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Optimization of nutritional factors and copper on laccase production by *Pleurotus florida*

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Abstract

Laccases as lignocellulolytic enzymes are commonly produced by submerged fermentation, but for more constructive production, it should be preceded by nutritional factors suitable for the fungi's growth. In the present study, the overproduction of laccase activity resulting from nutritional factor interactions was studied in *Pleurotus florida*, a white-rot fungi. Response surface methodology (RSM) based on the Box-Behnken factorial design (BBD) was performed to optimize the interaction of glucose and yeast extract concentrations to maximum enzyme activity with and without copper sulfate as an inducer. The results show a quadratic model with a very low p-value (<0.0001) to explain the changes in laccase production as a function of glucose, yeast extract, and copper sulfate concentrations. Based on the coefficient of determination (R^2) and mean absolute error, the RSM model provided a good quality prediction for the laccase production with all independent variables. The findings explain that a 4.4-fold increase in laccase activity occurs in the presence of copper compared to cultures without copper with an optimal concentration of glucose and yeast extract as carbon and nitrogen sources, respectively. Maximum laccase activity (5.28 UmL^{-1}) was obtained using optimized conditions (18.70 gL^{-1} glucose, 8.22 gL^{-1} yeast extract, and 0.93 mM copper sulfate). This finding could be used to induce high laccase production on a large scale for biomass changeover systems.

1. Introduction

Increasing population and economic growth have resulted in a common need for more energy. Therefore, energy and other valuable products from biomass supplies play a pivotal *role* in the overall energy system (Liu et al., 2009). Lignocellulosic biomass consists of cellulose, hemicellulose, and lignin and is the most abundant and renewable energy source in nature (Cardona & Sánchez, 2007). However, lignin *is* particularly difficult to biodegrade and reduces the bioavailability of the other lignocellulosic constituents *(*Bak et al., 2009*)*. Basidiomycetes, including white-rot fungi, are able to degrade lignin using several oxidoreductase enzymes, including manganese peroxidase, lignin peroxidase, and laccase (Minussi et al. 2002; Ryu et al. 2008).

Laccases (benzenediol oxygen oxidoreductase, EC1.10.3.2), a potent element of the ligninolytic system, are multi-copper oxidases that catalyze

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the oxidation of hydroxyl functional groups on a wide range of phenolic compounds and aromatic amines, with concomitant oxygen reduction to water (Giardina et al. 2010; Piscitelli et al. 2011; Valle et al. 2015). Due to their wide application, laccases are very attractive in several biotechnological areas, such as biofuel production, bioremediation, detoxification of toxic wastes, the food industry, pharmaceuticals, and wastewater therapy (Zeeb et al., 2014). Although laccases are common *enzymes* in nature and are present in plants, insects, and bacteria, fungi, particularly basidiomycetes, are a *more important source.* In fungi, the enzymes likely have different functions in lignin degradation, pathogenesis, cycling of soil organic substances, detoxification, fungal plant-pathogen interaction and defense, and fungal development and morphogenesis (Kues & Rühl, 2011). Therefore, fungal laccases have attracted significant attention from academics and industry. Unfortunately, in natural conditions, the low laccases production of different white-rot fungi cannot meet the practical demands of industrial biotechnology, and most of the chemicals needed for laccases inducers are expensive and ecologically toxic (Kudanga & Le Roes-Hill, 2014; Zhu et al., 2016).

There is a vast potential for microbial enzyme production to be used for industrial purposes. Different species of *Pleurotus* are the most prevalent *cultivable fungi* (Ohm et al., 2014; Sánchez, 2010) and have been used for studying and producing fungal laccases (Fernández-Fueyo et al., 2016). *Pleurotus* spp. efficiently utilizes various inexpensive agricultural residues to produce several intra and extracellular valuable enzymes. The productivity of continuous fermentation processes is affected by different nutritional (mainly carbon and nitrogen sources) and physicochemical conditions (temperature, pH, inoculum size, and agitation rates) (Giardina et al., 2010; Murugesan et al., 2009; Yadav, 2018). Besides genetic engineering technology, optimization of the culture medium has been used to enhance laccase production, especially by adding inducers (Dekker et al., 2007; Guo et al., 2017).

In previous reports, most researchers have used *P. ostreatus* and glucose and yeast extract as the best carbon and nitrogen sources for laccase production, respectively**.** Those studies evaluated one or two nutritional factors at a time to screen various process parameters during the fermentation process (Durán-Sequeda et al., 2022; Periasamy et al., 2010; Zhu et al., 2016), but this method requires much time, and the cultural component interactions were not studied. Response surface methodology (RSM) can be used to overcome this problem by designing experiments and exploring models to explain the relationships between the response and several independent variables. This method also reduces the number of trials and recognizes the importance of independent variables, alone or in combination. Considering these facts, we made an attempt to optimize the medium components (nutritional and inducer factors) to improve and increase laccase production by *Pleurotus florida*, as a commercial edible oyster mushroom. The results indicate that glucose (as a carbon source) and yeast extract (as a nitrogen source) availability influence laccase activity only when copper is present in a nutrient-sufficient condition.

2. Materials and Methods

2.1. Chemicals

Calcium chloride, $CaCl_2$ (99.9%); citric acid (99.5%); Copper (II) sulfate pentahydrate, $CuSO_4.5H_2O$ (>98.0%); magnesium sulfate heptahydrate, $MgSO_4.7H_2O$ (99%); phenol (>98.0%); potassium chloride, KCl (99%); potassium phosphate monobasic, K_2HPO_4 (99%); sodium hydroxide, NaOH (>98%); sulphuric acid (>98.0%); thiamine hydrochloride (99%) were supplied from Merck (Darmstadt, Germany). The yeast extract, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonate), ABTS (>98.0%), potato dextrose agar (PDA), and potato dextrose broth (PDB) were purchased by Sigma–Aldrich, St. Louis, MO, USA.

2.2. Organism and media

Pleurotus florida strain 285 (gau.1999.511) was kindly provided from the culture collection of the Department of Plant Protection, Gorgan University of Agricultural Sciences and Natural Resources. The fungal strain was preserved on PDA slants at 4°C, with periodic subculture for ten days of incubation at 25±2°C in the dark.

2.3. Culture conditions

Liquid PDB medium was used to determine maximum laccase production at different time intervals, 0-20 days (Zhu et al., 2016). Cultures were inoculated with three fungal agar discs (5 mm diameter) of 8 days old culture and incubated at 25±2°C in the dark. The experiments were performed in duplicate.

The submerged (SmF) fermentation cultures were performed in 250 mL flasks containing 50 mL of a basal medium composed of 0.5 gL^{-1} K_2HPO_4, 0.5 gL^{-1} KCl, 0.1 gL^{-1} $CaCl_2$, 0.25 gL^{-1} $MgSO_4.7H_2O$, 2.94 gL^{-1} sodium citrate, 2.1 gL^{-1} citric acid, 0.5 gL^{-1} thiamine with various glucose (0-3.3%, W/V), yeast extract (0-1.2%, W/V), or copper sulfate (0-1.2%, W/V) concentrations, as the best carbon, nitrogen, and inducer sources for laccase production in ***P. ostreatus***, respectively (Table 1). The experiment range was determined based on previous studies on laccase-increasing activities in SmF cultures for *P. ostreatus*, and zero in all cases were tested for limitation of each independent (Durán-Sequeda et al., 2022; Hazuchová et al., 2017; Zhu et al., 2016). The final pH was adjusted to 6.5 with 0.1M NaOH (Durán-Sequeda et al., 2022).

All SmF cultures were inoculated with three fungal agar discs (5 mm diameter) of 8 days old culture and incubated at 25±2°C in the dark for 14 days. All experiments were performed in duplicate.

2.4. Biochemical analyses

Laccase activity was monitored using ABTS (2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate) as the substrate. The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and was incubated for 5 min. One unit (U) of enzyme activity was determined spectrophotometrically at 436 nm as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min (Galhaup et al., 2002).

2.5. Optimization of laccase production using the RSM model

The Box-Behnken experimental design (BBD) was used to evaluate the influence of the three independent variables, namely carbon, nitrogen, and inducer concentrations (Table 1), as a 22-run experiment on laccase production in SmF cultures. The response was calculated from the following equation using Design-Expert version 10.0.4 (Sanei & Ahmad khan, 2019):

$$y_r = \alpha_0+\alpha_1G+\alpha_2Ye + \alpha_3Cs + \alpha_{12}GYe + \alpha_{13}GCs+\alpha_{23}YeCs + \alpha_{11}G^2 + \alpha_{22}Ye^2 + \alpha_{33}Cs^2 \quad (1)$$

Where y_r is the laccase production, and G, Ye, and Cs are the glucose, yeast extract, and copper sulfate concentrations, respectively. Analysis of variance (ANOVA) was used to determine the statistical significance of the independent variables and their interactions.

Mathematical methods were statistically evaluated by calculating the mean absolute error (MAE), the MSE, and the coefficient of determination (R^2) according to the following equations:

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(y_{a,i} - y_{p,i})^2 \quad (2)$$

$$R^2 = \frac{\sum_{i=1}^{n}(y_{p,i} - y_{a,i})^2}{\sum_{i=1}^{n}(y_{p,i} - y_m)^2} \quad (3)$$

$$MAE = \sum_{i=1}^{n}\left|\frac{y_{p,i} - y_{a,i}}{n}\right| \quad (4)$$

Where $y_{a,i}$ and $y_{p,j}$ are the measured and calculated values of the laccase activity, y_m is the mean value of laccase activity, and n is the number of experimental measurements. The *main*

effects and interactions between factors *were determined* on chosen variables of laccase activity (the response variable) by Factorial ANOVAs. The F-test was used for to determine statistical significance of the quadratic.

Table 1. Experimental range and levels of independent variables.

Variables	Symbols	Range and levels	
		-1	1
Glucose concentration (Carbon source*, gL^{-1})	G	0	33
Yeast extract concentration (nitrogen source, gL^{-1})	Ye	0	12.5
Copper sulfate concentration (Inducer, mM)	Cs	0	2

3. Results and Discussion

The laccase activity in *P. florida* dramatically increased at various time intervals (Fig. 1). The highest laccase activity in the PDB medium (1.56 UmL^{-1}) was obtained by the 14th day of cultivation and became partially constant thereafter. The relationship between time (x) and maximum laccase activity (y) was described by the $y= -0.0144x^2 + 0.4913x - 1.0578$ model ($R^2 = 0.9633$ and $p<0.001$). Considering the results, the effects of nutritional factors and inducers on laccase activity were screened on the 14th day.

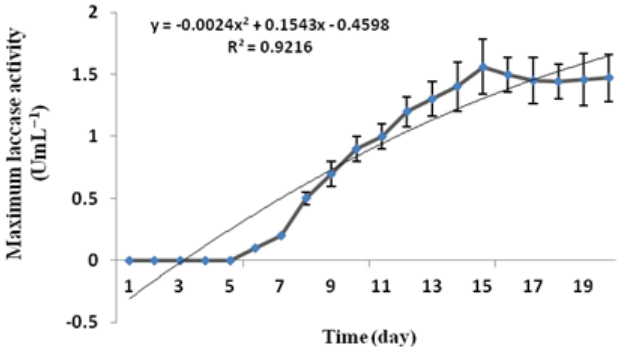

Figure. 1. Time course (days) of laccase activity (UmL-1) of Pleurotus florida cultivated in potato dextrose broth medium.

The responses of the laccase production carried out by RSM are listed in (Table 2). The model showed a second-order regression equation for the best description of the laccase production as a function of glucose (G), yeast extract (Ye), and copper sulfate (Cs) concentrations (Eq. 5):

$$Y_r = -0.1639 + 0.0895G + 0.7952Ye + 3.3436Cs + 5.2366\times10^{-3}GYe - 0.0164GCs - 0.0927YeCs - 3.4371\times10^{-3}G^2 - 0.471Ye^2 - 1.1774Cs^2 \quad (5)$$

The changes in response (laccase production) as a function of independent variables (glucose, YE, and copper sulfate concentrations) fit a quadratic model with a very low p-value (<0.0001). The natural logarithm of the residual sum of the square against the confidence interval showed a sudden dip, with a minimum in the region of the best optimum value (Fig. 2). As the current value of the confidence interval is close to the optimum value, a data transformation is not required. The maximum and minimum confidence interval value of the model is 2.29 and 0.89, respectively (Fig. 2).

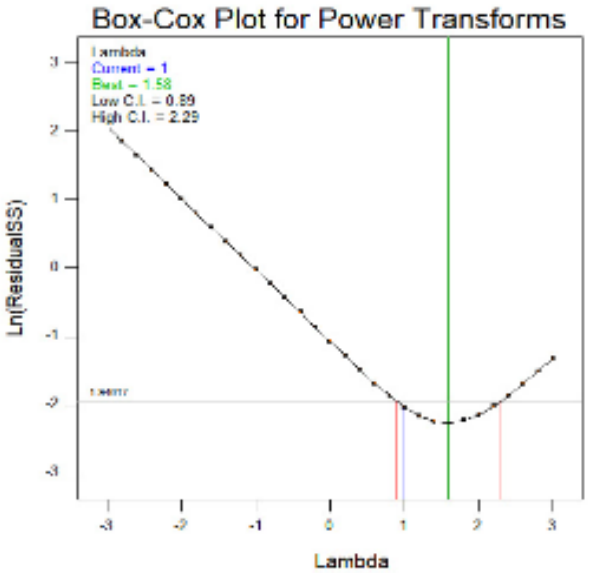

Figure. 2. Box–Cox plot for power transforms of glucose, yeast extract, and copper sulfate concentrations on laccase production by *Pleurotus florida*.

All individual independent variables (except for glucose) and other multiple interactions had a significant effect on the laccase production. A coefficient R^2 of 0.9906 showed a strong relationship between the laccase production (as a response) and independent variables. A Adeq precision value much higher than 4, the signal-to-noise ratio, refers to the navigation of the model for design space. A non-significant lack of fit value of 0.6885 shows that the quadratic model was valid for the present study. The coefficient value of copper sulfate is higher than other individual independent variables (except for glucose) and other multiple interactions (Eq. 5), which shows the positive and significant effect of variables on the laccase production.

Three-dimensional response surface graphs and their corresponding contour plots that show the effects of the independent variables on laccase activity are shown in (Figs. 3-5). These graphs, as optimization processes, allow defining the optimal conditions for maximum laccase production.

Table 2. Analysis of variance of the response surface methodology (RSM) method for glucose, yeast extract, and copper concentrations on laccase enzyme production in *Pleurotus ostreatus*.

Source of variance	Coefficient value	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
Model	-	13.78	9	1.53	140.25	<0.0001[a]
A-Glucose	0.0895	0.018	1	0.018	1.68	0.2199
B-Yeast extract	0.7952	1.81	1	1.81	165.62	<0.0001
C-Copper sulfate	3.3436	0.058	1	0.058	5.30	0.0401
AB	5.2366×10^{-3}	0.20	1	0.20	18.28	0.0011
AC	0.0164	0.076	1	0.076	6.99	0.0214
BC	0.0927	0.047	1	0.047	42.79	<0.0001
A^2	3.4371×10^{-3}	0.73	1	0.73	67.32	<0.0001
B^2	0.471	1.47	1	1.47	26.50	<0.0001
C^2	1.1774	3.38	1	3.38	309.86	<0.0001
Residual	-	0.13	12	0.011		
Core total	-	13.91	21			
Adeq Precision	43.299					
Lack of fit	0.6885					
R^2	0.9906					

[a] Statistically significant at the confidence level of 95%.

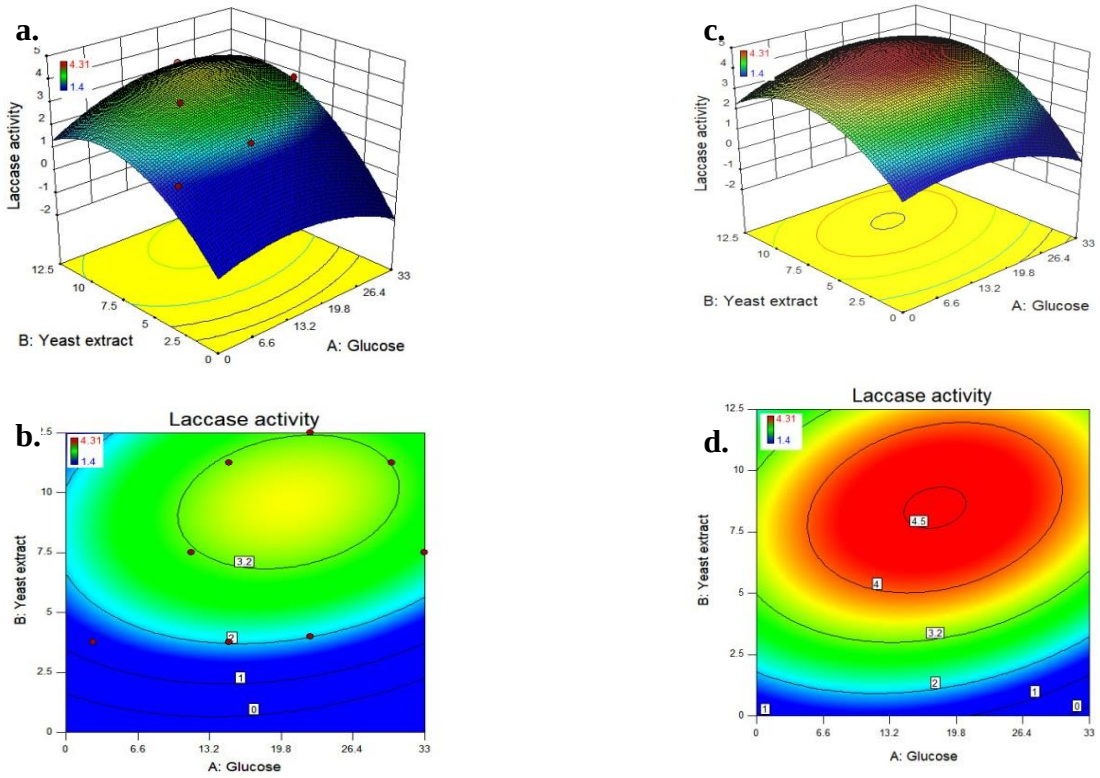

Figure. 3. The response surface (a, c) and corresponding contour (b, d) plots for the predicted laccase production as a function of the glucose (as carbon) and the yeast extract (as nitrogen) concentrations in the absence (0 mM; a, b) or presence (0.93 mM; c, d) mM of copper sulfate (as inducer). Two variables are considered simultaneously, while the third one remains constant.

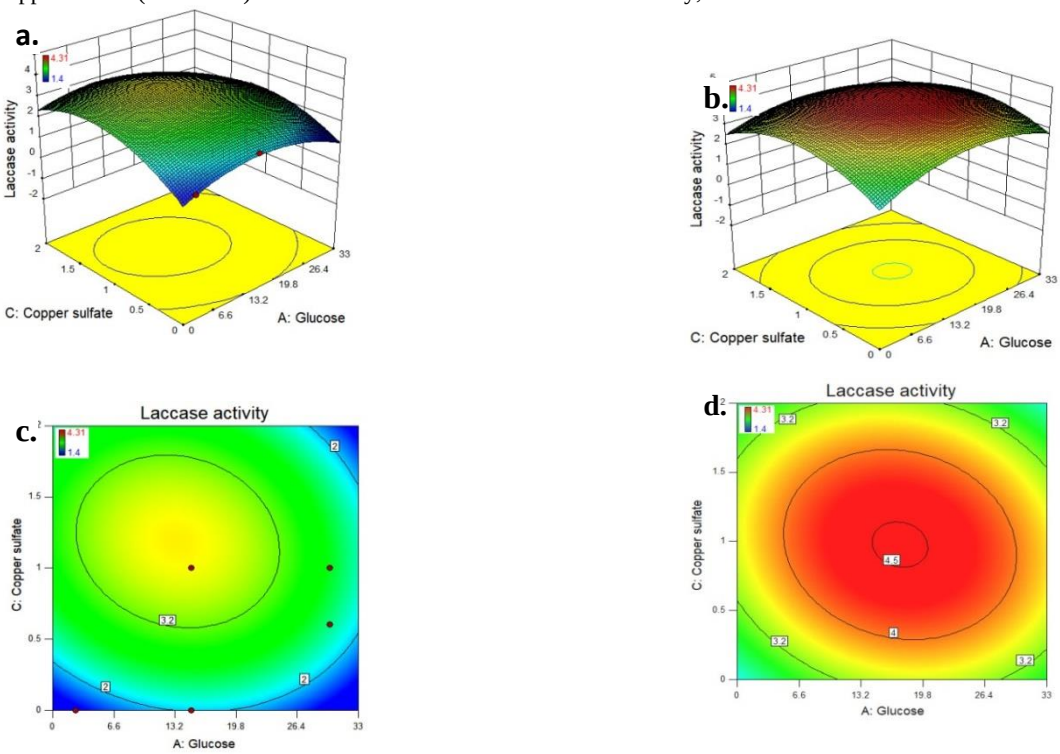

Figure. 4. The response surface (a, c) and corresponding contour (b, d) plots for the predicted laccase production as a function of the glucose (as carbon) and the copper sulfate (as inducer) concentrations for yeast extract (as nitrogen) concentration of 0 (a, b) or 8.22 (c, d) gL^{-1}. Two variables are considered simultaneously, while the third one remains constant.

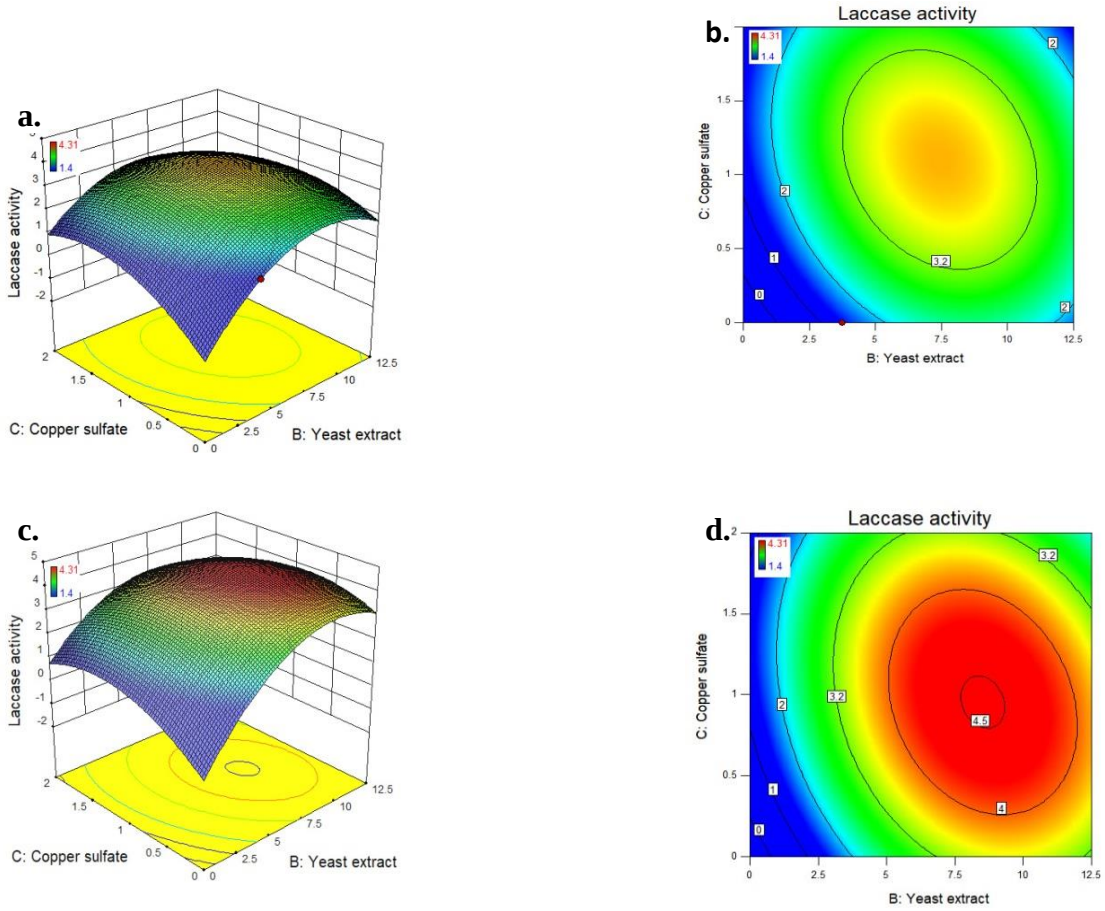

Figure. 5. The response surface (a, c) and corresponding contour (b, d) plots for the predicted laccase production as a function of the copper sulfate (as inducer) and the yeast extract (as nitrogen) concentrations for glucose (as carbon) concentration of 0 (a, b) or 18.70 (c, d) gL^{-1}. Two variables are considered simultaneously, while the third one remains constant.

The effect of the glucose and the yeast extract concentrations on the laccase production is shown in Fig. 3. The enzyme production gradually increased, reached a high, and then declined as each variable increased. The shape of the contours shows the significant effect of the interaction between the carbon and the nitrogen concentrations (Fig. 3). According to the BBD model (Eq. 5), the maximum laccase production was affected by the main effect of yeast extract concentration and the interaction between the glucose (18.70 gL^{-1}) and yeast extract (8.22 gL^{-1}) concentrations, both in the absence or presence of copper sulfate.

Optimum enzyme production with copper sulfate (0.93 mM) was 4.4-fold greater than the medium without copper sulfate (Fig. 2a and c). The maximum predicted laccase activities with this medium composition was 5.28 UmL^{-1}.

The shape of the contours in Fig. 4 shows the significant interaction of the glucose (as carbon) and the copper on the laccase production. The enzyme production generally increased, reached a plateau, and then decreased as the independent variables increased. The interaction can be changed by the presence of 3.75 or 8.20 gL^{-1} of yeast extract due to the positive effect of the concentration of this variable on laccase production.

(Fig. 4) shows that laccase production increased with the increase of the yeast extract and the copper concentrations at 18.70 gL^{-1} glucose concentration. The shape of the contours showed the significant interaction between the

concentration yeast extract and the copper (Fig. 5). According to the RSM model, the maximum production of laccase by *P. florida* can be obtained in 18.70 gL^{-1} glucose concentration under the conditions of action: the 8.22 yeast extract and the 0.93 mM copper sulfate

Microbial enzymes are widely used as cost-efficient and *eco*-friendly *alternatives* for chemical processing in different industries and bioremediation. Therefore the global demand for microbial enzymes is *drastically increasing*. Due to a broad range of substrates and biological characteristics, laccases have attracted much research attention. Under optimal propagation conditions, such as the fermentation process, the secretion of lignocellulolytic enzymes can be enhanced for commercial production (Chmelová & Ondrejovič, 2016; Kneževic et al., 2013. Gonzáles et al., 2013).

Liu et al. (2009) observed that although *P. ostreatus* was able to use several pentoses and hexoses, glucose was the best nutritional factor for fungal biomass and enzyme production. Similarly, nitrogen sources, especially organic alternatives, such as yeast extract, have been reported to affect laccase activity in *P. ostreatus* and other white-rot fungi (El-Batal et al., 2015; Kachlishvili et al., 2006). Induced laccase activity and isoform enzymes were also identified by copper in *Pleurotus* spp. (Palmieri et al., 2000; Piscitelli et al., 2011).

In this study, we evaluated the effect of glucose, yeast extract (as two nutritional components), and copper sulfate (as an inducer) on laccase production in *P. florida*. Our results propose that carbon- and nitrogen-sufficient conditions can improve enzyme activity, similar to reports with other species of *Pleurotus* (Durán-Sequeda et al., 2022; Karp et al., 2015; Zhu et al., 2016). However, a more than two-fold increase in laccase activity resulted from increasing the glucose concentration from 2.5 to 11 gL^{-1}; an additional increase to 30 gL^{-1} in glucose concentration did not enhance the laccase activity. Conversely, Periasamy et al. (2010) obtained lower enzyme activities in their study. In contrast, Hazuchová et al. (2017) and Yang et al. (2016) found that low nitrogen concentrations greatly decreased laccase production for different carbon concentrations. Our results show the positive effect of yeast extract concentrations on laccase activity. A similar effect was also described for both submerged- (Zhu et al., 2016) and solid-state fermentation (Karp et al., 2015). Our results also showed that different copper sulfate concentrations strongly improve the effect of the nutritional components on laccase activity. These findings explain the 4.4-fold increase in laccase activity (5.28 UmL^{-1}) that occurred in the **presence** of copper compared to cultures without copper with an optimal concentration of glucose and yeast extract as carbon and nitrogen sources, respectively. Similar results of copper stimulation of the laccase expression in *Pleurotus* spp. (Baldrian & Gabriel, 2002; Faraco et al., 2003; Soden et al., 2001; Stajic et al., 2011) and in *Trametes pubescens* (Galhaup et al., 2002a, b) were obtained. Although optimum enzyme production in the culture medium with copper was experimentally lower than in Durán-Sequeda et al. (2022) and Zhu et al. (2016), this may be explained by different species and strains of fungi.

4. Conclusions

The present study explains a partial analysis of the culture composition of laccase activity in *P. florida*. This analysis determined the positive synergistic effect of glucose and yeast extract concentrations for improved enzyme production induced by copper. The highest production was observed in the culture medium with 18.70 and 8.22 gl^{-1} of glucose and yeast extract concentrations, respectively. An increase in copper-dependent laccase production was optimized by adding 0.93 mM copper sulfate. Further experimental investigations are needed to focus on the regulation **mechanism** of the laccase expression, which may offer essential information to increase laccase production.

Abbreviation

HPV: Human papillomaviruses; MHC: Major histocompatibility complex; HLA: Leukocyte antigen; PC: Population coverage

Conflict of Interest

The authors declare that there is no conflict of interest.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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