase and MnP in co-cultures of the Mexican native white-rot fungus, *Trametes maxima*, and the filamentous soil microfungus, *Paecilomyces carneus*; b) investigate whether the development of the co-cultures, with or without inoculation with the soil microfungus, has an effect on enzyme activity increase and c) investigate different culture conditions using a PBED.

2. Materials and methods

2.1. Isolation of strains

Carpophores of *Trametes* spp. were collected in Vega del Pixquiac, San Ándres Tlalnelhuayocan, Veracruz, Mexico (location: 19°32′21.23″, 97°00′47.29″). In order to obtain the strain of *Trametes* spp., 0.5–1 cm fragments from the carpophores were washed in ethanol (70%) for 1 min, then in sodium hypochlorite (50%) for 3 min and finally in sterile distilled water. The washed and disinfected fragments were placed on potato-dextrose agar (PDA, BD Bioxon, Mexico) plates, supplemented with chloramphenicol (20 mg/L) (Sigma, st. Louis, MO, USA) in order to prevent bacterial contamination, and benomyl (3 mg/L) (Biesterfeld Co., Mexico), which inhibited mold growth. The soil microfungus, *Paecilomyces* spp., was isolated by Heredia and Arias [23] from an andic acrisol soil (texture: loam–silt loam) found on a coffee plantation located in Huatusco, Veracruz, Mexico (location: 19°12′57″, 96°53′7″).

The carpophores of *Trametes* spp. and the strain of *Paecilomyces* spp. were deposited in the herbarium (XAL) and in the Culture Collection of Micromicetos of the Instituto de Ecología A. C. Xalapa, Veracruz, Mexico. The studied strains were maintained and subcultured in PDA.

2.2. Molecular identification

The mycelia of the studied strains was grown in sterile petri dishes containing liquid complete yeast extract medium (standard CYM: dextrose, 20 g; peptone, 2 g; yeast extract, 2 g; $MgSO_4 \times 7\ H_2O$, 0.5 g; KH_2PO_4, 0.46 g; K_2HPO_4, 1 g; distilled water: 1000 mL), and incubated at 25°C. The mycelia were harvested using a sterile spatula, dried by squeeze-blotting between filter papers (Whatman, no. 1), placed in Eppendorf tubes (1.5 mL) and frozen in liquid nitrogen vapor (-130°C). The Eppendorf tubes were then freeze-dried overnight using vacuum apparatus (Labconco, Freezone 4.5, Kansas, Missouri, USA). Genomic DNA extraction and purification of the dried mycelial samples were carried out using Qiagen columns (Qiagen GmbH, Hilden, Germany), according to the methodology developed by Challen et al. [24].

DNA concentration and purity were determined by comparative agarose gel electrophoresis (1.5%), and ethidium bromide staining. PCR amplifications of the ITS1-5.8S-ITS2 region were carried out using the following primers: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [25]. Each amplification reaction (50 μL) contained: the master mix, 25 μL (AmpliTaq Gold PCR kit, Applied Biosystems, Foster, CA, USA), ITS-1 primer (10 μM, 5 μL), ITS-4 primer (10 μM, 5 μL), template DNA (50 ng, 2 μL) and PCR water (13 μL). All amplification reactions were performed using a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster, CA, USA). The temperature cycling protocol consisted of one initial denaturation cycle at 95°C for 1 min, followed by 25 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 5 min and a final cycle of 72°C for 5 min. The amplification products were analyzed by electrophoresis in 1.5% agarose gels, containing ethidium bromide, and purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) [26].

The purified PCR products were sequenced by SeqWright DNA Technology Services (Houston, Texas, USA). Each sample was sequenced in both directions. DNA sequence analyses were performed using the basic sequence alignment BLAST program, run against the NCBI database (www.ncbi.nlm.nih.gov). The Index Fungorum (www.indexfungorum.org) was used as a species authority.

2.3. Selection of the medium for co-culturing

In order to select the best medium for improving laccase and MnP activities by the *T. maxima–P. carneus* co-culture, the following three culture media were tested: Sivakumar et al. [27], Koroljova et al. [28] and Bose et al. [29]. The Sivakumar medium contained (g/L): glucose (20), yeast extract (2.5), KH_2PO_4 (1.0), $(NH_4)_2SO_4$ (0.05), $MgSO_4$ (0.5), $CaCl_2$ (0.01), $FeSO_4$ (0.01), $MnSO_4$ (0.001), $ZnSO_4$ (0.001) and $CuSO_4$ (0.002). The Koroljova medium contained (g/L): glucose (10), peptone (3), KH_2PO_4 (0.6), $ZnSO_4$ (0.001), K_2HPO_4 (0.4), $FeSO_4$ (0.0005), $MnSO_4$ (0.05) and $MgSO_4$ (0.5). Finally the Bose medium contained (g/L): glucose (20), KH_2PO_4 (1.5), $CaCl_2$ (0.37), $ZnSO_4$ (0.3), $MgSO_4$ (0.5), $(NH_4)_2SO_4$ (1) yeast extract (5) and peptone (2). All these culture media were adjusted to pH 4.5 (all reagents were purchased from J. T. Baker, Center Valley, PA, USA). Four mycelial disks (5 mm Ø) were taken from the active borders of the *T. maxima* PDA cultures (7 d old) and transferred to Erlenmeyer flasks (250 mL) containing 120 mL of one of the culture media. The flasks were incubated on a rotating shaker (120 rpm) at 25 ± 1°C for 12 d. There were four replicates for each evaluated culture medium. Culture samples were collected at regular intervals of 24 h and centrifuged (10,000 × *g*, for 10 min), the supernatant was used for enzyme activities and protein content measurements.

2.4. Enzyme and protein assay

Laccase activity was determined by measuring the oxidation of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at room temperature (25–27°C) in a reaction mixture containing 5.0 mM ABTS (Sigma, St. Louis, MO, USA) and 100 mM acetate buffer (pH 4.5). Absorbance changes in the presence of the enzyme were monitored for 5 min at 420 nm ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) [30]. One unit of laccase activity (U) was defined as the amount of enzyme required to oxidize 1 μmol ABTS/mL/min, laccase activities in the extract were expressed as a specific enzyme activity (U/mg protein).

MnP activity was measured according to Glenn and Gold [31]. The reaction mixture contained 25 mM sodium lactate (50 μL), 2 mM

$MnSO_4$ (50 μL), 0.1% egg albumin (50 μL), 0.2% phenol red (50 μL) and 0.7 mL culture filtrate in 20 mM lactate-succinate buffer (50 μL, pH 4.5). The reaction was started with the addition of 2 mM H_2O_2 (50 μL) in a total volume of 1 mL and stopped after 5 min by the addition of NaOH (50 μL, 2 N). The absorption at 610 nm was measured against a blank without any manganese in the reaction mixture. The molar extinction coefficient of the oxidized phenol red is 22 mM^{-1} cm^{-1}. One unit of MnP activity (U) is the amount of enzyme needed to form 1 μmol of oxidized phenol red/mL/min, MnP activities in the extract were expressed as specific enzyme activity (U/mg protein).

The protein content of the culture extracts was estimated according to the method used by Bradford [32] and BSA was used as a standard at known concentrations (0.0062, 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/mL). The standard curve was: $y = 0.1615c - 0.0125$ ($y = OD_{595}$, c = protein concentration in mg/mL, $R^2 = 0.998$).

2.5. Establishment of the co-culture

To estimate the effect of the *P. carneus* inoculation time on laccase and MnP activities, co-culturing with simultaneous (I0) and outdate inoculation were performed. For co-cultures with outdate inoculations, the mixed cultures were established by adding the filamentous microfungus *P. carneus* after 3 d (I3) and 5 d (I5) of *T. maxima* growth. Two monocultures: *T. maxima* and *P. carneus* were used as a control. The co-cultures were established in Erlenmeyer flasks (250 mL) with 120 mL of Sivakumar culture medium, which was the medium that was finally selected after testing. The *T. maxima* and *P. carneus* were inoculated with four mycelial agar disks (5 mm Ø) of active mycelia. All treatments, including the controls, were replicated four times. The co-cultures were incubated for 12 d under the same conditions mentioned previously. Laccase and MnP activities and protein content were evaluated daily.

2.6. Screening of important factors in enzyme activities under PBED

The PBED is a statistical technique used for screening experimental factors [20]. In this study the technique was used to identify the significant factors that had an impact on the laccase and MnP activities in *T. maxima–P. carneus* co-culture. The statistical screening was based on the main effects of the experimental factors, but not on their interaction effects. The Sivakumar medium was modified to test the influence of different factors for enzyme activities. Eleven factors were assessed: the components of Sivakumar medium (10 nutrients) and the inoculum amount (mycelial agar disks of *T. maxima*) (Table 1). Each factor was tested at two levels (coded): high level (+1) and a low level (-1), and four central points (0) were screened by running 16 experiments, as shown in Table 2. The factors which were significant at the 5% level ($P < 0.05$) from the regression analysis were considered to have a high impact on laccase and MnP activities. The experimental data were fitted according to [Equation 1], which includes the individual effects of each variable.

$$Y = \beta_0 + \sum \beta_i X_i (i = 1, 2, 3 \cdots\cdots k) \quad \text{[Equation 1]}$$

where:

Y response (enzyme activity),
β_0 model intercept,
β_i estimated variable.

Table 1
Variables and their levels for laccase and MnP activities under a PBED.

Code	Variable	Low level (-1)	Level 0	High level (+1)
G	Glucose (g/L)	10.0	20.0	30.0
Ye	Yeast extract (g/L)	1.25	2.5	3.75
K	KH_2PO_4 (g/L)	0.5	1.0	1.5
N	$(NH_4)_2SO_4$ (g/L)	0.025	0.05	0.075
Mg	$MgSO_4$ (g/L)	0.25	0.5	0.75
Ca	$CaCl_2$ (g/L)	0.005	0.01	0.015
Fe	$FeSO_4$ (g/L)	0.005	0.01	0.015
Mn	$MnSO_4$ (g/L)	0.0005	0.001	0.0015
Zn	$ZnSO_4$ (g/L)	0.0005	0.001	0.0015
Cu	$CuSO_4$ (g/L)	0.001	0.002	0.003
I	Inoculum (mycelial disks)	2.0	4.0	6.0

2.7. Data analysis

The selection of culture media and co-cultures was performed using four replicates. The differences among treatments were evaluated by analysis of variance (ANOVA) and a least significant difference (LSD) means comparison ($P = 0.05$) using GraphPad InStat. The results of the PBED were transformed by natural logarithm and analyzed using the software Design-Expert, ver. 8.1, Stat-Ease Inc., Minneapolis, MN, USA.

3. Results

3.1. Isolation and molecular identification

Fungi were isolated in Veracruz State, Mexico and identified at the molecular level. According to the sequence analysis, the ligninolytic fungus was identified as *T. maxima* (Mont.) (A. David & Rajchenb) with 99% similarity to the accessions: **JN164957.1** and **JN164932.1** [33]. In the same manner, the soil microfungus was identified as *P. carneus* (Duché & R. Heim) A.H.S. Br. & G. Sm. with 98% similarity to the accessions: **AB258369.1** [34], **DQ888728.1** [35] and **FN394726.1** [36]. Both DNA sequences were deposited in the GenBank under the accession numbers: **HF947516** (*T. maxima*) and **HF947521** (*P. carneus*).

3.2. Selection of the medium for co-culturing

Laccase and MnP activities in *T. maxima* were assessed in three culture media. The greatest laccase activity was found in Sivakumar medium (4560.9 U/mg protein) after 7 d of growth and was significantly ($P = 0.005$) higher than the Koroljova (302.2 U/mg protein) and Bose (12.1 U/mg protein) culture media. MnP activity was enhanced significantly ($P = 0.0003$) in Sivakumar medium (477.9 U/mg protein) on the 7th d of culturing and was higher than in the Bose (1.3 U/mg protein) and Koroljova (37.3 U/mg protein) culture media. Therefore, the Sivakumar culture medium was selected for use in the subsequent experiments.

3.3. Enzyme activity of T. maxima–P. carneus co-culture

The time at which *T. maxima* was inoculated with *P. carneus* had a significant effect on laccase activity (Table 3). The best time for inoculation with *P. carneus* was 3 d after *T. maxima* was established (I3). It had a maximum activity (12,382.5 U/mg protein) that was higher ($P = 0.0001$) than the monoculture (488.0 U/mg protein) and co-cultures: I0 (573.3 U/mg protein) and I5 (5,944.8 U/mg protein), on the 10th d.

The MnP activity showed a maximum activity (564.1 U/mg protein) on the 10th d in co-culture I5, which was higher ($P = 0.002$) than the monoculture (291.8 U/mg protein), and the co-cultures: I0 (9.0 U/mg protein) and I3 (417.4 U/mg protein). With regard to MnP maximum activity, 5 d after *T. maxima* establishment was the best time to inoculate *P. carneus* in the co-culture. No laccase activity was detected in *P. carneus* culture.

Table 2
PBED matrix for evaluating factors influencing laccase and MnP activities (U/mg protein) in the *T. maxima-P. carneus* co-culture.

Order	Run	G	Ye	K	N	Mg	Ca	Fe	Mn	Zn	Cu	I	Laccase		MnP	
													Observed	Predicted	Observed	Predicted
1	8	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	33,331.4	33,008.9	638.4	598.3
2	10	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	1785.9	1768.6	434.8	407.5
3	15	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	16,022.9	15,867.9	260.8	244.4
4	13	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	25,461.7	25,215.3	328.8	308.1
5	4	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	13,374.0	13,244.6	220.9	207.0
6	14	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	7037.8	6969.7	50.7	47.5
7	1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	14,621.2	14,479.7	216.5	202.8
8	5	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	16,625.1	16,464.3	371.8	348.4
9	2	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	12,513.7	12,392.6	211.5	198.2
10	6	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	13,098.5	12,971.8	177.5	166.3
11	12	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	13,085.8	12,959.2	84.1	78.8
12	7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8082.8	8004.6	88.4	82.8
13	3	0	0	0	0	0	0	0	0	0	0	0	12,113.5	11,960.7	155.3	193.4
14	16	0	0	0	0	0	0	0	0	0	0	0	11,942.8	11,960.7	147.9	193.4
15	11	0	0	0	0	0	0	0	0	0	0	0	13,547.5	11,960.7	217.9	193.4
16	9	0	0	0	0	0	0	0	0	0	0	0	9292.4	11,960.7	128.6	193.4

3.4. Screening of important factors in enzyme activities under PBED

The data in Table 2 indicate that, there was a wide variation in laccase and MnP activities during the 16 runs. This variation reflects the importance of medium optimization if high enzyme activity yields are to be realized. During the kinetics, the highest laccase activity (33,331.4 U/mg protein) was seen in the 8th trial run, whereas the lowest activity (1,785 U/mg protein) was seen in the 10th trial run. There was a 1.8-fold increase in laccase activity in the 8th trial run compared to the central points (runs: 3, 16, 11 and 9) and was improved by high glucose (30 g/L), $(NH_4)_2SO_4$ (0.075 g/L) and $MnSO_4$ (0.0015 g/L) concentrations and low KH_2PO_4 (0.5 g/L), $FeSO_4$ (0.005 g/L) concentrations and inoculum amount (two mycelial disks) (Fig. 1).

The *P*-value was used to evaluate the significant factors and parameters when the confidence levels were greater than 95%. Glucose ($P = 0.006$), KH_2PO_4 ($P = 0.02$), $(NH_4)_2SO_4$ ($P = 0.01$), $FeSO_4$ ($P = 0.02$), $MnSO_4$ ($P = 0.01$) and inoculum amount ($P = 0.01$) had significant influences on laccase activity (Table 4). The polynomial equation for laccase activity is represented by [Equation 2]. The significance of [Equation 2] was checked using a *F*-test and the value was highly significant [(*P*-value > *F*) = 0.017]. Coefficient R^2 indicates the variability of the predicted response compared to the experimental results (Table 2). The predicted response in the model becomes more reliable as R^2 approaches one. The R^2 of the model [Equation 2] was 0.986, which indicated that 98.6% of the variability in the experimental data could be explained by the estimated model. Furthermore, the adjusted determination coefficient (Adj. R^2) was high (95%) and that confirmed that the model was highly significant. Additionally, this model had a low coefficient of variation (1.51%).

$$\begin{aligned}\text{Ln(laccase)} = {} & 9.39 + 0.32\,\text{G} + 0.048\,\text{Ye-}0.22\,\text{K} + 0.28\,\text{N} \\ & + 0.037\,\text{Mg-}0.12\,\text{Ca-}0.20\,\text{Fe} + 0.29\text{Mn} \\ & + 0.12\,\text{Zn} + 0.15\,\text{Cu-}0.30\,\text{I}\end{aligned} \quad \text{[Equation 2]}$$

Regarding to the MnP activity, the maximum value was seen in the 8th trial run (638.4 U/mg protein), as was found for the laccase activity. However, the lowest value was found in the 14th trial run (50.7 U/mg protein). There was a 2.9-fold increase in MnP activity in the 8th trial run compared to the central points (runs: 3, 16, 11 and 9). According to the Pareto chart (Fig. 2), the MnP activity was improved by high concentrations of yeast extract (3.75 g/L), $MgSO_4$ (0.75 g/L), $CaCl_2$ (0.015 g/L) and $MnSO_4$ (0.0015 g/L).

The yeast extract ($P = 0.01$), $MgSO_4$ ($P = 0.03$), $CaCl_2$ ($P = 0.04$) and $MnSO_4$ ($P = 0.04$) had significant effects on MnP activity (Table 5). The polynomial [Equation 3] was obtained from the multiple regression analysis and describes the effect of the culture conditions on MnP activity. The statistical significance of [Equation 3] was checked

Table 3
Kinetic of laccase and MnP specific activity (U/mg protein) in *T. maxima–P. carneus* co-cultures, inoculated at 0, 3 and 5 d.

Culture Time (d)	Laccase activity (U/mg protein)				MnP activity (U/mg protein)			
	T. maxima monoculture	Co-culture I0 (0 d)	Co-culture I3 (3 d)	Co-culture I5 (5 d)	*T. maxima* monoculture	Co-culture I0 (0 d)	Co-culture I3 (3 d)	Co-culture I5 (5 d)
1	6.35 b	28.97 **a**			n.d.	n.d.		
2	6.12 b	75.82 **a**			15.75 a	13.02 a		
3	5.45 b	298.10 **a**	P. c. inoculation		18.87 b	141.35 **a**	P. c. inoculation	
4	62.6 b	1107.43 **a**	184.42b		16.19 b	43.96 b	118.95 **a**	
5	673.82 b	2301.75 **a**	1648.88 **a**	P. c. inoculation	16.28 c	106.44 b	242.81 **a**	P. c. inoculation
6	3962.43 b	3950.43 b	6724.65 **a**	1653.30 c	89.39 b	29.75 c	306.78 **a**	n.d.
7	4404.00 b	3860.92 b	8290.23 **a**	5118.93 b	116.97 b	118.24 b	207.10 **a**	117.64 b
8	4218.82 b	1073.90 c	6841.07 **a**	6183.57 ab	368.63 a	81.83 b	487.26 a	401.98 a
9	4127.48 b	832.10 c	8574.45 **a**	4811.15 b	460.97 a	23.99 b	552.51 a	444.26 a
10	4881.07 b	573.38 c	12,382.5 **a**	5944.85 b	291.80 b	9.07 c	417.43 b	564.12 **a**
11	4392.95 b	544.80 c	8949.80 **a**	3901.63 b	309.88 a	n.d.	400.05 a	294.90 a
12	4506.93 b	431.17 c	7243.88 **a**	4636.12 b	248.66 a	n.d.	233.57 a	220.63 a

Bold script indicates values significantly different from *T. maxima* monoculture (LSD, $P < 0.05$); n.d. = no detected.
Co-culture I0 = *T. maxima* cultures inoculated at the same time with *P. carneus*.
Co-culture I3 = *T. maxima* cultures inoculated with *P. carneus* 3 d after settling *T. maxima* in the culture medium.
Co-culture I5 = *T. maxima* cultures inoculated with *P. carneus* 5 d after settling *T. maxima* in the culture medium.

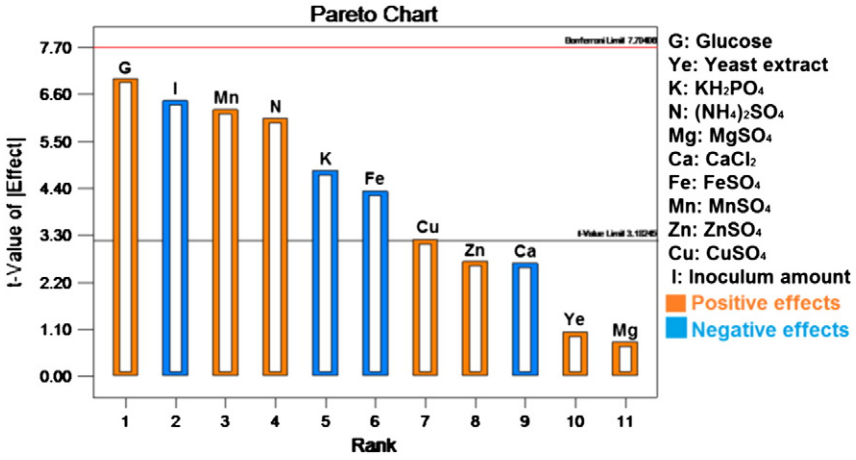

Fig. 1. Pareto chart of eleven-factors standard effects on laccase activity in *T. maxima*–*P. carneus* co-culture.

using a F-test [(*P*-value > F) = 0.043] and the R^2 and Adj. R^2 were close to one (R^2 = 0.94 and Adj. R^2 = 0.79).

$$\begin{aligned}\mathrm{Ln(MnP)} = {} & 5.27 + 0.19\,\mathrm{G} + 0.47\,\mathrm{Ye} + 0.003\,\mathrm{K} - 0.10\,\mathrm{N} \\ & + 0.26\,\mathrm{Mg} + 0.23\,\mathrm{Ca} + 0.019\,\mathrm{Fe} \\ & + 0.21\,\mathrm{Mn} - 0.19\,\mathrm{Zn} - 0.048\,\mathrm{Cu} - 0.18\mathrm{I}\end{aligned} \qquad \text{[Equation 3]}$$

4. Discussion

Enzymatic activity in the genus *Trametes* has been widely studied. However *T. maxima*, although in the genus *Trametes*, has not been studied to any great degree. Laccase and MnP activities in *T. maxima* were influenced by the culture media; the best culture medium was Sivakumar, which achieved maximum laccase and MnP activities on the 7th d of incubation. In contrast, the Bose medium was the worst. The Bose medium was different from Sivakumar medium because it lacked $CuSO_4$, $FeSO_4$ and $MnSO_4$; it is well known that these metal ions play an important role in laccase synthesis and activity [37]. Kumarasamy et al. [38] suggested that low amounts (<1 mM) of Cu^{2+} and Fe^{2+}, like those present in the Sivakumar medium, enhance laccase activity in white-rot fungi. Moreover, the Sivakumar medium showed a 14- and 12-fold increase in laccase and MnP activities, respectively compared with the Koroljova medium. This significant difference could be due to the absence of Cu^{2+} and low amounts of glucose (10 g/L) and Fe^{2+} (0.0005 g/L) in the Koroljova medium.

The laccase activity of *T. maxima* (4,560.9 U/mg protein or 16,433.9 U/L) was higher than has been seen in other strains of *T. maxima* previously studied by Songulashvili et al. [39] and Elisashvili et al. [37] (13,500 U/L and 8600 U/L, respectively). Other species of *Trametes* have shown a low laccase activity compared with *T. maxima*, for example: *T. versicolor* [40], *Trametes pubescens* [41], *Trametes ochracea* [37] and *Trametes trogii* [42] (1200 U/L, 750 U/L, 3900 U/L and 14,200 U/L, respectively).

The highest MnP activity was found in Sivakumar culture medium, this could be due to low yeast extract and Mn^{2+} concentrations; previously Kamitsuji et al. [43] reported that a high MnP activity in ligninolytic fungi occurred when the yeast extract (2 g/L) concentration in the culture medium was low. In other studies, Songulashvili et al. [39] found a low MnP activity (460 U/L) in *T. maxima* when using different waste/by-products from the food industry to stimulate MnP activity and Elisashvili et al. [37] found a low MnP activity (610 U/L) in *T. maxima* when using a culture medium supplemented with mandarin peel. MnP activity in *T. maxima* (477.9 U/mg protein or 1700.8 U/L) was higher in this study than has been reported in other *Trametes* spp. such as, *T. versicolor* [44], *Trametes zonata* [39], and *Trametes unicolor* [37] with 150, 360, 420 and 590 U/L, respectively.

The ligninolytic enzymes in *Trametes* spp. are highly regulated by several nutrients, such as: glucose, nitrogen and metal ions (Cu^{2+}, Mg^{2+}) [43], and their production is also affected by many typical fermentation factors, such as: medium composition and type, concentrations of the carbon and nitrogen sources, pH, temperature and the presence of inducers (2,5-xylidine, ethanol, veratryl alcohol, polychlorinated biphenyls, etc.) [40]. However, the biology of the fungus is the most important factor in ligninolytic enzyme production because ligninolytic metabolism is strain-dependent. This means that the selection of new strains with significant laccase or MnP activity is possible [4].

Recently there has been an increased interest in producing white-rot fungal enzymes in co-culture with microorganism, specifically with soil microfungi [4]. However, few studies have been undertaken [9,12,13,18,19]. Therefore, this study investigated the improvement of

Table 4
Analysis of variance for laccase activity.

Source	Sum of squares	d.f.	Mean square	*F*-ratio	*P-value*	Percentage *P* (%)
Glucose (G)	1.23	1	1.23	48.72	0.006	20.51
Yeast extract (Ye)	0.027	1	0.027	1.07	0.38	0.45
KH_2PO_4 (K)	0.59	1	0.59	23.28	0.02	9.80
$(NH_4)_2SO_4$ (N)	0.92	1	0.92	36.47	0.01	15.36
$MgSO_4$ (Mg)	0.017	1	0.017	0.65	0.49	0.28
$CaCl_2$ (Ca)	0.18	1	0.18	7.05	0.08	2.97
$FeSO_4$ (Fe)	0.48	1	0.48	18.83	0.02	7.93
$MnSO_4$ (Mn)	0.99	1	0.99	39.03	0.01	16.43
$ZnSO_4$ (Zn)	0.18	1	0.18	7.26	0.07	3.06
$CuSO_4$ (Cu)	0.26	1	0.26	10.30	0.05	4.34
Inoculum (I)	1.06	1	1.06	41.67	0.01	17.54
Residual	0.076	3	0.025			1.33
Total (corrected)	6.02	15				100

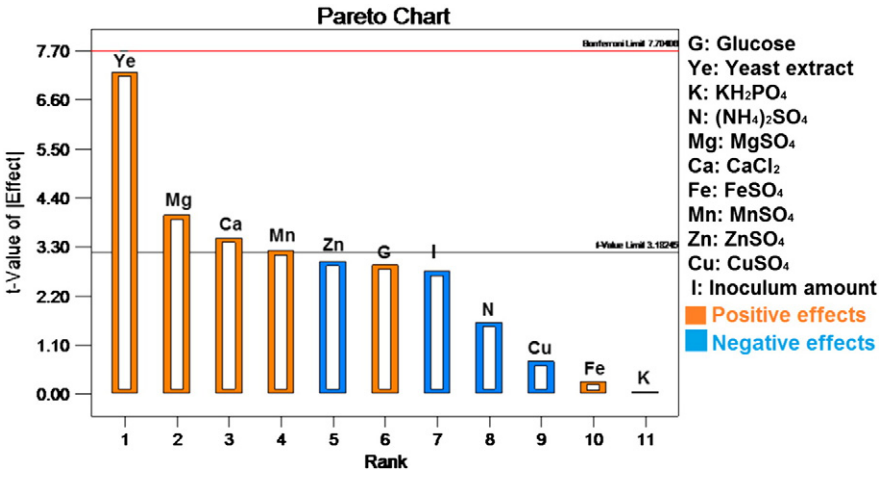

Fig. 2. Pareto chart of eleven-factors standard effects on MnP activity of the *T. maxima–P. carneus* co-culture.

laccase and MnP activities in *T. maxima* by the inoculation of *P. carneus* in a co-culture system. The results showed that laccase and MnP activities were affected by the time of inoculation of *P. carneus* on the *T. maxima* cultures. Inoculation of both fungi simultaneously (I0) only enhanced laccase activity to a high extent during the first five days. On the 6th and 7th d, the laccase activity did not decrease, but was statistically lower than the laccase activity found in the control (*T. maxima* monoculture).

With regard to the MnP activity in the co-culture I0, it was higher only on the first 3 d after inoculation. Thereafter, only low MnP activities were detected. This effect could be due to increased biomass accumulation and nutrient consumption in co-culture I0, compared to the monoculture. A similar effect was described by Flores et al. [14] in co-cultures of *Pleurotus ostreatus* with *Trichoderma viride* when they were simultaneously inoculated.

The greatest increase in enzyme activity was when *T. maxima* were inoculated with *P. carneus* on the third day of growth (co-culture I3). The laccase activity was significantly higher in comparison to the monoculture from the 5th to the 12th d and the maximum activity was found on the 10th d when the activity was 1.5 times higher than the *T. maxima* monoculture. The MnP activity increased between d 4th–7th of incubation. On the other days, the MnP activity in co-culture I3 was less than or equal to the control. Co-culture I5 did not induce laccase activity and only on the 10th d did the MnP activity increase by 0.9 times compared to the control. Laccase increases caused by *P. carneus–T. maxima* co-culture I3 lasted for 7 d, which was similar to previous results reported by Baldrian [12], who found laccase increases after 3, 8, 10, 12, and 14 d for *T. versicolor* in co-culture with *Trichoderma harzianum*, *Acremonium sphaerospermum*, *Fusarium reticulatum*, *Humicola grisea* and *Penicillium rugulosum*, respectively. The inoculation of the microfungi was made on the 11th d of *T. versicolor* establishment and the increase varied from 1.5 to 49 times, compared to the control. Despite this increase, the levels of laccase activity in these co-cultures (12.0–223.2 U/L) were lower than that produced by *T. maxima–P. carneus* co-culture I3 (12,382.5 U/mg protein or 27,033.7 U/L).

The use of co-cultures in laccase activity induction is an environmentally safe and low cost strategy. Several attempts have been made to increase laccase activity by co-culturing white-rot fungi with an antogonic fungi. The laccase activities of *L. edodes* [10], *T. versicolor* [12], *Pleurotus eryngii* [45], *P. ostreatus* [5,46], *Pleurotus pulmonarius*, *Pleurotus djamor* [13] and *Trametes* spp. [19] were improved when they were co-cultured with *Trichoderma* spp. However, the laccase yields in these studies were relatively low in comparison with those obtained in this study.

The induction of MnP in co-culture systems has been less studied than laccase. Only Chi et al. [47] and Qi-He et al. [45] have found MnP induction in *P. ostreatus–Ceriporiopsis subvermispora* (7.3 times increase, 250 nkat/L) and *P. ostreatus–Phlebia radiata* (1 times increase, 800 nkat/L) co-cultures, respectively. However the fungi used in their co-cultures were not soil microfungi, which probably led to the low MnP induction. The interactions between different microorganism play a critical role in co-cultures because cell growth by one species could enhance or inhibit the enzyme activities of the other strain present in the medium [19]. Therefore, it is necessary to study other genera and species of soil microfungi in co-culture systems in order to find those that are able to induce laccase activity in white-rot fungi.

The PBED is a statistical method used to select culture parameters and it has been proved to be a powerful and useful tool in biotechnology [21,22]. This study has undertaken the first screening of important factors affecting laccase and MnP induction in a co-culture system. The main effects by 11 independent factors were screened in co-culture I3. The results of the PBED experiment demonstrated that glucose,

Table 5
Analysis of variance for MnP activity.

Source	Sum of squares	d.f.	Mean square	*F*-ratio	*P-value*	Percentage *P* (%)
Glucose (G)	0.42	1	0.42	8.43	0.06	6.67
Yeast extract (Ye)	2.61	1	2.61	52.25	0.01	41.34
KH_2PO_4 (K)	1.56E^4	1	1.56E^4	0.003	0.96	0.002
$(NH_4)_2SO_4$ (N)	0.13	1	0.13	2.60	0.21	2.06
$MgSO_4$ (Mg)	0.81	1	0.81	16.14	0.03	12.77
$CaCl_2$ (Ca)	0.62	1	0.62	12.32	0.04	9.75
$FeSO_4$ (Fe)	4.15E^3	1	4.15E^3	0.083	0.79	0.07
$MnSO_4$ (Mn)	0.52	1	0.52	10.43	0.04	8.25
$ZnSO_4$ (Zn)	0.45	1	0.45	8.90	0.06	7.04
$CuSO_4$ (Cu)	0.027	1	0.027	0.55	0.51	0.43
Inoculum (I)	0.38	1	0.38	7.65	0.07	6.06
Residual	0.15	3	0.050			5.57
Total (corrected)	6.32	15				100.00

KH_2PO_4, $(NH_4)_2SO_4$, $FeSO_4$, $MnSO_4$ concentrations and inoculum amount had significant effects on laccase activity in the co-cultures. In order of significance for the variables affecting laccase activity, the glucose and the inoculum amount had the most significant effect on laccase activity in *T. maxima–P. carneus* co-cultures according to the $P > F$ values.

The laccase yield slightly increased as the glucose concentration rose in the culture medium and the yield slightly increased as the inoculum amount decreased. Some authors suggest that the high yields of laccase in white-rot fungi occur when a high concentration of glucose is used as a carbon source [48,49,50]. In addition, Jang et al. [51] suggested that the optimum amount of glucose required for maximum laccase production by *Trametes* spp. (monoculture) in a submerged culture was 20 g/L of glucose. Mikiashvilil et al. [48] reported that 20 g/L glucose was the optimum carbon resource for laccase production in a monoculture of *T. versicolor*, which was similar to the amount used in this study. However, Pazarlioglu et al. [52] found a low laccase activity in *T. versicolor* during submerged fermentation using 1% glucose (10 g/L), but they used a single monoculture and the laccase activity was low (3,000 U/L) compared with the *T. maxima* used in this study (4,560.9 U/mg protein or 16,433.9 U/L).

The low inoculum amount (two mycelial disks) enhanced laccase activity in *T. maxima* after co-culturing with *P. carneus*. This result is different from that obtained by Nandal et al. [53], who reported that laccase activity in *Coriolopsis caperata* increased as the inoculum concentration rose. It is important to consider that there are many ways to prepare the inoculum for the production of laccase. These include: spore suspension, mycelium, homogenized mycelium and fungal colonized agar plugs [12,13,21], and there are no general recommendations with regard to the best inoculation method. Dekker et al. [54] suggests that the use of mycelial disks agar induced fungal growth and enhanced laccase production. According to our PBED results, the amount of mycelial disks used can affect enzyme activity in co-cultures.

$CuSO_4$ is a metal ion that is necessary for the synthesis and induction of laccase by white-rot fungi [37,40]. However in *T. maxima–P. carneus* co-cultures, it seems that it is not a key factor, because it's *P*-value was within specified limits ($P > F\text{-}value = 0.05$) and its percentage *P* was low at 4.3% (Table 3). We suggest that the increase in laccase activity is due to the metabolites and enzymes produced by *P. carneus* and not by the $CuSO_4$ in the culture medium. In addition, the amount of $CuSO_4$ present in the Sivakumar culture medium was lower (0.06 mM) than that usually used to induce laccase activity (>1.5 mM) [50].

According to the ANOVA, the most significant factor for MnP activity was yeast extract concentration, with a $P > F\text{-}value$ of 0.005 and a percentage *P* of 41.3 (Table 5). The importance of the yeast extract concentration for MnP activity by white-rot fungi has been previously reported by Mikiashvilil et al. [48]. In a previous study, Kamitsuji et al. [43] observed that the highest MnP activity from *P. ostreatus* was after 8 d of culture in a peptone–glucose–yeast extract medium (0.2 g/L of yeast extract). This amount was lower than that used in the Sivakumar medium, so the high yeast extract levels used in this study could explain the high MnP activity in *T. maxima* seen in this study.

Regarding to the $CaCl_2$ its importance in laccase activity has been described previously [55], but not its importance in MnP activity. According to our PBED results, $CaCl_2$ is an essential factor because there was a slight increase in MnP activity as $CaCl_2$ levels rose in the culture medium. The function of $CaCl_2$ in the culture medium is to maintain protein structures and stabilize the activities of several enzymes [56]. Another factor affecting MnP activity was the manganese (Mn) concentration, because Mn is fundamental to the catalytic activity of MnP during the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2. In this study, the highest MnP activity was found when the amount of Mn^{2+} present was lowest (8th trial run). The amount of Mn^{2+} added, in the form of $MnSO_4$, was 3.3 μM (0.0005 g/L). This metallic ion is essential for the synthesis of MnP by white-rot fungi. Kamitsuji et al. [43] did not find any MnP activity in *P. ostreatus* in the glucose–yeast extract and peptone–glucose–yeast extract media without the addition of $MnSO_4$, but when $MnSO_4$ was added, at a concentration of 270 μM (0.04 g/L), high MnP activity was seen (800 U/L). However, this MnP activity by *P. ostreatus* was lower than the activity seen in this study using a *T. maxima–P. carneus* co-culture (564.12 U/mg protein or 1225.4 U/L).

The laccase and MnP activities in the genus *Trametes* depends on the physiological, nutritional and biochemical nature of the species used, and the strain of the species chosen [37]. However, this study demonstrates that both enzymes can be induced by the presence of soil microfungi, such as *P. carneus*, and the optimal requirements for enzyme induction in a co-culture system may be different from those required for a monoculture system. Therefore it is necessary to elucidate the mechanism used to increase enzymatic activity in a *T. maxima–P. carneus* co-culture system and to optimize enzyme production using the most appropriate experimental design, based in the results of this study. Over all it is concluded that the interaction in liquid fermentation, between indigenous *T. maxima–P. carneus* in co-culture improves laccase and MnP activities. Both the chemical composition of the medium and the timing of when the *T. maxima* culture is inoculated with the soil microfungus (*P. carneus*) are important factors for improving enzymatic yield, and finally these results give a basis for further studies into large scale fermentation and production of laccase and MnP in a co-culture system.

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Author contributions

Proposed the theoretical frame: WCC, GHA, RRV; Conceived and designed experiments: WCC, GHA, RRV; Contributed reagents/materials/analysis tools: GHA, RRV, DMC; Wrote the paper: WCC, GHA, RRV; Performed the experiments: WCC; Analyzed the data: WCC, RRV, DMC.

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