UDK 577.15
Original scientific paper

APPLICATION OF CRUDE FUNGAL LACCASE FROM *GANODERMA SPP.* IN DECOLORIZATION OF TRIPHENYLMETHANE DYE CRYSTAL VIOLET

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Abstract

Industrial dye effluents that contain triphenylmethane dyes are environment-threatening problem. The triphenylmethane dyes are one of the largest dye's group that is discharged in large quantities to water bodies. Removing of them with fungal enzymes is big scientific challenge. The fungal laccases are promising tool for removing of these dyes from water bodies. In this study, the potential of crude fungal laccase from genus Ganoderma in decolorization of triphenylmethane dye, crystal violet was investigated. The crude fungal enzyme was produced using agroindustrial lignocellulosic waste, cereal mix. The effect of different substrate masses (15g, 25g and 50 g) and Cu^{2+} ions concentrations (0.25 mM, 0.75 mM, 1.0 mM, 2.5 mM u 5.0 mM) on lccase production were investigated. The obtained crude fungal laccase with the highest activity was used for decolorization of crystal violet at different concentrations from 20 mg/L to 50 mg/L. The crude fungal laccase from genus Ganoderma had the highest activity of 47.43 U/g, when the substrate mass of 50 g was used for enzyme production. The lower masses of substrate (15g and 25g) induced lower laccase activities of 45.07 U/g and 46.69 U/g, respectively. The initial addition of Cu^{2+} ions in concentration of 0.25 mM to agroindustrial waste led to decrease of laccase activity compared to the laccase activity of laccase obtained from agroindustrial waste that didn't contain Cu^{2+} ions. On the other hand, the increase of Cu^{2+} concentration to 1.0 mM in the substrate led to increase the laccase activity. The highest laccase activity of 47.53 U/g was obtained when the 1.0 mM Cu^{2+} was added to substrate, while the decrease in laccase activity was observed when 5.0 mM Cu^{2+} was added to the agroindustrial waste. Decolorization of crystal violet at different concentrations (20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L) was carred out at pH 5 and temperature of 50 ºC for 120 min. The highest decolorization efficiency of 14.42 % was obtained in the case of the lowest dye concentration (20 mg/L), while the lowest decolorization efficiency of 3.76 % was obtained when the highest dye concentration of 50 mg/L was decolorized with crude fungal laccase for 120 min. The obtained results show that fungal crude laccases can be used for decolorization of triphenylmethane dyes, but the detailed optimization is very important for obtaining relatively high decolorization efficiencies for short time.

Key words: *white rot fungi, laccase, agroindustrial waste, decolorization, triphenylmethane dye, crystal violet*

Introduction

Triphenylmethane dyes, such as crystal violet, brilliant green, and malachite green, are among the most widely used synthetic dyes. They're widely used in a variety of industries, including textile, leather, paper, cosmetics, and pharmaceuticals (Cheriaa & Bakhrouf, 2009; Ogugbue & Sawidis, 2011). Without prior treatment, these dyes are released into the environment, primarily into aquatic ecosystems, disrupting the biological balance of the ecosystem. Specifically, the presence of these dyes in water reduces light penetration to aquatic plants responsible for photosynthesis, and it can have a toxic effect on aquatic animals (Kaur & Bera, 2020).

Triphenylmethane dyes can be removed from water bodies using a various physical and chemical methods, including flocculation, coagulation, adsorption, membrane filtration, precipitation, and irradiation/ozonization (Ledakowicz & Pazdzior, 2021; G. Li et al., 2014). However, the most significant disadvantage of these methods is the production of toxic by-products, which is why biological methods of removing synthetic colors are increasingly being sought (G. Li et al., 2014). Because of their ability to produce a diverse range of enzymes, many fungi are capable of decolorizing synthetic dyes (Zhang et al., 2012).

Laccases are one of the most important fungal enzyme groups that have found use in the dye removal process. Laccases (EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of a wide range of aromatic substrates as well as the reduction of molecule oxygen to water (Zhang et al., 2012). Furthermore, they can also oxidize nonphenolic substrates in the presence of the proper redox mediators (K. Li & Xu, 1999).

Among fungi, white-rot fungi are unquestionably the most significant laccase producers due to their highly developed and effective ligninolytic system (Yang et al., 2017). Several lignocellulosic materials that in their composition possess a suitable concentration of lignin, the primary inducer of laccase formation, can be used by white rot fungi to produce laccase. The waste from cereal crops, which accumulates to a significant degree and pollutes the environment, is the most popular lignocellulosic material used for these purposes (Wang et al., 2019).

White-rot fungi may produce laccases with low activity, thus in order to make up for this shortcoming, the primary substrate is supplemented with other chemicals. The primary substrate is frequently supplemented with various agents including aromatic and phenolic compounds, and different inorganic substances (copper or manganese) in specific quantities in order to boost the yield as well as the activity of the produced laccase (Bertrand et al. 2017, Chmelova et al. 2014).

Metal ions frequently affect laccase gene transcription in fungi due to the ubiquitous regulation of laccase expression by metals. Copper acts as a transcription inducer and cofactor in the catalytic center of some laccase genes. The addition of Cu^{2+} in the form of $CuSO_4$ to substrates can result in a significant increase in total laccase activity and the production of certain laccase isoenzymes, though this effect can be negligible in some cases (Chmelova et al. 2014).

The goal of this study was to see how different substrate masses of cereal mix, and copper ion concentrations affected the production and activity of laccase from the genus *Ganoderma*. Laccase was also tested after production for its ability to decolorize triphenylmethane dye, crystal violet.

Materials and Methods

Chemicals and sample materials

Copper-sulfate was purchased from Zorka Pharma, Šabac, Serbia. Guaiacol was purchased from Fischer Scientific Acro. The malt extract broth was purchased from Torlak, Serbia. The triphenylmethane dye, crystal violet was purchased from Sigma-Aldrich. Other chemicals were the highest commercial grades purchased from Merck and Lach-Ner. The cereal

mix was kindly donated by local agricultural cooperative.

Microorganism

Fungus *Ganoderma spp.* was used for laccase production. *Ganoderma spp.* was from the culture collection from the Department of Biochemical Engineering and Biotechnology of the Faculty of Technology and Metallurgy, Belgrade, Serbia.

Inoculum preparation

Mycelium was grown on malt extract agar plates to make the inoculum. Malt extract agar was previously sterilized and poured into Petri dishes for inoculum preparation. It was made up of malt extract broth (20 g/L) and agar (15 g/L) (Petri dishes 90mm in diameter).

Laccase production

Agro-industrial lignocellulosic waste (cereal mix) was used as a nutrient medium for mushroom mycelium cultivation and laccase production. The dry matter content of this waste was 71.96%. In 300 mL Erlenmeyer flasks, 50 g of waste substrate were measured. Nutrient media were sterilized in an autoclave at 121 ºC for 30 min. The substrates were cultivated the mycelium of the fungus that had previously grown on malt agar. Five (1x1 cm) squares of malt agar containing mycelium of higher fungi were sterilely cut and transferred to an Erlenmeyer flask with waste substrate. Nutrient media prepared in this manner were incubated in a thermostat at 30 ºC for 6 days. Laccase extraction was carried out in each Erlenmeyer flask by adding 0.1 M Na-acetate buffer (pH 5.0). After that, the samples were shaken on an orbital shaker (170 rpm, 25 ºC, 50 min). The samples were centrifuged at 6000 rpm after shaking. Laccase activity was determined by spectrophotometric analysis of guaiacol oxidation at 470 nm using the resulting supernatant, which contained crude enzyme.

Laccase activity (U/mL) was calculated using equation:

$$E.A.[U/mL] = (A_{470} \times V_t)/(t \times V_e \times \varepsilon),$$ where is

E.A. laccase activity [U/mL], A_{470} absorbance at 470 nm, Vt total volume of reaction mixture (mL), t incubation time, ε extinction coefficient of guaiacol (0.6740 μM/cm), V_e laccase volume (mL).

Laccase activity [U/g] was calculated according to the equation:

$$E.A.[U/g] = E.A.[U/mL] \times V \div m_s,$$ where is

E.A. laccase activity [U/g], E.A. laccase activity [U/mL], V total volume of buffer (mL), m_s mass of cereal mix (g).

Examination of the influence of Cu^{2+} concentration on the laccase production

The effect of various Cu^{2+} ion concentrations added to lignocellulosic waste (cereal mix) on laccase production was investigated. For this experiment, 50 g of cereal mix was mixed with Cu^{2+} ion solutions of 0.25 mM, 0.75 mM, 1.00 mM, 2.50 mM, and 5.00 mM concentrations. Erlenmeyer flasks were cultivated with the fungal mycelium in the previously described manner after sterilizing the medium in an autoclave at 121ºC for 30 min. Nutrient media prepared in this manner were incubated in a thermostat at 30ºC for 6 days. Laccase activity was then determined

in the previous described way.

Examination of the influence agroindustrial waste masses on the laccase production

The effect of different weights of agro-industrial waste on laccase enzyme production was investigated. Agro-industrial waste weighing 15 g, 25 g, and 50 g was placed in 300 mL Erlenmeyer flasks. Each Erlenmeyer flask received 0.15 mL, 0.25 mL, and 0.50 mL of 1mM copper ($CuSO_4 \cdot 5H_2O$), and the flasks were sterilized in an autoclave at 121ºC for 30 minutes. After sterilization, the fungal mycelium was cultivated into Erlenmeyer flasks in the previously described manner. Nutrient media prepared in this manner were incubated in a thermostat at 30ºC for 6 days. Following that, laccase activity was determined in the previous described way.

Decolorization of dye crystal violet

When investigating the decolorization potential of *Ganoderma spp.* laccase, crystal violet dye was used. The dye concentrations were 20 mg/L, 30 mg/L, 40 mg/L, and 50 mg/L. The reaction mixture included 1.5 mL of 0.1 M Na-acetate buffer (pH 5.0), 0.5 mL of dye, and 0.5 mL of crude *Ganoderma spp*. laccase, whereas the control included a dye solution without enzyme. The prepared samples were incubated at 50ºC, and the change in color intensity was measured at 30 min, 60 min, 90 min, and 120 min. The process was spectrophotometrically monitored by measuring absorbance at 584 nm. The percentage of decolorization was calculated using the following equation based on the obtained absorbances:

$$Decolorization\ (\%) = \frac{absorbance_{initial} - absorbance_{time}}{absorbance_{initial}} \times 100$$

Results and Discussion

Laccase production

Cereal waste is not only treated and used to lessen its environmental impact, but also to fully utilize and convert it into biotechnologically important products such as biofuels, biofertilizers, microbial enzymes, and organic acids (Hassan et al., 2021).

Laccase, a ligninolytic enzyme produced by *Ganoderma spp*., a white rot fungus, is capable of degrading lignin in lignocellulosic biomass and can be produced on cereal wastes using SSF (Kahraman & Yeilada, 2001). Laccase produced by *Ganodema spp.* on cereal mix had activity of 47.43 U/g (31.62 U/mL) after 6 days of fermentation. On the other hand, indigenously isolated *Ganoderma spp.* produced laccase with a minimum laccase activity of 142 U/gds when sugarcane bagasse was used as the substrate, and a maximum laccase activity of 974 U/gds when wheat bran was used as the substrate (Revankar et al., 2007). When grown on commercial substrate, enzyme-producing medium, and potato dextrose medium, *Ganoderma lucidum* produced laccase with lower activity than in our study (Qin et al., 2019).

The influence of various Cu^{2+} ion concentrations on laccase production

The effect of Cu^{2+} ion concentrations in the medium on the *Ganoderma spp* laccase production was investigated. The concentrations were 0.25 mM, 0.75 mM, 1.00 mM, 2.50 mM, and 5.00 mM. The enzyme's activity was determined, as well as which concentration of Cu^{2+} ions had the greatest influence on laccase production.

When compared to the laccase from the control that did not contain these metal ions, the different concentrations of Cu^{2+} ions tested had no significant effect on laccase activity (Fig. 1). Laccase activity was 47.43 U/g in the control, but 0.25 mM and 0.75 mM solutions of Cu^{2+} ions reduced laccase activity to 45.65 U/g and 46.88 U/g, respectively. An increase in the

concentration of added ions resulted in an increase in enzyme activity, which achieved maximum in the presence of at 1 mM Cu^{2+}ions concentration. Laccase demonstrated the greatest activity (47.53 U/g) in the presence of a 1 mM Cu^{2+} ion solution.

The activity of the enzyme then decreased as the concentration of Cu^{2+} ions increased, so that the lowest activity of laccase (42.73 U/g) was measured in the presence of a 5 mM solution of copper ions. Based on the results, it can be concluded that a copper ion concentration of 1mM had the greatest effect on laccase enzyme production.

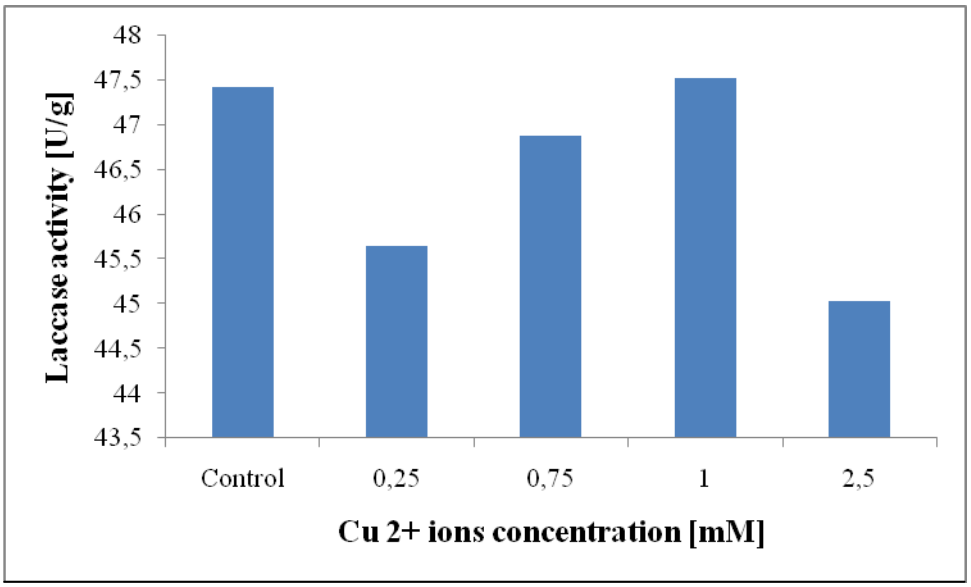

Figure 1. Effect of various Cu^{2+} ion concentrations on Ganoderms spp. laccase activity

Gomaa et al. (2015) reported the same trend of increasing and decreasing laccase activity due to the addition of various concentrations of Cu^{2+} ions in the case of laccase from *Aspergillus flavus*. When 1 mM copper sulfate was added to the cultivation medium, the laccase activity increased initially from 5.10 U/mL to 51.84 U/mL. This was followed by a gradual decrease to 21.6 U/mL at 5 mM copper sulfate and a further decrease to 6.91 U/mL at 10 mM copper sulfate (Gomaa & Momtaz, 2015).

The optimal Cu^{2+} ion concentration for *Lentinus tigrinus* laccase activity was 1.5-2.0 mM. Laccase activity was higher in the presence of 2.5 mM $CuSO_4$ in the culture medium than in the absence, but lower than in the presence of 2 mM $CuSO_4$ (Shutova et al., 2008). On the other hand, the effect of copper on laccase activity in *G. lucidum* E47 was more pronounced in the presence of ferulic acid, with an optimum of 1 mM (Kuhar & Papinutti, 2014).

The influence of agroindustrial waste masses on the laccase production

The effect of different amounts of agro-industrial waste (cereal mix) on laccase enzyme production was investigated. Waste substrate masses of 15 g, 25 g, and 50 g were tested. The optimal mass for the production of laccase with the highest activity was determined using the laccase activities obtained.

Although the increase in laccase activity was not significant, it was caused by an increase in the mass of agro-industrial waste (Fig. 2). Laccase activity was highest at 47.43 U/g when 50 g of cereal mix was used and lowest at 45.07 U/g when 15 g of this lignocellulosic material was used.

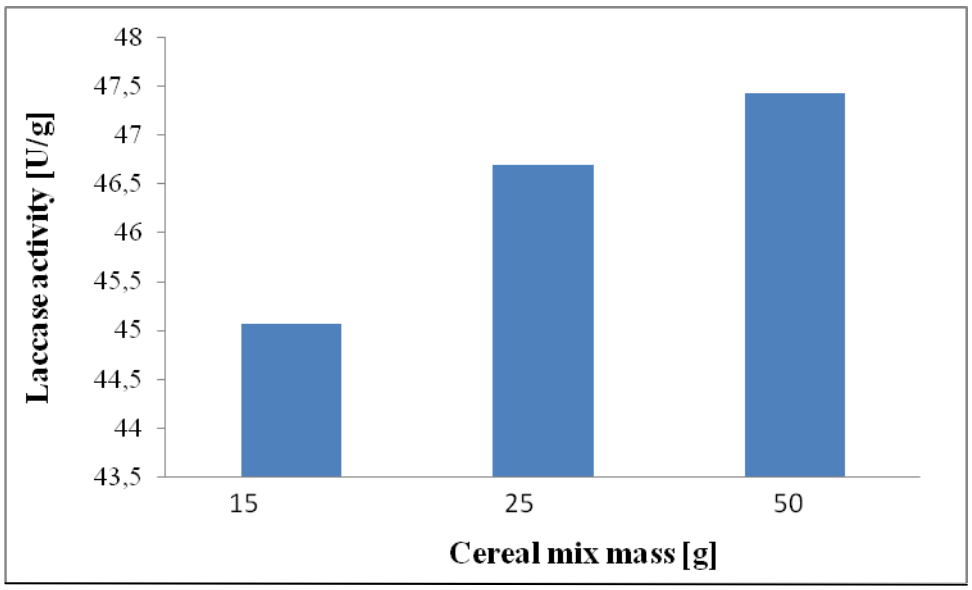

Figure 2. Effect of various cereal mix masses on Ganoderms spp. laccase activity

Decolorization of crystal violet

The ability of *Ganoderma spp.* laccase to decolorize the triphenylmethane dye crystal violet was investigated. Four different dye concentrations (20 mg/L, 30 mg/L, 40 mg/L, and 50 mg/L) were used in the decolorization process with *Ganoderma spp*. laccase (47.43 U/g) for 120 min (Fig. 3).

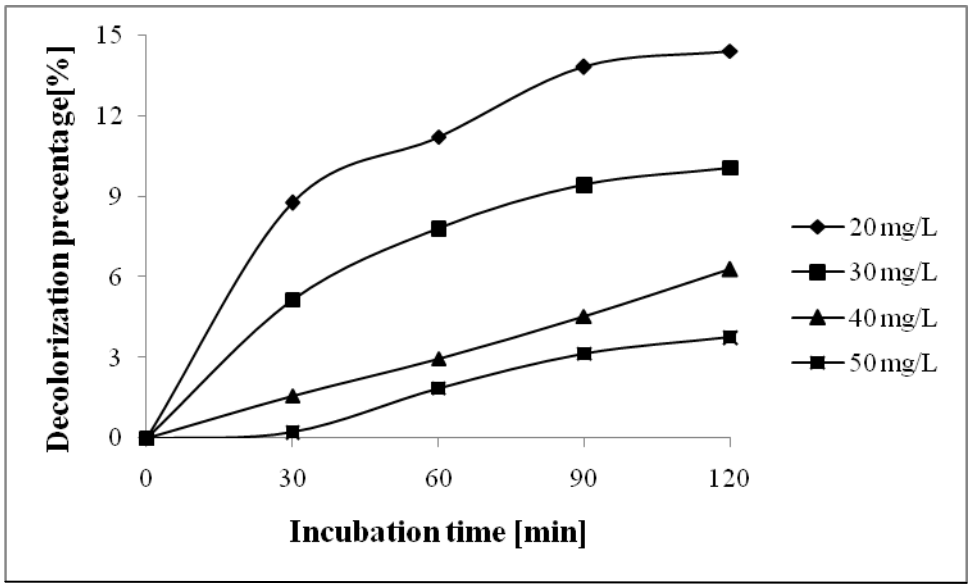

Figure 3. Decolorization of crystal violet dye with crude fungal laccase from Ganoderma spp. for 120 min

In all four dye samples tested, dye intensity decreased during decolorization. The greatest decrease in color intensity, i.e. the highest percentage of decolorization, occurred after 30 min of incubation, after which color decolorization occurred at a lower intensity, which can be explained by the decrease in laccase activity at 50ºC during incubation time.

The lowest concentration of 20 mg/L resulted in the greatest decrease in the intensity of the crystal violet dye, with the highest decolorization percentage of 14.42% after 120 min (Fig. 4). The percentage of decolorization of crystal violet decreased as the dye concentration increased, so the percentage of decolorization was low at 30 mg/L and was 10.09% (Fig. 4). In the case of the highest dye concentration (50 mg/L), the decolorization percentage was the

lowest (3.76%) (Fig. 4).

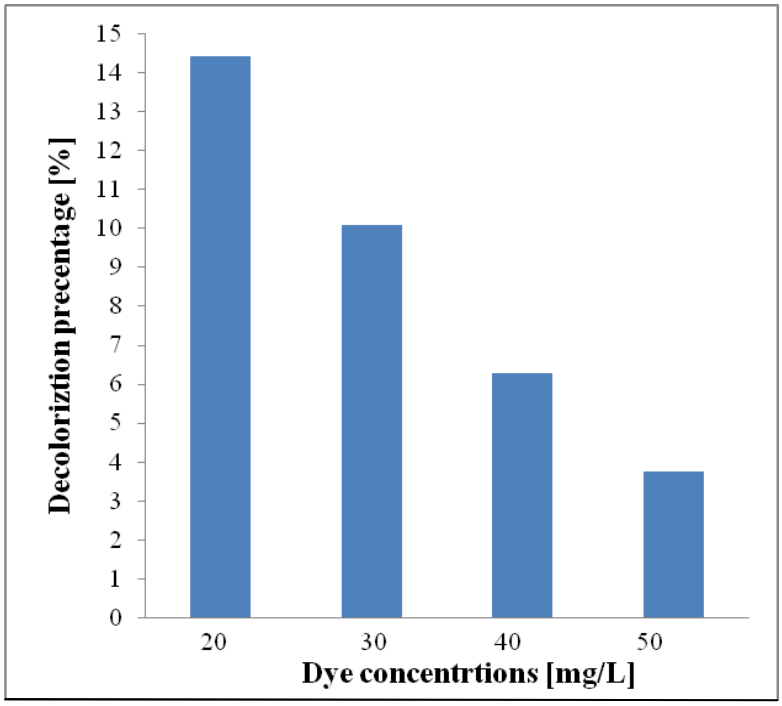

Figure 4. Decolorization percentage of different crystal violet dye concentrations with crude fungal laccase from Ganoderma spp. after 120 min

In comparison to our study, Morales-Alvarez et al (2017) reported lower percentages of crystal violet decolorization. Crystal violet dye at a concentration of 5.00 mg/L had a decolorization percentage of only 23.6% after 24 hours of decolorization with *Ganoderma lucidum* laccase (Morales-álvarez et al., 2017). The crude laccase *Trametes sp*. LH-3 decolorized crystal violet (5 mg/L) by 50% in 24 hours (Huang et al. 2011).

Conclusions

Different cereal mix masses had no effect on crude laccase activity, but 50 g of waste mass was optimal for laccase production. The activity was 47.43 U/g when 50 g of this substrate was used to produce crude laccase by *Ganoderma spp*.

Cu^{2+} ion concentrations influenced crude laccase activity. Laccase activity was inhibited by Cu^{2+} ion concentrations ranging from 0.25 to 0.75 mM, as well as 2.50 mM, whereas it was stimulated by concentrations of 1.00 mM.

Ganoderma spp. crude laccase decolorized triphenylmethane dye crystal violet for 120 minutes at concentrations ranging from 20 to 50 mg/L, with the highest decolorization percentage at 20 mg/L.

Acknowledgments

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No. 451-03-47/2023-01/200287 and 451-03-47/2023-01/200135).

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