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SOME FACTORS INFLUECING *TRICHODERMA* LACCASE PRODUCTION IN SUBMERGED CULTURE

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Abstract

The main goal of this study was to optimise the laccase production on laboratory scale, under submerged cultivation, in synthetic media. For this purpose, two Trichoderma spp. strains were isolated from different soil and compost samples. These isolates were then inoculated onto culture media under different pH conditions (4, 5, 6, 7 and 8). To induce laccase production, the culture media were supplemented with two different inductors such as guaiacol 0.03% and 2 - 2.5mM CuSO4. In the first 48 hours of submerged fungal culture, the media with initial pH values in the neutral-basic range (7 and 8), recorded drastically low of pH (between 4 and 5), which suggests an acidification of the culture medium. After 14 days from the experiment initiation, Trichoderma TdMI2 isolate grown on media supplemented with guaiacol, at an initial pH value of 8, had a maximum production of fungal biomass and recorded a slight increase in enzymatic activity (from 0.5 to 0 , 6 U / ml). Between the two isolates of Trichoderma spp - TdCP and TdMI2 - cultured on media supplemented with 2.5 mM CuSO4 as inducer for laccase, only TdMI2 had an enzymatic activity of 0.62 U / ml after 3 days of culture.

Key words*: Trichoderma spp., laccase activity, acidification, guaiacol, copper sulphate inducer.*

INTRODUCTION

The laccase enzyme (benzenediol: oxidoreductase) is an oxidoreductase capable of oxidizing phenolic and non-phenolic compounds that have been considered an essential tool in the fields currently known as white biotechnology and green chemistry. Laccase is one of the most robust biocatalysts due to its wide applications in different environmental processes such as detecting and treating chemical pollutants and dyes, pharmaceutical removal or lignin degradation for biofuel production (Popa et al., 2018; Burlacu et al., 2018; Alvarado-Ramirez et al., 2021). Laccases are produced by a wide range of organisms, including fungi, plants, bacteria, or insects (Albu et al., 2019).

Most of the studied laccases are of fungal origin, especially from white rot fungi, *Tramates versicolor, Pycnoporus sanguineus*, and *Trichoderma* spp. (Ranimol et al., 2018). Among *Trichoderma,* different species and isolates of diverse origin, were tested for their laccase production, purification and biotechnological application (Kalra et al., 2013; Ahmed et al., 2015). Most of the application relates to textile dye decolorisation and make use of *T. harzainum* (Bagewadi et al., 2017; Ranimol et al., 2018). Other studied species are *Trichoderma atroviride* and *Trichoderma longibrachiatum* (Bagewadi et al., 2017), while very recent publications refers to the use of *Trichoderma asperellum* (Shanmugam et al., 2020). Basically, this fungus may produce lacasse without any inductor; however, some authors reported that the addition of guaiacol or copper may increase the laccase activity under different pH and temperature conditions (Singh et al., 2014; Bagewadi et al, 2017; Ranimol et al., 2018).

Previously, our team has performed a screening for laccase production among different macro an micromycetes. As results, two strains of *Trichoderma* spp., one variety of *Pleurotus ostreatus* and two of *Agaricus bisporus* originating from supermarket wastes, were detected, by on-plate assay, as important

laccase producers, in the presence of different guaiacol concentrations (Albu et al., 2020).
The present work deals with the optimisation of laccase production under submerged cultivation conditions, in synthetic media, targeting the two isolates of *Trichoderma* spp. As fermentation factors, were chosen the enzyme inductors (guaiacol and copper sulphate) and the pH variation.

MATERIALS AND METHODS

Microbial strains. Two isolates of *Trichoderma* spp., TdCP (composting source) and TdMI2 (soil source) were used for the experiments. The fungal isolates were selected as a result of the agar-plate screening where they proved to be good producers of laccase. Both species were identified by sequencing as being *Trichoderma asperellum* (data not published).

Media. The fungal strains were cultivated 10 days at 28ºC for the spore/inoculum production on PDA (Potato Dextrose Agar) from VWR Chemical, UK. The laccase production was performed on PDB (Potato Dextrose Broth; VWR Chemicals, UK).

Experimental variants for laccase production. Two different inductors (guaiacol and cooper sulphate) were employed in the experiments conducted at different pH values (4, 5, 6, 7, and 8).
The guaiacol test was performed only for the TdMI2 isolate. After autoclaving, the PDB culture medium was supplemented with 30 µL guaiacol. To initiate the experimental variants, 100 ml of PDM was used, distributed in 250 ml Erlenmeyer flasks. The inoculation was done with a suspension of 10^6 spores/mL and the cultures were shanked at 120 rpm during 15 days at 28ºC. During incubation, variations in the pH of the culture medium were periodically monitored.
In the second test, for inducing laccase production by fungal cultures was added as inducer CuSO4 at different concentrations (2-2.5 mM). For this test were used both *Trichoderma* isolates (TdCP and TdMI2).
For each test, one flask containing 100 ml medium was taken as a sample periodically every two days, and filtrated using filter paper No. 1 to determine the growth dry weight (g/100 ml) and laccase enzyme activity in filtrate.

Assay for laccase activity
The laccase activity was determined by using guaiacol as substrate (Kalra et al., 2013). Oxidation of guaiacol by laccase is used to measure enzyme activity. The reaction mixture contained: 1 ml guaiacol (2 mM), 3 ml sodium acetate buffer (10 mM) and 1 ml enzyme source (fungal supernatant). The mixture was incubated at 30°C for 15 min and the absorbance was read at 450 nm using UV spectrophotometer. One unit of laccase activity (U) was defined as the amount of the enzyme required to oxidize 1 µM of guaiacol per min. The laccase activity in U/ml is calculated by this formula:
E.A. (U/mL) = A x V/ t x e x v,
where:
E.A = Enzyme activity; A = Absorbance;
V = Total mixture volume (ml); v = enzyme volume (ml); t = incubation time; e = extinction coefficient for guaiacol (0.6740 µM/ cm).

RESULTS AND DISCUSSIONS

Two isolates of *Trichoderma* spp., TdCP (composting source) and TdMI2 (soil source) were used to optimize laccase production under submerged culture with the participation of two inducers such as guaiacol and copper sulphate (CuSO4). Also, different levels of initial pH values ranged from 4 and 8 were applied.

The pH evolution during *Trichoderma* cultivation
The influence of guaiacol addition in PDB medium (0.3%) on the laccase production was initially tested on the isolate TdMI2, originating from soil. The tests were performed under different initial pH conditions (4, 5, 6, 7 and 8) and it was measured constantly (each two days) during the process. It was noticed that, in the absence of guaiacol, even from the second day of incubation, the medium with an initial pH of 8, has drastically decreased at 5, while the medium with an initial pH of 5 showed a less significant decrease, reaching

levels around 4.5. No changes were noticed in the case of the initial pH of 4 (Figure 1).

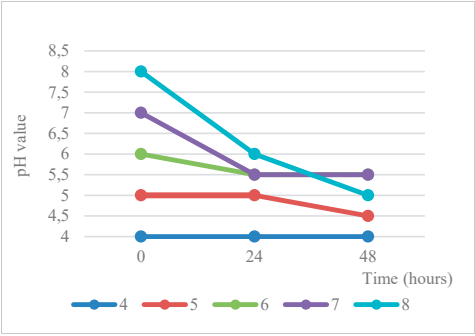

Figure 1. Evolution of pH during incubation of TdMI2 isolate on PDB medium; w/o guaiacol at different pH values

Similarly, in the presence of 0.3% guaiacol, the initial pH 8 has drastically decreased after 48 hours of cultivation, to even lower level, respectively 4; initial value of pH 7 also decreased to lower levels (pH 5) (Figure 2). The acidic pH values have kept their initial range (4 and 5).

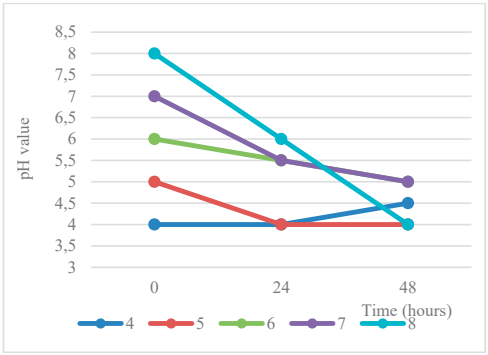

Figure 2. The evolution of pH during incubation of TdMI2 isolate in PDB medium with 0.3% guaiacol at different pH levels

As described by Pelagio-Flores et al. (2017), *Trichoderma* species isolated from soils have the property to induced the acidification of the environment. A few other fungi, mainly pathogenic, acidify their culture media such as: *Penicillium* spp., *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, and *Phomopsis mangiferae* (Prusky et al., 2016). *Trichoderma* spp. grows better in acidic conditions with an optimal growth at pH ranging from 4 to 6, and they can modify the pH of the environment (Singh et al., 2014), but the consequences of fungal-mediated pH changes for plant root growth and development have not yet been deepen analysed (Pelagio-Flores et al., 2017). They produce different secondary metabolites such as non-ribosomal peptides, terpenoids, pyrones and indolic-derived compounds (Contreras-Cornejo et al. 2016) which may induce such acidification, but further studies should be conducted in this regard.

The influence of pH and guaiacol on laccase production

The laccase activity was measured in evolution, every two days, for all the pH values (4, 5, 6, 7 and 8). As described above, the initial pH has decreased in the case of neutral and basic pH (7 and 8), and, after 24-48 hours, actually, all media had a pH on the range 4-5, which was reported as optimal for the *Trichoderma* development. The production of laccase was estimated based on the incubation time.

However, in our case, with or in the absence of guaiacol, the higher laccase activity was noticed in the case of an initial pH of 8, after 14 cultivation days, reaching 0.55-0.6 U/mL as enzymatic activity (Figure 3 versus Figure 4).

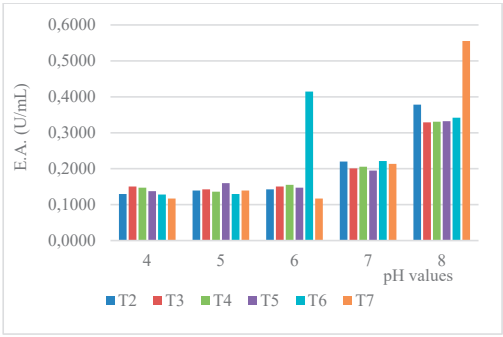

Figure 3. The laccase activity evolution in the presence of 0.3% guaiacol measured every 2 days in the culture of TdMI2 isolate

The guaiacol presence has not significant influence in the laccase production on an initial pH of 8, while for initial acidic pH (4 and 5) its presence influenced positively, but in a lower degree, the enzyme production. The activity measured for the acidic pH was more than 50% lower than in the case of an initial basic pH.

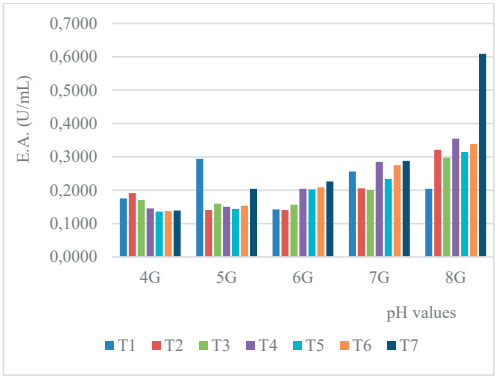

Figure 4. The laccase activity evolution in guaiacol absence for TdMI2 isolate (measured each 2 days)

In this attempt, we were trying to correlate also the biomass production during the cultivation. A correlation was established between the growth of the fungus (biomass weight) and pH with respect to time (Figure 5). The isolate showed maximum growth on 14th day at pH 8 with biomass to be maximum (1270 g). These results clearly suggest the ability of the fungus to grow on an initial pH of 8 which actually decreases after 48 hours in acidic range (4-5). This is close to the values reported by Ranimol et al. (2018) on *Trichoderma harzianum* on pH of 7-7.5.

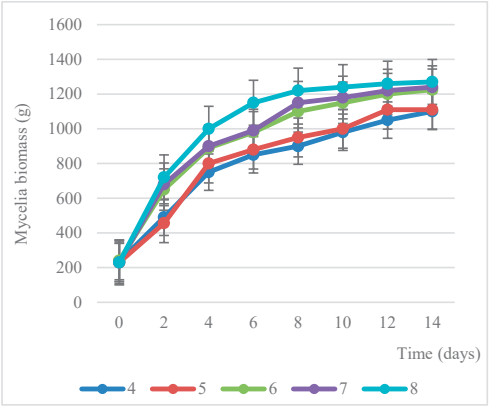

Figure 5. The mycelial biomass evolution of TdMI2 isolate in PDB with 0.03% guaiacol at different pH

The influence of CuSO4 presence on laccase activity

On visual level it was noticed that samples w/o copper sulphate (the control) kept a light yellow color constantly, during all 10 cultivation days, while in the presence of the CuSO4 as inducer, the media color gained different brown overtones, even from the 3rd cultivation day (Figure 6).

The samples with higher concentration of copper sulfate (2.5 mM) have a darker color; among the two isolates, the most intense color was noticed in the case of TdMI2. This results indicate also that both isolates are tolerant to the copper presence, which is an important property when using *Trichoderma* in application like heavy metals (copper) bioaccumulation in soils. For instance, Jovicic-Petrovic et al. (2014) have tested different *Trichoderma* soils isolates for their resistance to Cu (II) concentrations ranging from 0.25 to 10 mmol/L.

Regarding the enzymatic activity, the evolution is quite different. In the first 3 days, the copper inductor has a positive and equal influence on both isolates, being registered laccase activity increases of 0.15-0.2 U/mL against the control.

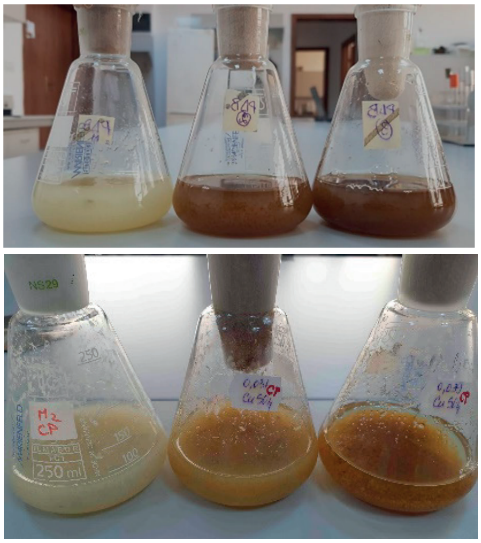

Figure 6. Cultural aspects of *Trichoderma* isolates in PDB additionated with CuSO4 at different concentrations; (up) TdMI2; (down) TdCP

A change was registered after 7 and 10 cultivation days, when, in the case of the isolate TdCP the enzymatic activity has decreased with 0.25 U/mL, while for the isolate TdMI2 the laccase activity increases, reaching double levels against the 3rd cultivation day when adding 2.5mM CuSO4 (Figure 7).

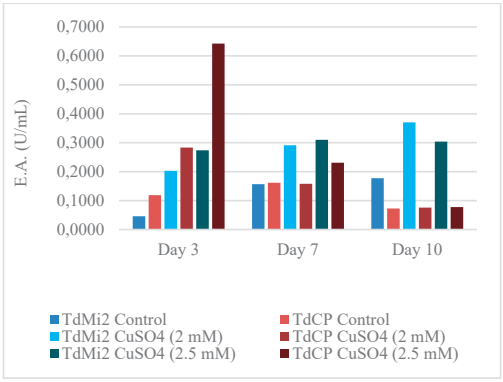

Figure 7. The influence of CuSO4 on laccase activity for two *Trichoderma* isolates

Khambhaty et al. (2015) showed that *Trichoderma atroviridae* could efficient produce laccase without addition of any supplement. However, the addition of CuSO4 increased the activity by almost 25% proving that $Cu2^{+}$ catalytically enhances the action of laccases. Copper is known as a constituent in the laccase's catalytic site; still, its presence greatly influences the enzyme action. Other studies have also shown that copper not only induces laccase by the expression of laccase genes, but it also positively affects the activity and stability of the enzyme (Baldrian et al., 2002) in other fungi (*Pleurotus ostreatus*). Even more, very recently a novel method for simultaneous enhancement of catalytic activity and reusability of laccase was carried out to overcome the limitations on industrial application of laccase, like lignin removal. The immobilization of laccase onto copper ferrite magnetic nanoparticles (CuMNPs); the increased catalytic activity of laccase was observed at 15 mM CuSO4 and the laccase immobilized CuMNPs exhibited 18% higher enzymatic activity when compared to that of laccase in free and immobilized MNPs form (Muthuvelu et al. 2020).

CONCLUSIONS

Different trials were performed on two *Trichoderma* isolates to test the influence of the pH and the inducers presence (guaiacol and CuSO4) on the laccase production in submerged culture, in synthetic media.

The maximum fungal biomass was obtained after 14 cultivation days at an initial pH of 8.

The results reveal the fact that, probably due to secondary metabolites production, the initial pH from neutral-basic range (7 and 8) are drastically decreasing in the first 48 hours reaching pH level of 4-5. Such situation was registered both, in the presence or absence of guaiacol (0.03%). The highest laccase activity was registered after 14 cultivation days at an initial pH of 8; the addition guaiacol has slightly increased the enzymatic activity (from 0.5 to 0.6 U/mL).

In the presence of copper sulphate as inducer, the two isolates behaviour in terms of laccase activity was quite different. While the isolate from the arable soil (TdCP) had a maximum activity after 3 cultivation days with 2.5 mM copper (0.62 U/mL) the isolate from the compost (TdMI2) reached the maximum activity only after 10 cultivation days (0.38 U/mL).

Further, it is expected to use the strain TdMI2 (identified as *Trichoderma asperellum*) for the laccase production on bioreactor scale, to purify and characterise the enzyme and to tests its potential biotechnological application, like lignin degradation or tumours inhibition. Considering the copper positive influence on its laccase production, potential immobilisation in copper based nanoparticle may be considered for the investigations.

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