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SCREENING AMONG MICRO AND MACROMYCETES FOR LACCASE PRODUCTION

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Abstract

Laccases show multiple biotechnological application and different fungal groups have been widely reported as laccase producers. The main aim of our research it was to perform on-plate screening for laccase production among different macro and micromycetes, while optimising the screening protocol in the presence of different guaiacol concentrations. From our collection were taken into account filamentous fungi belonging to the following species: Aspergillus clavatus, A. aculeatus, Botrytis cinerea, Neurospora crassa, Trichoderma sp., Penicillium digitatum. Among the macromycetes were tested Laetiporus sulphureus, Ganoderma lucidum, Agaricus bisporus and Pleurotus ostreatus. The screening was performed on PDA added with guaiacol. The positive microorganisms belong to two strains of Trichoderma spp. isolated from soils, one variety of P. ostreatus and two of A. bisporus originating from supermarket wastes. In the case of P. ostreatus, the use of guaiacol higher than 0.1% has inhibited the fungal growth, as well as the halo-formation. In the case of Trichoderma spp. strains the use of guaiacol above 0.1% and till 1% dind't lead to any halo formation, while the mycelial growth was not inhibited; relevant halos were registered for the concentrations of 0.01% and 0.025%, when the maximum was reached after 6-7 cultivation days.

Key words*: fungi, laccase, guaiacol, on-plate screening optimisation.*

INTRODUCTION

In the past decade, enzymes like laccases, have gained great importance for their biotechnological applications. Laccases play an important role in bioremediation and biodegradation, dye decolorization, paper and pulp industry, as well as in the food industry (Couto et al., 2006; Burlacu et al., 2018). Laccases belongs to the enzyme family of copper-containing oxidases catalysing a variety of oxidations and having a broad substrate specificity.

Fungi, macro and micromycetes, have been widely reported as laccase producers. Different Ascomycetes and Basidiomyceste species, like *Lentinus edodes*, *Coprinus comatus, Oxyporus obducens, Fomes fomentarius*, *Ganoderma lucidum*, *Fomitopsis pinicola, Flamulina velutipes, Pleurotus eryngii*, *Pleurotus ostreatus, Agaricus bisporus* or *Trametes versicolor* were reported to have potential for laccase production (Popa et. al., 2018; Albu Proca et al., 2019). Among the filamentous fungi, several species were reported to produce laccase, respectively *Aspergillus nidulans, Botrytis cinerea, Melanocarpus albomyces, Chalara paradoxa, Chetomium thermophilum, Magnaporthe grisea, Podospora anserina, Neurospora crassa, Rhizoctonia solani* or *Trichoderma harzianum* (Albu Proca et al., 2019). Yet, among the fungi group, the laccase production studies are far to be completed and there is still room to optimise the screening methodology and the enzyme production.

Different methods for on-plate lacasse production screening have been reported by now by adding different substances or colour indicators in the media, like ABTS [2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] (Sodent et al., 2002), bromphenol blue (Tekere et al., 2001) or guaiacol (Kiiskinen et al., 2004; Lopez et al., 2006).

Guaiacol is a phenolic natural product first isolated from Guaiac resin and the oxidation of lignin. Nowadays is commonly derived from guaiacum or wood creosote as yellowish aromatic oil. From a biochemical point of view guaiacol is a monomethoxybenzene that consists of phenol with a methoxy substituent

at the ortho position (ChEBI database). When screening the production of laccases for bioremediation purposes of various xenobiotics, analyses revealed that guaiacol is much better associated with the decolorization of multiple structurally different dyes (Wong et al., 2013). In this respect, when approaching laccase production for bioremediation application the use of guaiacol may be a successful solution. The reported data are making reference to the use of different guaiacol concentration in the screening for laccase production, and the employed amounts are quite different, starting from 0.01% (Kiiskinen et al., 2004) to increased concentration (1%) for selecting high tolerance laccase producing strains (Devasia & Nair, 2016).

The main aim of our research was to perform on-plate screening for laccase production among different macro and micromycetes, while optimising the screening protocol in the presence of different guaiacol concentrations.

MATERIALS AND METHODS

Microorganisms

In the screening were used different macro and micromycetes isolated and conserved in the microbial collection of the Faculty of Biotechnology from USAMV Bucharest or procured from the market as wastes (Table 1).

Table 1. Mycetes used for laccase's production on-plate screening

Mycetes group	Species/Strain/ Variety	Origin
Micromycetes	*Aspergillus clavatus*	Grapes
	Aspergillus aculeatus	Grapes
	Botrytis cinerea	Grapes
	Neurospora crassa	Cacao beans
	Trichoderma spp. MI2	Soil
	Trichoderma spp. CP	Soil
	Penicillum digitatum	Soil
Macromycetes	*Laetiporus sulphureus* DD	Forest tree from Danube Delta
	Laetiporus sulphureus B	Urban tree Bucharest
	Ganoderma lucidum	Forest tree from Danube Delta
	Agaricus bisporus white variety	Supermarket waste
	Agaricus bisporus brown variety	Supermarket waste
	Pleurotus ostreatus	Supermarket waste

Media

For the inoculum preparation, as well as for the on-plate screening was used a fungal basal medium, respectively PDA (Potato Dextrose Agar). For the visualisation of the laccase production, the PDA was added with guaiacol, as described below.

On-plate screening method for laccase production

For the initial screening of laccase production was employed PDA supplemented with 0.04% guaiacol (Roth Werke GmbH), according to Kalra et al. (2013). Guaiacol was added to the media before autoclaving. The positive test is indicated by the formation of a brown-reddish halo around the fungal culture. In the case of the filamentous fungi (micromycetes), the strains were cultivated on PDA and spore suspensions (10^6 spores/ml) were prepared as inoculum; 100 µl spore suspension was inoculated in the centre of the PDA + guaiacol plate. In the case of the macromycetes, a fragment of 0.5 cm^2 from the mushroom's cap was added on PDA after a partial sterilisation in 70% ethanol. The fungi were cultivated at 28ºC during 8 to 14 days (depending on how fast the mycelium invaded the plate). The cultivation temperature in the case of *Botrytis cinerea* was lower, respectively 21.4ºC which is considered as optimal for the specie (Judet-Correia et al., 2010). For the positive strains the laccase production was monitored further on PDA plates supplemented with different guaiacol concentrations (0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 0.75%, 1%) when the halo formation was daily measured (cm in diameter). The tests were performed in duplicate and the mean values were compared; no significant deviations from the mean were registered in the halo size.

RESULTS AND DISCUSSIONS

In our initial on-plate screening for laccase production (PDA added with 0.04% guaiacol) were tested seven filamentous fungi and six macromycetes, of which only two filamentous fungi and three macromycetes tested positive (Table 2).

Among the six tested macromycetes, only the commercial strains of *Pleurotus ostreatus* and *Agaricus bisporus* have produced distinct

brown-reddish halo in the first step screening on medium supplement with 0.04% guaiacol. Comparing the two species, *P. ostreatus* produced a bigger halo under the same cultivation condition and was taken into account for further testing targeting the optimisation of the screening procedure under different guaiacol concentrations (0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 0.75%, 1%).

Table 2. Qualitative results of the on plate initial screening for fungal laccase production on medium supplemented with 0.04% guaiacol

Specie/Strain/Variety	Screening result
Aspergillus clavatus	-
Aspergillus aculeatus	-
Botrytis cinerea	-
Neurospora crassa	-
*Trichoderma sp.*MI2	+
Trichoderma sp. CP	+
Penicillum digitatum	-
Laetiporus sulphureus DD	-
Laetiporus sulphureus B	-
Ganoderma lucidum	-
Agaricus bisporus white variety	+
Agaricus bisporus brown variety	+
Pleurotus ostreatus	++

Legend: (-): negative; (+): 0-3 cm halo; (++): 3-9 cm halo

They were noticed two different pattern groups in the *Pleurotus ostreatus* mycelia development (Figure 1). When using small concentration of guaiacol (0.01% to 0.05%) the plate was invaded by the mycelia after 11 cultivation days at 28°C, while for higher guaiacol concentrations (0.1 to 1%) the mycelial growth was inhibited, and, even after 14 incubation days, the mycelia didn't invade the entire plate. This can be related to the fact that under higher guaiacol concentration more laccase is produced and few species/strains have been reported to have high laccase tolerance (Devasia & Nair, 2016).

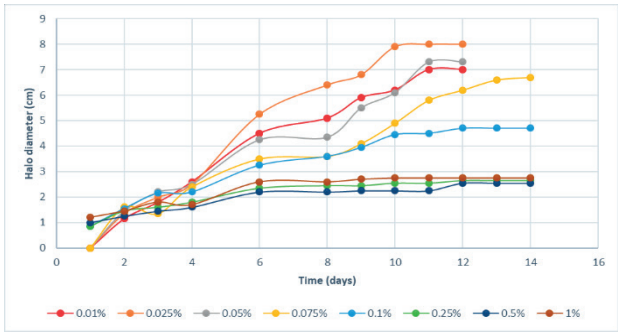

Figure 1. Laccase halo formation evolution for *Pleurotus ostreatus* cultivation at 28°C in PDA supplemented with different guaiacol concentrations

The halo formation followed the mycelial growth evolution and, similarly, two patterns were noticed. In the case of low guaiacol concentrations (0.01% to 0.05%) the halo was not visible in the first incubation day, becoming clearly visible in the third incubation day; from the 10th incubation day, when the maximum was achieved, no significant changes were registered. In the second pattern, of the high guaiacol concentrations (0.1 to 1%), the brown-reddish halo was formed even from the first incubation day (in the first 4 hours), but its development didn't register a major increment starting with the 5th incubation day, being constant till the end of monitoring (14th incubation day). In the case of the 0.075% guaiacol content it was noticed an intermediate pattern between the two groups.

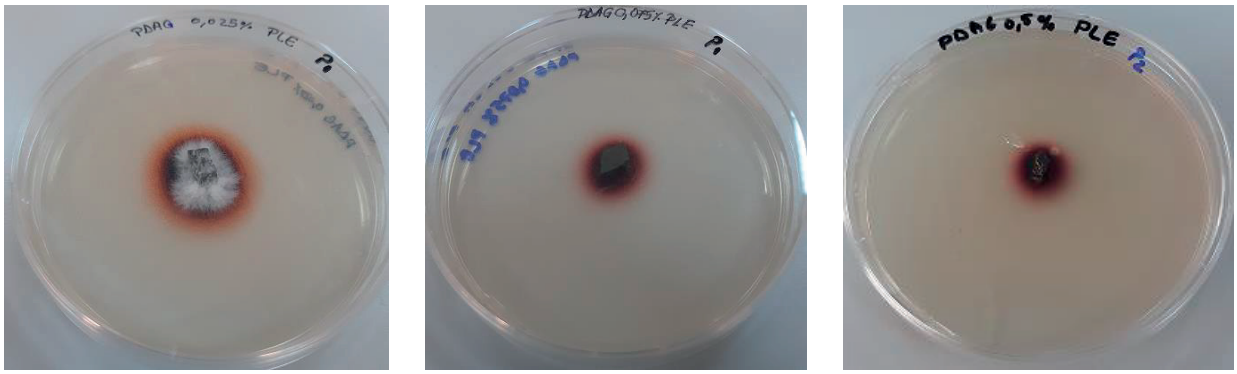

Figure 2. Aspects of the laccase halo formation for *Pleurotus ostreatus* at different guaiacol concentrations in the 3rd incubation day (from left to right: 0.025%; 0.075%; 0.5%)

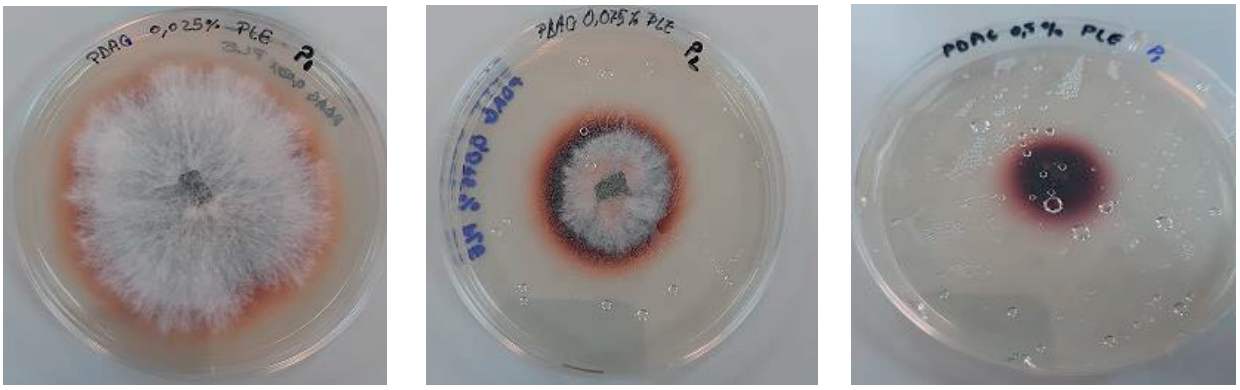

Figure 3. Aspects of the laccase halo formation for *Pleurotus ostreatus* at different guaiacol concentrations in the 8th incubation day (from left to right: 0.025%; 0.075%; 0.5%)

Most of the reported data emphasize that in the case of the plate assay method for fungal laccase test, using guaiacol as substrate in the medium, the brown-reddish zone developed by the isolated strains may appear even from the first incubation day, but can be clearly visible in the 3rd incubation day (Kiiskinen et al., 2004; Kalra et al., 2013; Fu et al., 2013), which is in line with our findings (Figure 2). In the case of *Pleurotus ostreatus* the halo was clearly visible for all tested concentrations, in accordance to other reports; the difference in the halo size between the 3rd and the 8th incubation days can be visualized comparing Figure 2 with Figure 3.

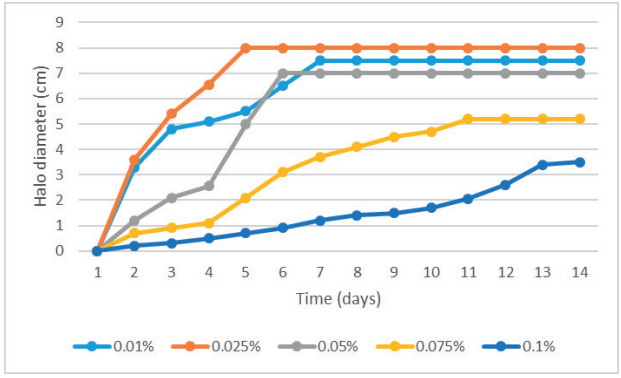

Figure 4. Laccase halo formation evolution for *Trichoderma* spp. MI2 cultivatated at 28°C in PDA supplemented with different guaiacol concentrations

Among the seven tested micromycetes (filamentous fungi), only two isolates of the same genus, namely *Trichoderma*, formed visible halo for the laccase production. This is only partially in line with other authors reports which listed species like *Neurospora crassa, Botrytis cinerea* (Gochev & Krastanov, 2007) or *Penicillium digitatum* (El-Shora et al., 2008) as high potential laccase producers; that may be expalined due to the used strains or to the cultivation conditions.

Both *Trichoderma* isolates were tested further to optimise the screening procedure in different guaiacol concentration. Surprinsingly, when using guaiacol above 0.1% and till 1% no halo formation was detected, while the mycelial growth was not inhibited as was the case of the macromycetal *Pleurotus ostreatus*. It can be noticed that both *Trichoderma* isolates registered similar halo formation evolution for all guaiacol concentrations (Figure 4 and Figure 5).

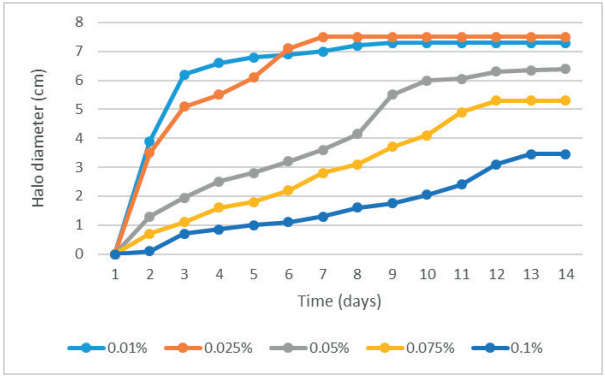

Figure 5. Laccase halo formation evolution for *Trichoderma* spp. CP cultivated at 28°C in PDA supplemented with different guaiacol concentrations

A slight exception was noticed in the case of the 0.05% concentration when the strain MI2 exhibited a pattern closer to the smaller concentration group, while in the case of the strain CP the pattern was closer to the higher concentration group. The higher halos (7-8 cm) were registered for the concentrations of 0.01% and 0.025 %, when the maximum was reached after 6-7 cultivation days. This is in line with

data reported by Ahmed & Siddiqui (2015). Aspects of the halo formation at 0.025% for both *Trichoderma* isolates after three cultivation days are visible in Figure 6.

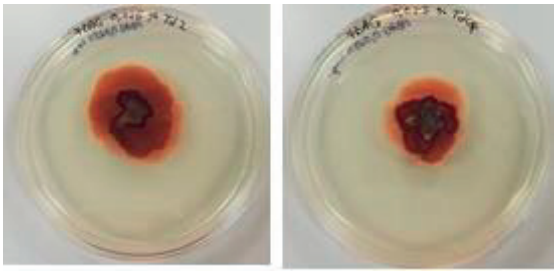

Figure 6. Aspects of the laccase halo formation in the presence of 0.025% guaiacol after 3 incubation days at 28ºC (left: *Trichoderma* spp. MI2; right: *Trichoderma* spp. CP)

Reported data (Ranimol et al., 2018) on *Trichoderma harzianum* suggested as optimal guaiacol concentration for laccase on-plate screening as 0.05%, which is only partially in accordance with our results.

CONCLUSIONS

In our attempt to screen among different fungal species for laccase production, we have identified positive strains belonging to macro or micromycetes groups, respectively two strains of *Trichoderma* spp. isolated from soils, one variety of *Pleurotus ostreatus* and two of *Agaricus bisporus* originating from supermarket wastes. Among the macromycetes, *Pleurotus ostreatus* exhibited the highest potential for laccase production, while in the *Trichoderma* spp. strains, their potentials were close.

In terms of guaiacol concentration used in the screening, some conclusions are to be taken into account, depending on the fungal group. In the case of the macromycete group, respectively *Pleurotus ostreatus*, using guaiacol concentration higher than 0.1% is inhibiting the fungal growth, as well as the halo–formation; this may be correlated to the strain tolerance to the presence of laccase in the medium. Also, in small guaiacol concentrations (0.01-0.05%) the halo formation is clearly visible in the third cultivation day, while for higher concentrations (0.1-1%) in the first 4 hours the halo is visible. In the case of *Trichoderma* spp. isolates, when using guaiacol above 0.1% and till 1% no halo formation was detected, while the mycelial growth was not inhibited; the higher halos sizes were registered for the concentrations of 0.01% and 0.025%, when the maximum was reached after 6-7 cultivation days.

For both fungal groups, when on-plate screening is performed it is recommended to be used lower guaiacol concentrations, starting with 0.01% to 0.075%. Higher guaiacol concentration (0.1% to 1%) may induce the laccase formation in a very first step, but may inhibit the mycelial growth and are recommended only when screening for fungal strains tolerant to high laccase formation. Guaiacol was confirmed, in small concentrations (0.01-0.05%), as useful indicator when screening for both laccase producers and laccase tolerant fungi of bioremediation use.

Further investigations are taken into account on how guaiacol concentration induces the laccase production and tolerance under fungal submerged culture.

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