

SHORT COMMUNICATION

***Botryosphaeria* spp. as Producers of Laccase When Cultivated on Vegetable Oils as Sole Carbon Source: Optimizing Laccase Production by *Botryosphaeria rhodina* MAMB-05 on Soybean Oil**

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Abstract:

Eight vegetable oils and glycerol were examined as sole carbon source to screen nine isolates of *Botryosphaeria* spp. Among these, *B. rhodina* MAMB-05 produced highest laccase titres on all carbon sources evaluated followed by *B. rhodina* MMLR isolated (from orange, *Citrus* sp.). The influence of inorganic nitrogen (NH_4Cl , NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3), organic nitrogen (yeast extract (YE), urea, peptone, and corn steep liquor) sources, inorganic phosphate (KH_2PO_4 , Na_2HPO_4 , NaH_2PO_4 , K_2HPO_4), as well as increasing concentrations of copper (from zero to $100 \mu\text{g mL}^{-1}$), as nutritional requirements for enhancing laccase production by *B. rhodina* MAMB-05 were also evaluated. Soybean oil was the only carbon source in a central composite factorial design (2^3) to optimize laccase production with the selected variables NaNO_3 , KH_2PO_4 and copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), using the response surface methodology. The optimal conditions obtained were NaNO_3 (100 g L^{-1}), KH_2PO_4 (125 g L^{-1}) and $50 \mu\text{g mL}^{-1}$ of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The specific laccase activity obtained (2.95 U mg^{-1}) in the validation experiments using the optimal condition for laccase production by *B. rhodina* MAMB-05 was in agreement with the predicted value (3.07 U mg^{-1}), and demonstrated the fitness of the proposed model.

Keywords: *Botryosphaeria rhodina* MAMB-05; laccase; soybean oil, glycerol; response surface methodology

1. Introduction

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are multi-copper oxidases that reduce molecular oxygen to water, simultaneously performing an one-electron oxidation of various aromatic substrates in the reduced state such as diphenols, monophenols, and aromatic amines [1-3]. Fungal laccases have been studied and applied to several biotechnological processes as a treatment of

agro-industrial wastes, in fruit juice processing, clarification of wine and beer, and biobleaching of cellulose pulps for paper [4-8]. These enzymes are also implicated in the pathogenicity and morphogenesis of a diversity of organisms, including ligninolytic fungi [9].

A strain of *Botryosphaeria* sp. isolated from an eucalypt stem canker [10] and classified as *Botryosphaeria rhodina* MAMB-05 [11] has been described as a constitutive producer of laccase

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[12], and enzyme titres could be enhanced through induction by veratryl alcohol [13, 14, 15] and copper [16], and was also influenced by aeration [17], and the type of carbon and nitrogen (N) sources used [15-18].

A study of the conditions of cultivation is essential for optimization of processes in the production of enzymes such as laccases, in order to produce large amounts of enzyme in media supplemented only with cheap and accessible substrates, making scale-up production economically viable [19]. Palm oil fibre and olive oil mill wastewater have been described as raw resources used in the production of laccases by filamentous fungi [20, 21]. In previous work on laccase production by *B. rhodina* MAMB-05, Dekker et al. [16] found that soybean oil when incorporated into basal medium was able to produce laccase on this simple and inexpensive fermentation substrate. However, the effect of concentration of soybean oil and other fermentation parameters were not optimized in that work.

Response surface methodology (RSM) is an effective statistical technique to investigate complex processes and has been used in the optimization of laccase production by different fungal species [13, 22-24]. The advantage of statistical factorial design is the reduced number of experimental runs required to provide sufficient information for statistically-acceptable results. Furthermore, it is a faster and less-expensive method for gathering research data than the classical experimental methods [25].

In the work reported herein, eight vegetable oils and glycerol were examined as sole carbon sources by nine isolates of *Botryosphaeria* spp. for the production of laccases. Among these were five teleomorphs morphologically identified as *Lasiodiplodia theobromae* isolated from rotting tropical fruits [11]. *B. rhodina* MAMB-05 was found to be the best laccase producer. The influence of inorganic and organic nitrogen sources, phosphate and copper to increase laccase production using soybean oil as sole carbon source was also evaluated. Laccase production by *B. rhodina* MAMB-05 on soybean oil was optimized examining the composition of the nutrient medium by means of factorial design and RSM. This information will contribute to knowledge on the production, properties and

applications of the enzymes produced by *B. rhodina* MAMB-05, and the development of new biotechnological processes.

2. Results and Discussion

The use of surfactants and soybean oil as carbon sources to produce laccases by *B. rhodina* MAMB-05 was previously reported when these were added to basal medium [16]. In the present work, different plant seed oils and glycerol were examined as sole carbon sources to produce laccase by nine isolates of *Botryosphaeria* spp. (Table 1).

B. rhodina MAMB-05 was the best laccase-producing isolate among those examined with highest enzyme titers (expressed as specific activity) resulting from glycerol (25.8 U mg⁻¹), followed by babassu (14.7 U mg⁻¹) and maize (9.3 U mg⁻¹) oils. Among all of the *Botryosphaeria* spp. examined, only *B. rhodina* isolates MAMB-05 and MMLR produced laccase in significant amounts. Both isolates were also considered the best laccase producers when cultivated on glucose as sole carbon source [11]. Earlier studies [12] revealed that glycerol (1.5 % w v⁻¹) was an important carbon source in enhancing laccase activity by *B. rhodina* MAMB-05, and this was also found in our work (Table 1). Dijskstra et al. [26] demonstrated that olive oil and soybean oil separately stimulated growth and laccase production by *Agaricus bisporus* using a casein-malt extract medium. The use of an emulsion with soybean oil and Tween-80 and glucose as carbohydrate substrate also positively influenced laccase and biomass production by a basidiomyceteous fungus, *Trametes villosa* CCB176 [27].

Nutritional requirements for laccase production by *B. rhodina* MAMB-05 on soybean oil (1 % v v⁻¹) were examined, as different N and P sources have been reported to enhance laccase production by some microorganisms [16, 28]. In the present work, the best N source for laccase production by *B. rhodina* MAMB-05 grown on soybean oil as sole carbon source was sodium nitrate (2.49 U mg⁻¹), while for ammonium nitrate (the N source present in VMSM), the activity was considerably lower (0.05 U mg⁻¹) (Figure 1A).

Cavallazzi et al. [29] investigated the effect of ammonium tartrate on the production of laccase by *Lentinula edodes*, and showed that the

appropriate N source greatly influenced the production of this enzyme. The same was found for laccase production by *Pestalotiopsis* sp., where ammonium tartrate was chosen as the best N source among urea, YE and ammonium chloride [30]. Dekker et al. [16] reported that ammonium chloride reduced laccase titres in *B. rhodina* MAMB-05 when cultured only on glucose as carbon source, even in the presence of veratryl alcohol, an inducer of laccase production [17]. According to the authors, this effect may be explained by the potential inhibitory action that chloride ion exerted on the activity of laccase [17, 26]. The assimilation of different nitrogen sources for each microorganism varies, and is strongly influenced by the characteristics of cultivation [31]. Yeast extract and corn syrup liquor that are rich in organic N sources also enhanced laccase activity (Figure 1A).

According to Figure 1B, potassium phosphate was the best P source for laccase production by *B. rhodina* MAMB-05 on soybean oil. Fungal biomass was positively affected (9.76 g L^{-1}) by the absence of P when compared to VMSM medium containing potassium phosphate (6.12 g L^{-1}) or sodium phosphate (4.43 g L^{-1}). In *Streptomyces psammoticus* MTCC 7334, magnesium phosphate was found as the optimal P source to enhance laccase yields [24]. In a series of studies to determine the influence of P on laccase production, Cavallazzi et al. [29, 32] observed that potassium phosphate was the best P source for laccase production by *Lepistasordida* and *Lentinus edodes* when compared to other salt sources.

Production of laccase by different fungi is also regulated by the presence of copper, which is present in the active site of the enzyme structure. Dekker et al. [16] tested different concentrations of copper ($0 - 40 \text{ } \mu\text{g Cu mL}^{-1}$) for the production of laccase by *B. rhodina* MAMB-05 growing on glucose, and found that in the absence of copper, the fungus produced only minor levels of laccase, suggesting that this fungus was copper-dependent for the production of this enzyme.

Palmieri et al. [33] reported that copper induced laccase production in cultures of *Trametes versicolor* and *Phanerochaete chrysosporium* when these strains were grown on glucose. The production of this enzyme by *Pleurotus ostreatus* also significantly increased

with higher concentrations of copper added to the nutrient medium. Copper also enhanced laccase production in *Trametes pubescens* MB89 [34], and *Phanerochaete chrysosporium* NCIM 1197 [35] using glucose-based medium.

Although the amount of copper required for the production of laccase by *B. rhodina* MAMB-05 in the presence of glucose varied between 0.003 and $40 \text{ } \mu\text{g mL}^{-1}$ [16], the present study found that in the presence of soybean oil as sole carbon source, the appropriate concentration of copper was higher, $50 \text{ } \mu\text{g mL}^{-1}$. In a related study, laccase production by *B. rhodina* MAMB-05 on poplar hemicellulose prehydrolyzate was optimized by a 3^3 -factorial design, resulting in optimal enzyme titers (36.37 U mL^{-1}) by varying the concentration of xylose in the prehydrolyzate to $0.06 \text{ } \%$ w v⁻¹, together with adding copper ($2 \text{ mM CuSO}_4 \cdot 5\text{H}_2\text{O}$) as laccase inducer, and glycerol ($1.5 \text{ } \%$ w v⁻¹) to increase the level of fermentable substrate [12].

Biomass production was found to be negatively affected (reduced by $\sim 16 \text{ } \%$) at concentrations of copper in the culture medium greater than $41 \text{ } \mu\text{g mL}^{-1}$. Baldrian and Gabriel [36] similarly found that the biomass produced by *Pleurotus ostreatus* was affected by the level of copper being less in the presence of $5 \text{ mM CuSO}_4 \cdot 5\text{H}_2\text{O}$.

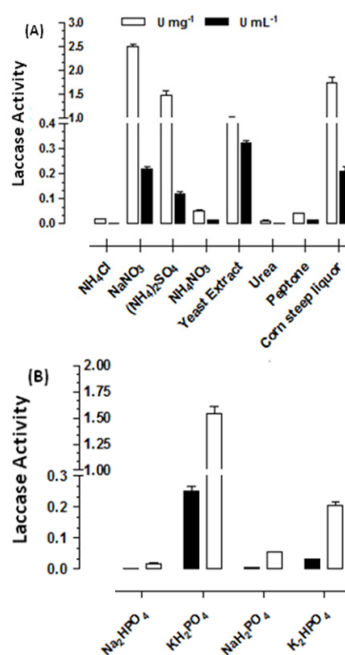


Figure 1. Production of laccases by *Botryosphaeria rhodina* MAMB-05 grown on soybean oil as sole carbon source: Effect of different (A) nitrogen and (B) phosphate sources.

Table 1. Specific activities of laccase produced by nine isolates of *Botryosphaeria* spp. cultivated on vegetable oils and glycerol as sole carbon sources.

Isolates of <i>Botryosphaeria</i> spp.	Specific laccase activity (U mg ⁻¹)								
	Vegetable oils								Glycerol
	Soybean	Olive	Sunflower	Maize	Canola	Babassu	Sesame	Cotton	
<i>B. rhodina</i> MAMB-05	5.1 ± 1.60	2.4 ± 0.09	4.4 ± 3.16	9.3 ± 2.77	6.9 ± 6.27	14.7 ± 4.74	7.7 ± 2.67	7.4 ± 1.45	25.8 ± 0.96
<i>B. ribis</i> EC-01	0.01 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.02
<i>B. rhodina</i> MC-01	0.1 ± 0.06	0.1 ± 0.01	0.2 ± 0.02	0.1 ± 0.00	0.2 ± 0.07	0.1 ± 0.00	0.3 ± 0.05	0.2 ± 0.10	0.7 ± 0.40
<i>B. rhodina</i> MMBJ*	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.01	0.2 ± 0.04	0.1 ± 0.01	0.2 ± 0.10
<i>B. rhodina</i> MMGR*	0.1 ± 0.02	0.05 ± 0.04	0.02 ± 0.00	0.06 ± 0.03	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.03	0.04 ± 0.01
<i>B. rhodina</i> MMLR*	2.7 ± 0.48	2.7 ± 2.48	2.6 ± 1.46	1.8 ± 0.85	1.4 ± 0.33	0.3 ± 0.06	3.9 ± 1.39	2.0 ± 1.37	0.9 ± 0.00
<i>B. rhodina</i> MMMFO*	0.03 ± 0.01	0.2 ± 0.14	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.04	0.04 ± 0.00	0.02 ± 0.00	0.1 ± 0.01
<i>B. rhodina</i> MMMFR*	0.1 ± 0.03	0.03 ± 0.00	0.1 ± 0.02	0.3 ± 0.22	0.1 ± 0.01	0.08 ± 0.04	0.4 ± 0.28	0.1 ± 0.03	0.2 ± 0.03
<i>B. rhodina</i> MMPI*	0.1 ± 0.01	0.03 ± 0.01	0.09 ± 0.02	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.08	0.1 ± 0.06	0.2 ± 0.05

* classified morphological as the teleomorphic form, *Lasiodiplodia theobromae* [11].

Besides these advantages, Tychanowicz et al. [37] and Hao et al. [30] reported that high concentrations of copper and aromatic compounds increased the production of laccase by the ascomycete *Podospora anserina*, and that this enzyme acted in a defense mechanism against oxidative stress caused by free radicals. The mechanism of action was based on the formation of a chelate between Cu^{2+} ion during the synthesis of laccase. For *Trichoderma harzianum* ZF-2, the interactions between different fermentation parameters for laccase production were characterized using a Plackett-Burman design and the response surface methodology.

Wheat straw powder, soybean meal, and CuSO_4 were all found to have a significant influence on laccase production, and the resulting optimal medium components were determined as follows: wheat straw powder 7.63 g L^{-1} , soybean meal 23.07 g L^{-1} , $(\text{NH}_4)_2\text{SO}_4$ 1 g L^{-1} , CuSO_4 0.51 g L^{-1} , Tween-20 1 g L^{-1} , MgSO_4 1 g L^{-1} , and KH_2PO_4 0.6 g L^{-1} [38].

Based upon the above findings, the laccase production by *B. rhodina* MAMB-05 using soybean oil as carbon source was optimized based upon response surface methodology using different concentrations of copper, KH_2PO_4 and NaNO_3 as indicated in Table 2.

Table 2. Central-composite experimental-design matrix defining conditions for concentrations of copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), KH_2PO_4 and NaNO_3 on laccase production by *Botryosphaeria rhodina* MAMB-05.

Variables In Coded Levels				Responses			
Runs	x_1	x_2	x_3	Laccase activity (U mL^{-1})	Specific activity (U mL^{-1})	Final pH	Biomass (g L^{-1})
1	-1	-1	-1	0.02	0.02	8.2	7.54
2	+1	-1	-1	0.01	0.007	8.6	7.16
3	-1	+1	-1	0.62	1.52	6.6	6.50
4	+1	+1	-1	1.22	2.26	6.7	8.31
5	-1	-1	+1	0.02	0.01	8.7	7.57
6	+1	-1	+1	1.11	0.57	7.8	3.95
7	-1	+1	+1	0.99	1.82	6.6	7.89
8	+1	+1	+1	1.88	2.02	7.3	8.80
9	+1.68	0	0	0.02	0.01	8.4	7.37
10	-1.68	0	0	0.003	0.004	9.0	8.21
11	0	-1	0	0.004	0.001	9.3	2.64
12	0	+1.68	0	0.60	0.90	6.5	7.21
13	0	0	+1.68	0.003	0.001	8.7	3.06
14	0	0	-1.68	0.57	0.29	6.8	5.14
15 (0)	0	0	0	1.61	3.04	6.9	7.24
16 (0)	0	0	0	1.68	3.11	7.1	8.44
17 (0)	0	0	0	1.66	3.07	6.9	6.53
Factors				Real values			
	-1.68	-1	0	1	1.68		
x_1 , $[\text{Cu}^{2+}]$ ($\mu\text{g mL}^{-1}$)	8	25	50	75	100		
x_2 , $[\text{KH}_2\text{PO}_4]$ (g L^{-1})	0	0	125	250	335		
x_3 , $[\text{NaNO}_3]$ (g L^{-1})	16	50	100	150	184		

In the present work, the results presented in the Pareto chart showed the relative importance of the variable effects studied. Figure 2 shows a graphical depiction of each of the effects in the above table, where the effects were displayed in decreasing order after each effect was converted to a t-statistic by dividing it by its standard error [25].

These results clearly demonstrate that the effect of copper is the most significant factor in the production of laccase, followed by N source. The

Pareto chart also showed that the interactions of copper versus N and P were not significant at the 95% confidence level.

The R-squared value implied that 72% of the variability in the observed response values could be explained by the model. The analysis of variance (ANOVA) (Table 3) showed the lack-of-fit ($p > 0.05$) was not significant, indicating that the model was predictive. The pure error was low, indicating good reproducibility of the experimental data.

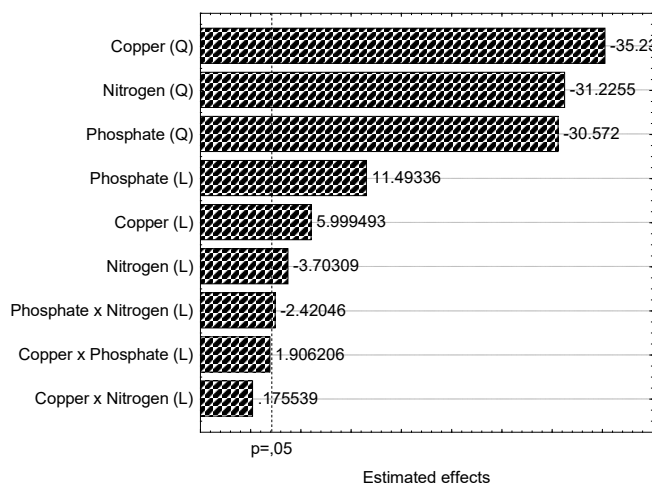


Figure 2. Pareto chart representing the analysis of variance for linear (L) and quadratic (Q) components of the parameters studied for the production of laccase by *Botryosphaeria rhodina* MAMB-05 grown on soybean oil as sole carbon source. Results are significant for $p > 0.05$.

Table 3. ANOVA data for the laccase production by *Botryosphaeria rhodina* MAMB-05 grown on soybean oil as sole carbon source, and test of significance for the regression coefficients.

Source of Variation	SQ	gf	QM	F-test	p
Copper (L)	0.3343	1	0.3343	32.69	<0.0001
Copper (Q)	12.6918	1	12.6918	1241.29	<0.0001
Phosphate (L)	1.3506	1	1.3506	132.09	<0.0001
Phosphate (Q)	9.5564	1	9.5564	934.64	<0.0001
Nitrogen (L)	0.1402	1	0.1402	13.71	0.007
Nitrogen (Q)	9.9693	1	9.9693	975.02	<0.0001
Phosphate versus Nitrogen (L)	0.0599	1	0.0599	5.85	0.02
Lack-of-fit	13.2282	7	1.8897	184.82	
Pure error	0.1942	19	0.0102		
Total	47.8235	33			

SQ, sum of squares; gf, degrees of freedom; QM, means square; L, linear components; Q, quadratic components.

Based upon the values of laccase activities observed in the different experimental runs, the optimal concentrations of copper, KH_2PO_4 and NaNO_3 for maximum production of laccase (3.07 U mg^{-1}) observed for *B. rhodina* MAMB-05 grown on soybean oil as carbon source, occurred exactly in the central point in the factorial design, which was composed of the following real values of each component KH_2PO_4 (125 g L^{-1}), NaNO_3 (100 g L^{-1}) and Cu^{2+} (50 g mL^{-1}), respectively.

Regression coefficients obtained by multiple regression analysis in terms of coded variables and their values for standard error are shown in Equation 1. All values represented are significant ($p < 0.05$), therefore, when possible, the obtained models were simplified by the elimination of terms that were not statistically significant. The second-order polynomial model was presented in its reduced form:

$$\hat{Y} = 2.747 + 0.11x_1 - 0.73x_1^2 + 0.816x_2 - 0.803x_2^2 - 0.061x_3 - 0.664x_3^2 - 0.061x_2x_3 \quad (1)$$

where specific laccase activity (Y), copper (x_1), KH_2PO_4 (x_2) and NaNO_3 (x_3) were encoded. According to this model, a decrease in the concentration of NaNO_3 (negative values of x_3) must be accompanied by a slight increase in the concentration of KH_2PO_4 ($+0.816x_2$) to get an increase in laccase titres.

Figure 3 represents the contour surfaces of the studied interactions. All three contour surfaces exhibited similar behavior for the predicted specific activity of laccase (Y), reaching maximal activity at 3.07 U mg^{-1} . Maximal experimental activities found for laccase by *B. rhodina* MAMB-05 grown on soybean oil was observed for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at $48 - 52 \text{ g mL}^{-1}$; NaNO_3 , $99 - 101 \text{ g L}^{-1}$; and KH_2PO_4 , $124 - 126 \text{ g L}^{-1}$. Vasconcelos et al. (2000) [13] optimized the production of laccase

by *Botryosphaeria* sp. in the presence of veratryl alcohol and found that the concentration of VA and time of cultivation had significant effects.

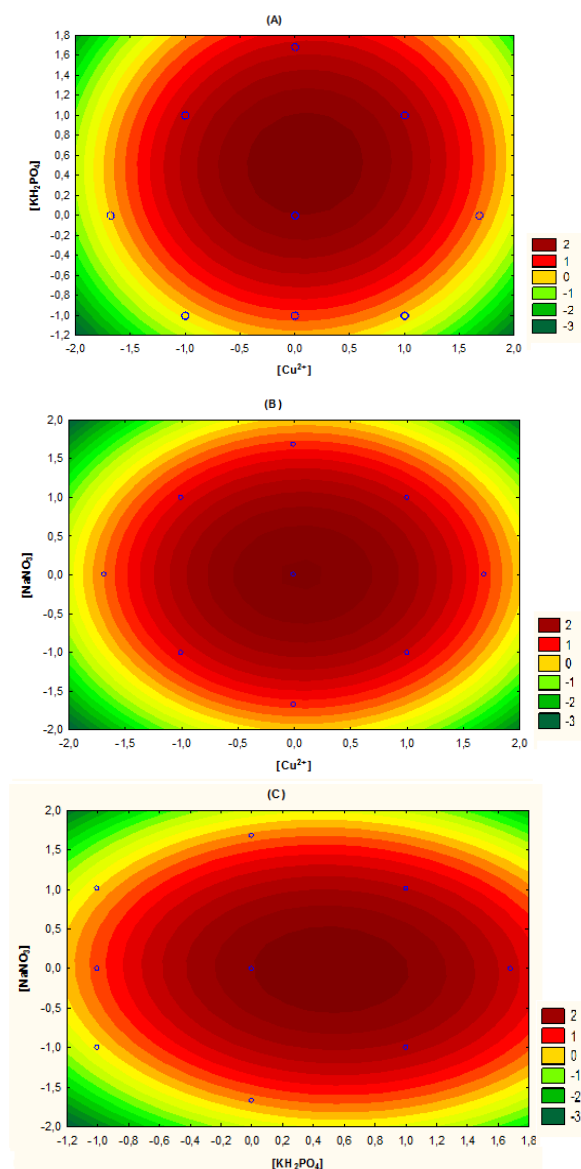


Figure 3: Contour surface plots of laccase production by *Botryosphaeria rhodina* MAMB-05 grown on soybean oil as sole carbon source showing the interactions between (A) KH_2PO_4 versus copper; (B) NaNO_3 versus KH_2PO_4 ; and (C) NaNO_3 versus copper.

Tavares et al. [39] optimized the production of laccase in *Trametes versicolor* by studying glucose concentration, initial pH and agitation rates, and obtained a mathematical model that showed that only glucose concentration and initial pH, and the interaction between both variables, were significant in the production of the enzyme.

Medeiros et al. [40] optimized the production of laccase by *Pleurotus ostreatus* studying initial pH, YE concentration and the action of some inducers, which resulted in an observation that only initial pH and YE concentration were statistically significant in enzyme production.

From the analysis of variance (ANOVA), the model was concluded to be significant ($p < 0.0001$), with a coefficient of determination $R^2 = 0.72$. This suggested that the model generated provided good correlation between experimental and predicted values. The predicted values versus the values observed confirmed that the proposed model described correlated well with the experimental data. To confirm the possible applicability of the model obtained by RSM, validation experiments were performed using the optimal conditions obtained, producing enzyme activities of laccase of 2.95 U mg^{-1} , which were in agreement with the predicted value of 3.07 U mg^{-1} , thereby demonstrating the fitness of the proposed model.

3. Material and Methods

Materials

All vegetable oils used in this study were food grade. Canola, cotton seed, maize and sunflower oils were purchased from Salada, Bunge Alimentos S/A (Brazil); sesame seed oil was from Kenko, Sakura Nakaya Alimentos S/A; olive oil from Andorinha, Sociedade Portuguesa de Azeites S/A; castor oil (mamona) from Remy Comércio e Beneficiamento de Mamona (Londrina-PR, Brazil); soybean oil from Liza, Cargil do Brazil. Babaçu oil (from babassu palmnuts (*Attalea speciosa*, sin. *Orbignya phalerata*) was kindly provided by Dr. Heizir F. de Castro, Escola de Engenharia de Lorena, USP, Lorena-SP, Brazil). Corn steep liquor (Milhocina®) was provided by Corn Products Brazil (Mogi Guaçu-SP, Brazil).

Botryosphaeria isolates

An isolate of *Botryosphaeria* sp., viz., MAMB-05, was isolated from a canker on the stem of an eucalypt tree, and was molecularly identified as *Botryosphaeria rhodina* [11]. *B. rhodina*MC-01 [41] was isolated from curupixá wood, *Micropholis* spp., in the Amazon region of Pará

(Brazil). *B. ribis* EC-01 was isolated from the stem of an *Eucalyptus citriodora* tree in São Paulo (Brazil) [41]. Isolates from five rotting Brazilian tropical fruits and an infected mango tree leaf were all morphologically identified as *L. theobromae* and molecularly classified as *B. rhodina* [11]. They are represented by *B. rhodina* MMGR (from soursop (graviola), *Annona muricata*); *B. rhodina* MMPI (from custard-apple (pinha), *Annona squamosa*); *B. rhodina* MMLR (from orange, *Citrus* sp.), *B. rhodina* MMMFR and MMMFO (respectively from mango fruit and mango tree leaf, *Mangifera* sp.), *B. rhodina* MMBJ (from eggplant (berinjela), *Solanum* sp.). All nine isolates of the *Botryosphaeria* spp. were maintained on potato-dextrose agar (PDA) slants at 4.0 ± 2 °C, and sub-cultured at three-monthly intervals.

Cultivation conditions

Inocula of the fungal isolates were prepared by growing the fungi on agar plates containing minimal salts medium (VMSM, [42]), agar (20 g L⁻¹) and glucose (10 g L⁻¹) for 120 h at 28 ± 2 °C. Each fungal isolate was grown in submerged liquid cultivation in 125-mL baffled Erlenmeyer flasks (4 baffles per flask) containing 25 mL nutrient medium comprising VMSM and vegetable seed oils (1.0 %, v v⁻¹) or glycerol (1.0 %, v v⁻¹), as sole carbon source (pH 6.0), using four 7-mm diam. agar plugs for inoculum. Cultures were incubated at 28 ± 2 °C in a rotary shaker at 180 rpm for 120 h.

In experiments where different nitrogen (N) sources were examined: inorganic N (NaNO₃, NH₄Cl, (NH₄)₂SO₄, and NH₄NO₃ - the N source of VMSM) and organic N (yeast extract (YE), peptone, urea and corn steep liquor), a modified VMSM was prepared incorporating the N sources described above, each added separately. Corn steep liquor was used as a suspension according to Messias et al. [43]. A modified VMSM was also prepared to evaluate the effect of phosphate (P) on the production of laccase by *B. rhodina* MAMB-05 by incorporating the following: K₂HPO₄, NaH₂PO₄, Na₂HPO₄ and KH₂PO₄ (the latter being the source of P in VMSM). To evaluate the effect of copper (Cu) in the nutrient medium, a VMSM was prepared omitting copper from the trace element solution. Instead a CuSO₄.5H₂O solution was added directly to VMSM to give final copper

concentrations ranging from 0 – 100 µg Cu mL⁻¹ in the nutrient medium. All experiments were performed in duplicate, and the results represent the mean value \pm SD.

Laccase assay

The cell-free extract used as the source of laccase was obtained on harvesting the fungal cultures following the removal of the mycelium by centrifugation (2,240 x g at 4 °C for 15 min). Fungal mycelium (biomass) was determined gravimetrically after drying to constant weight at 70 °C.

Laccase activity was determined using 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich, St. Louis, MO, USA) as substrate at pH 3.0 using 120 mM citrate-phosphate buffer and incubated at 50 °C for 5 min, and the absorbance measured at 420 nm (ξ = 36,000 mol⁻¹ cm⁻¹) [44]. Laccase activity is expressed in units as the number of µmols of oxidized ABTS formed per min.

Extracellular protein was determined by a modified Lowry procedure according to Hartree [45] using bovine serum albumin as standard.

Experimental Design

To optimize the production of laccase by *B. rhodina* MAMB-05 grown on soybean oil as sole carbon, a 2³ factorial central-composite design was developed, summarizing 17 experimental runs conducted in duplicate with 3 replicates at the central point (Table 2). Statistical analyses including the Pareto chart, estimated effects and 2³ factorial central-composite calculations were obtained from Experimental Design (DOE) analysis using StatisticaV. 6. www.statsoft.com (StatSoft, Inc. 2001). (Page 10).

4. Conclusions

In conclusion, this work demonstrated that *B. rhodina* MAMB-05 was able to produce laccase with an alternative substrate, soybean oil, as sole carbon source. Under optimal conditions, the enzyme activity predicted by the model agreed well with the experimental data, confirming the validity of use of RSM to optimize enzyme

production by microorganisms. Our results strongly suggest that laccase production by *B. rhodina* MAMB-05 may present interesting properties for industrial applications.

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