Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v42i1.52699

Solid-state fermentation with orange waste: optimization of Laccase production from *Pleurotus pulmonarius* CCB-20 and decolorization of synthetic dyes

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ABSTRACT. Laccases are oxidoreductase enzymes that have the ability to oxidize phenolic substrates. Its biotechnological potential has been greatly explored in many areas as biotechnology industry, bioremediation of dyes, food industry and environmental microbiology. The aim of this study was maximize the laccase production by *Pleurotus pulmonarius* (Fr.) Quélet in solid-state fermentation (SSF) using orange waste as substrate. After optimization the capability of the crude laccase to decolorize dyes was analyzed. The fermentation medium in the solid-state was optimized by applying a factorial design. After statistics optimization, laccase activity increased two times. The laccase activity appears to be correlated with the ability of crude extract to decolorize some industrial dyes. The optimized laccase was characterized with respect to optimum pH, influence of temperature and salts. Our results demonstrate that *P. pulmonarius* was an efficient producer of an important industrial enzyme, laccase, in a cheap solid-state system using orange waste as substrate.

Keywords: factory design; edible mushroom; decoloration; enzyme; *P. pulmonarius*.

Received on March 19, 2020.
Accepted on April 21, 2020.

Introduction

The orange fruit is one of the widely produced and consumed fruits in the world. Its production exceeds 80 million tons per year. Around 34% of orange production is usually made into juice, however in countries where orange is widely produced (Brazil and USA) this number rises to 96%, generating a huge amount of citric residue (approximately 50% of the total fruit weight) which may be potentially applied to biotechnology (Alexandrino, Faria, Souza, & Peralta, 2007; Giese, Dekker, & Barbosa, 2008). Orange residues are rich in insoluble polymers, including hemicellulose, cellulose, and pectin (La Torre et al., 2017).

The genus *Pleurotus* includes species of white-rot *fungi* that have ability to degrade lignin, cellulose and hemicellulose of wood due to the production of various enzymes, such as cellulase, xylanase, MnP and laccase (Freitas et al., 2017; Inácio et al., 2018). Laccases (ρ-difenol: oxygen oxidoreductase, EC 1.10.3.2) are enzymes that oxidize phenolic and non- phenolic compounds and are produced by plants, bacteria, insects and fungal species such as *Pleurotus pulmonarius* (Fr.) Quélet. (Cantele et al., 2017a).

In bioremediation, the laccases degrade phenolic compounds that are important environmental pollutants. These polyphenol oxidases have been obtained from the fermentation of agricultural waste, for example, sugarcane bagasse (Menezes, Silva, & Durrant, 2009), soybean (Sherief, El-Tanash, & Temraz, 2010) and rice straw (McCue, Horii, & Shetty, 2004). Some of these residues appear to stimulate the production of laccase (Cantele et al., 2017b) and, besides acting as cheap sources of nutrients for fungi, they help to minimize the cost of enzyme production. The use of laccase on a commercial scale is limited by the ability to obtain this enzyme in high yields via microbial processes (Brugnari et al., 2018).

Several approaches can be used to develop technologies for the production of enzymes with commercially significant cost (Oliveira, Amorim, Azevêdo, Godoy, & Freire, 2018). The response-surface

methodology (RSM) evaluates the relationship between a group of controlled experimental factors and the results observed of one or more selected variables, allowing the optimization of a process (Othman, Elsayed, Elshafei, & Hassan, 2018). The production of enzyme and the effects of several variables in the laccase production by *P. pulmonarius* developed in solid-state system using orange waste as substrate were evaluated in this work as well as the capability of the crude laccase after optimization to decolorize dyes.

Material and methods

Chemicals

ABTS, syringaldazine, DMP, starch and yeast extract, Remazol brilliant blue (RBBR), Poly R-478 and Congo red were obtained from Sigma-Aldrich, USA. Methyl violet was obtained from Merck and Brilliant Blue Reafix BFNG (Reactive Blue 268), a dye obtained from a laundry textile in Maringá, Paraná, Brazil. All other reagents were of analytical grade.

Microorganism and culture conditions

P. pulmonarius CCB-20 was obtained from the Culture Collection of the Botany Institute of São Paulo. The stock culture of the fungus was maintained at 4 ºC through periodical transfer in Petri dish containing wheat bran (20 g L^{-1}), agar (18 g L^{-1}) and mineral medium Vogel (Vogel, 1956). Orange (*Citrussinensis* (L.) Osb.) residue was obtained at the local businesses and consisted of orange waste, including membrane tissue as well as peel, after extracting the juice (solid waste). The material was dried at 40 ºC and milled (2 mm). Wheat bran was also obtained in the local businesses.

Factorial design

A central composite design (CCD), 2 ** (5-1), was employed to evaluate the influence of different variables in the production of laccase and to obtain the best conditions in citrus residue (Table 1). Five independent variables were evaluated: orange waste (X1), wheat bran (X2), starch (X3), moisture (X4) and yeast extract (X5). The experiment was carried out with a total of 30 combinations (Table 2), including 16 cubic points, 10 axial points and four central points. The results were adjusted by a second order polynomial equation. The substrates were added in Erlenmeyers flasks of 125 mL and moistened with mineral medium Vogel (Vogel, 1956) to obtain the necessary moistures. The Erlenmeyers flasks were autoclaved at 120°C for 15 min. Two disks with mycelium (7 days and 15 mm of diameter) of *P. pulmonarius* were inoculated in each Erlenmeyer flask, which were incubated at 28°C in the dark, for 12 days. Cultivation was interrupted with the addition of 20 mL of distilled water, followed by agitation for 20 min. at 8 ºC, filtration and centrifugation (10 min.; 10,000g; 4 ºC). The supernatant was used as crude enzymatic solution.

Table 1. Experimental range of the five variables studied using CCD in terms of actual and coded factors.

Independent variables	Coded levels				
	-2	-1	0	1	2
Orange waste (g) (X1)	2	2.5	3	3.5	4
Wheat bran (g) (X2)	0.5	1	1.5	2	2.5
Starch (g) (X3)	0.5	1	1.5	2	2.5
Moisture (%) (X4)	50	60	70	80	90
Yeast extract (g) (X5)	0.1	0.2	0.3	0.4	0.5

Enzymatic activity

The ligninolytic enzyme activities, laccase (EC 1.10.3.2), Mn peroxidase (EC 1.11.1.13) and lignin peroxidase (EC 1.11.1.4) were measured as described previously by Freitas et al. (2017). The aryl-alcohol oxidase (EC 1.1.3.7) was estimated as Guillén, Martínez, and Martínez (1990). The enzymatic activities were expressed in international unities per milliliter (U L^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute.

Characterization of fungal laccase after optimization

The culture filtrates were precipitated by drop-wise addition of chilled acetone and the acetone-precipitated enzymes were used in the following experiments. The molecular mass of the laccase was

determined by SDS/PAGE (Laemmli, 1970). Protein bands were visualized by silver staining. The following standards (MW-70 kit-Sigma) were used to estimate the molecular weight of the enzyme: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glucose-6-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14. 2 kDa). The laccase activity was visualized in the gel after washing with 0.05 mol L^{-1} sodium acetate buffer (pH 5.0) and incubation with 1 and 10 mmol L^{-1} ABTS for 30 min. at 4°C.

For determination of the pH optimum, the enzyme was assayed with malonate (pH 3.0, 50 mmol L^{-1}), citrate (pH 4.0–6.0, 50 mmol L^{-1}), phosphate (pH 6.0–7.5, 50 mmol L^{-1}), Tris-HCl (pH 8.0–9.5, 50 mmol L^{-1}) and glycine buffer (pH 10.0-11.0, 50 mmo L^{-1}) at 40. The effect of pH on stability of the crude laccase was determined after 24 hours incubation of laccase in 50 mmol L^{-1} sodium acetate buffer (pH 4; 4.5 and 5.0) without substrate at room temperature (25°C). The remaining activities were measured under standard conditions.

The optimum temperature of laccase was determined between 35°C and 70°C. Thermal stability was investigated by incubating the enzyme in 50 mmol L^{-1} sodium acetate buffer (pH 5.0) at 40, 50 and 60°C for different times. Immediately afterwards, the enzyme was immersed in an ice bath and then activity was tested at 40°C.

The kinetic constants was calculated using syringaldazine (ε_{525}= 65 L $mmol^{-1}$ cm^{-1}) in 100 mmol L^{-1} sodium phosphate buffer, pH 6.5, ABTS (ε_{420}= 36 $mmol^{-1}$ cm^{-1}) in 100 mmol acetate buffer, pH 5.0 and DMP (ε_{477}= 14.8 $mmol^{-1}cm^{-1}$) in 50 mmol L^{-1} malonate buffer, pH 4.5 from a Lineweaver-Burk plot. One unit of enzymatic activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per min. at 40°C. The influence of chemicals reagents $CuSO_4$, NaF, HgCl, NaCl, Na_2SO_4, NaN_3, β-mercaptoethanol were also tested in different concentrations in laccase activity.

Decolorization of synthetic dyes

To study of crude laccase capacity (after optimization) in decolorization the following dyes were used: Remazol brilliant blue (RBBR), Ethyl violet, Methyl violet, Methyl green, Methylene blue, Poly R-478, Congo red, Bromophenol blue, Green bromocresol, Methyl red and Brilliant Blue Reafix BFNG. A reaction mixture containing 2.0 mL of dye in 0.05 mol L^{-1} sodium acetate buffer (pH 5.0) and 2.0 mL crude laccase (diluted to give a final activity of 3 U) was incubated at 28°C in the dark. After 24 hours, the decrease in absorbance was measured at the maximum absorbance of each dye in a UV and VIS Shimadzu Spectrophotometer and expressed in terms of percentage. In parallel, a boiled crude laccase preparation was used as negative control.

Statistical analysis

All the analysis was carried out in triplicate. The data was expressed as the means ± standard deviations. Differences of 5% ($p < 0.05$) among the means were considered significant according to Dunnett test. The results were analyzed using the Statistica 7.0 software (StatSoft, Tulsa, Oklahoma) and GraphPad Prism® 5.0 (San Diego, USA).

Results

Optimization of laccase production

A factorial design was developed in the pursuit of studying the influence of five variables and also to verify the interactions between them on the production of laccase by *P. pulmonarius*. The selected variables were a certain amount of orange waste (g), wheat bran (g), starch (g), yeast extract (g) and moisture (%). The experimental results are shown in Table 2.

The data for the dependent variable were also analyzed and converted into natural logarithm (Lambda value equal to 0) according to the Box-Cox transformation system. The adjustment of the quadratic model to the data with second order interactions resulted in a coefficient of determination (R^2) of 0.84, indicating that 84 % of the variability in response can be explained by the model. To validate the regression coefficient, an analysis of the laccase production variance (ANOVA) was performed (Table 3). The maximum laccase activity of 25,447 U L^{-1} was observed in the experiment under the following conditions: orange waste, 3.5 g; wheat bran, 1.0 g; starch, 2.0 g; yeast extract 0.2 g,

and 80% moisture. The treatment validation confirmed the maximum activity. The variables considered significant by the model were moisture and starch, which provided a positive effect ($p < 0.05$) in the laccase production and are highlighted in Table 3. The optimal values (predicted by the model) for these variables were starch, 2.5 g and 90% moisture. Therefore, the increased amount of starch and the initial moisture content of the medium may result in higher laccase titers. However, the combination of starch with lower amount of orange waste resulted in lower colonization and low enzymatic activity (Table 2 - treatment Nº 4). Figure 1 represents the response surface showing the effect of interaction between starch and orange waste in the laccase production.

Table 2. Results of experimental range of variables studied in the optimization of laccase production using CCD in terms of actual and coded factors.

Run	A: Orange pulp (g)	B: wheat bran (g)	C: starch (g)	D: moisture (%)	E: yeast extract (g)	Laccase activity ($U\ L^{-1}$)
1	2.5	1.0	1.0	60	0.2	716
2	2.0	1.0	1.0	80	0.2	2.056
3	2.5	1.0	2.0	60	0.2	2.212
4	2.5	1.0	2.0	80	0.4	16.650
5	2.5	2.0	1.0	60	0.2	1.143
6	2.5	2.0	1.0	80	0.4	22.844
7	2.5	2.0	2.0	60	0.4	3.524
8	2.5	2.0	2.0	80	0.2	22.344
9	3.5	1.0	1.0	60	0.2	1.002
10	3.5	1.0	1.0	80	0.4	5.827
11	3.5	1.0	2.0	60	0.4	14.791
12	3.5	1.0	2.0	80	0.2	25.447
13	3.5	2.0	1.0	60	0.4	3.502
14	3.5	2.0	1.0	80	0.2	7.193
15	3.5	2.0	2.0	60	0.2	1.224
16	3.5	2.0	2.0	80	0.4	9.474
17	2.0	1.5	1.5	70	0.3	1.194
18	4.0	1.5	1.5	70	0.3	3.025
19	3.0	0.5	1.5	70	0.3	4.112
20	3.0	2.5	1.5	70	0.3	1.037
21	3.0	1.5	0.5	70	0.3	1.690
22	3.0	1.5	2.5	70	0.3	5.583
23	3.0	1.5	1.5	50	0.3	213
24	3.0	1.5	1.5	90	0.3	4.340
25	3.0	1.5	1.5	70	0.1	1.819
26	3.0	1.5	1.5	70	0.5	2.284
27	3.0	1.5	1.5	70	0.3	1.800
28	3.0	1.5	1.5	70	0.3	1.812
29	3.0	1.5	1.5	70	0.3	1.723
30	3.0	1.5	1.5	70	0.3	1.820

Table 3. ANOVA for response surface quadratic model. $R^2 = 0.84$; Adjusted $R^2 = 0.70$.

Factors	SS[a]	DF[b]	SQM[c]	F[d]	p[e]
(1) Orange waste (L)	0.32899	1	0.32899	0.84958	0.370363
Orange waste (Q)	1.11647	1	1.11647	2.88318	0.108869
(2) Wheat bran (L)	0.14854	1	0.14854	0.38359	0.544407
Wheat bran (Q)	1.35726	1	1.35726	3.50502	0.079578
(3) Starch (L)	3.55612	1	3.55612	9.18338	0.007958
Starch (Q)	2.83811	1	2.83811	7.32918	0.015540
(4) Moisture (L)	13.58469	1	13.58469	35.08129	0.000021
(5) Yeast extract (L)	0.99190	1	0.99190	2.56149	0.129052
Yeast extract (Q)	1.31488	1	1.31488	3.39556	0.083980
1Lby 2L	1.44599	1	1.44599	3.73415	0.071214
2Lby 3L	2.39673	1	2.39673	6.18936	0.024260
2Lby 4L	0.54566	1	0.54566	1.40912	0.252529
4Lby 5L	0.43118	1	0.43118	1.11347	0.307002
Experimental error	6.19575	16	0.38723		
Total SS	37.31501	29			

[a]SS – sum of squares; [b]DF – degrees of freedom; [c]SQM – sum squares mean; [d]F – F value; [e]p– significance probability ($p < 0.05$).

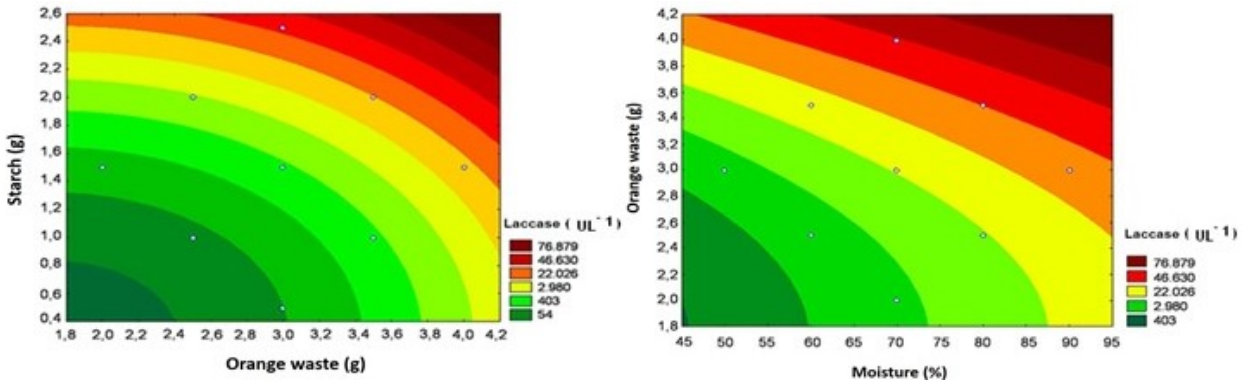

Figure 1. In a: Response surface plots showing the effect of interaction between orange waste (X1) and starch (X3) on the yield of laccase. In b: Response surface plots showing the effect of interaction between moisture (X4) and orange waste (X1) on the yield of laccase.

As a feature of the RSM, the interaction between the variables in the laccase production was easily noticed, because even in experiments where there were large pools of carbon, the enzyme activities were low when the initial moisture of the culture was at or below 70%, for example, in treatments numbers 11, 18 and 22 in Table 2.

Characterization of fungal laccase after optimization

No aryl-alcohol oxidase, Mn peroxidase and lignin peroxidase activity was detected in this crude extract. A small range of optimum pH for the oxidation of ABTS was observed, pH 4.5–5.0, whereas a relative activity of 33.1% and 8.5% at pH 6.0 and 8.0, respectively, were found (Figure 2a). The crude laccase was more stable in pH 5.0 and maintained about 60% of its initial activity after 24 hours (Figure 2b). The effect of temperature on the activity of the crude laccase is shown in Figure 2c and 2d. The temperature optimum of the laccase was 60°C with ABTS as a substrate in acetate buffer, pH 5.0, and relative activities at 70°C were 77%. The enzyme retained about 68%, 35% and 18% of its initial activity when heated for 2 hours at 40, 50 and 60°C, respectively (Figure 2d). Crude laccase retaining 100% activity after 15 min. incubation at 40°C and presented a half-life of 100 min. and 40 min. at 50 and 60°C, respectively (Figure 2d). At 4°C, the enzyme was stable for several days.

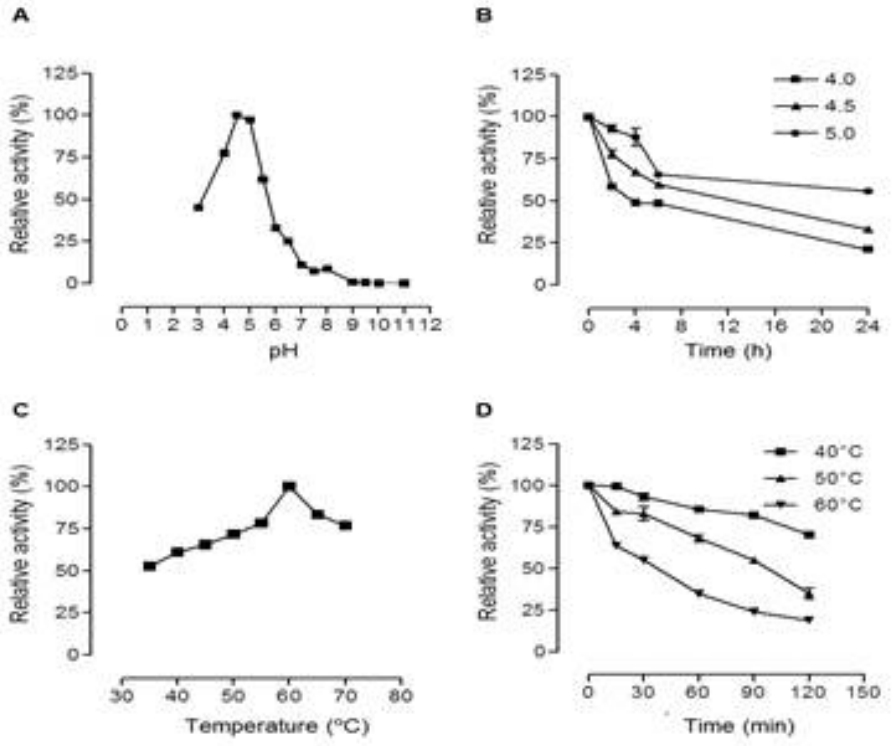

Figure 2. Effect of pH (a and b) and temperature (c and d) on the crude laccase *Pleurotus pulmonarius*.

The SDS-PAGE indicates a main band in the crude laccase with molecular weight of 23 kDa and 46.5 kDa, confirmed by the zymogram. The kinetic studies were carried out using ABTS, syringaldazine and DMP as substrate. The kinetic parameters suggest that the order of affinity toward the tested substrates was syringaldazine > ABTS > DMP. The apparent K_M value of the enzyme for syringaldazine determined from the Lineweaver-Burk plot was estimated to be 0.16 mmol L^{-1}, and the corresponding V_{max} value was 8.1 mmol $L^{-1}min^{-1}$. The K_M value of the enzyme for ABTS was estimated to be 0.43 mmol L mmol L^{-1}, and the corresponding V_{max} value was 15.9 mmol $L^{-1}min^{-1}$. For DMP, the values of K_M and V_{max} were 2.10 mmol L^{-1} and 89.84 mol $L^{-1}min^{-1}$, respectively.

The effects of several chemicals in the laccase activity were determined with ABTS as substrate (Table 4). Crude laccase was strongly inhibited by HgCl and slightly inhibited by $CuSO_4$. NaF and the reducing agent 2-mercaptoethanol caused complete inhibition of laccase activity. The potential laccase inhibitor NaN_3 stimulated laccase activity in the concentrations studied: 0.375; 1.5 and 3.0 mmol L^{-1}.

Table 4. Effect of several reagents on crude *Pleurotus pulmonarius* laccase.

Reagent	Residual activity (%)		
	0.375 mM*	1.5 mM*	3mM*
Control	100	100	100
$CuSO_4$	71	81.3	89.5
NaF	0	0	0
HgCl	22.2	12.4	5.9
NaCl	113.8	78.8	88.7
Na_2SO_4	108.9	76.1	72.3
NaN_3	118.1	129.2	122.8
β-mercaptoethanol	0	0	0

*Final concentration.

The enzyme was activated by NaCl and Na2SO4at 0.375 mmol L-1, but at higher concentrations there was inhibition.

Decolorization of synthetic dyes

The crude extract free peroxidase and aryl-alcohol oxidase activity, prepared from optimizated culture, was evaluated for enzymatic decolorization activity using ten different groups of reactive dyes (Table 5). Great decolorization occurred to bromophenol blue, green bromocresol, methyl green and RBBR at 0.05%. Methyl violet, congo red and methyl red were partially decolorized. Low decolorization was observed to methylene blue and Poly R-478. Ethyl Violet showed better decolorization in lower concentrations.

Table 5. Decolorization of some synthetic dyes by the laccase of *P. pulmonarius*.

Dye	λnm	Decolorization (%)		
		Dye concentration 0.05%	Dye concentration 0.01%	Dye concentration 0.005%
Methyl Violet	584	65.67	52.51	51.75
Congo Red	498	29.68	34.25	49.72
Poly R – 478	520	29.29	32.69	33.18
Bromophenol blue	610	98.03	91.37	82.39
Green bromocresol	640	98.43	86.06	68.35
RBBR	595	90.44	66.19	53.03
Ethyl Violet	596	19.74	71.07	73.42
Methyl Green	629	97.31	94.39	90.87
Methylene blue	665	52.51	41.71	42.61
Methylred	520	49.87	55.82	57.93
Blue Reafix BFNG	635	81.12	77.65	75.20

Discussion

The conventional methods of experimental optimization involve changing one variable, keeping the others constant. These methods do not represent the combined effect of all variables and are time consuming, because they require a large number of experiments to determine the optimal levels (Othman et al., 2018). A factorial design not only allows us to evaluate the influence of a single variable on a system, but also interactions between variables. With the new culture medium, the laccase activity obtained in this

study was two times higher than with the former medium (consisting only of orange waste and mineral solution) and the culturing time was reduced by eight days. The good correlation of the determination coefficient (R^2= 0.84) indicated that the design data were properly adjusted to the model used. Revankar and Lele (2006) studied the effect of six carbon sources in the laccase production by *Ganoderma* sp. and found that the starch showed the highest enzyme production. This effect was also observed in the culture of *P. pulmonarius* when the starch 30% (w w^{-1}) was combined with the orange waste 52% which was evidenced by the increased colonization of the substrate and high laccase activity.

The moisture is a key parameter to control the growth of microorganisms and the production of metabolites in SSF (Niladevi, Sukumaran, & Prema, 2007). High moisture was needed so that laccase activity increased significantly.

The application of laccase in biotechnological processes requires the production of large amounts of enzyme at low cost and, therefore, the current focus of research with laccase is oriented toward the identification and optimization of this efficient production system.

The activity of many laccases decrease rapidly beyond optimum pH (Téllez-Téllez, Fernández, Montiel-González, Sánchez, & Díaz-Godínez, 2008), which occurred in this study (Figure 2a). Souza and Peralta (2003) observed a large range of optimum pH (4.0–5.5) for the oxidation of ABTS in study with purified laccase of *P. pulmonarius* CCB-19. This result is in agreement with the literature, which reported pH optimum values for fungal laccases range between 4.0 and 7.0, depending on the substrate used (Neifar, Jaouani, Ghorbel, Chaabouni, & Penninckx, 2009). The temperature optimum of the laccase crude was 60°C but the stability at this temperature decreased after fifteen minutes. In studies with other white-rot fungi there was found lower temperature optimum for the laccases (Souza & Peralta, 2003; Souza, Zilly, & Peralta, 2002).

Laccases from basidiomycetes are generally monomeric protein with a molecular mass between 50 and 80 kDa, but the production of isoforms may be mainly influenced by the culture conditions (Mechini et al., 2006). Souza and Peralta (2003) found an isoform of purified laccase of *P. pulmonarius* CCB-19 after denaturing and non-denaturing SDS-PAGE with a molecular mass of 22.4 kDa, similar to this study.

Laccases can be sensitive to denaturing conditions typically found in dye containing effluents, such as high salts concentrations. These authors found that Na_2SO_4 slightly stimulated the laccase activity, even at the high concentration of 1.0 mol L^{-1} and NaCl inhibited the enzyme even at low concentrations. This behavior is not usual in fungal laccases, which are usually sensitive to several cations (Souza et al., 2002).

White rot fungi have been considered as a biological alternative for decolorization of synthetic dyes, which possess a structure similar to lignin (Zilly et al., 2011; Mota et al., 2015; Vikrant et al., 2018). The ability of *P. pulmonarius* to decolorize industrial dyes was demonstrated in several studies and is frequently correlated with the presence of ligninolytic enzymes (Souza et al., 2002; Zouari-Mechichi et al., 2006). The results on dye decolorization with the crude enzyme, without peroxidase activity, also indicated that laccase is the enzyme involved in the process, as observed by Zouari-Mechichi et al. (2006).

Conclusion

Our results support the use of orange waste as substrate for production of important industrial enzymes, in special, laccases by *P. pulmonarius*. This abundant residue is a cheap alternative for cultivation of filamentous fungi.

Acknowledgements

This Project was supported by *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES).

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