Lignin Degradation by a Novel Thermophilic and Alkaline Yellow Laccase from *Chitinophaga* sp.

Eliana Gertrudes de Macedo Lemos[1,2,3*], Bárbara Bonfá Buzzo[1,2], Natália Sarmanho Monteiro Lima[1,2], Pâmela Aparecida Maldaner Pereira[1,2], Elisângela Soares Gomes-Pepe[1,2], Camila Cesario Fernandes Sartini [2]

[1] *Department of Agricultural, Livestock and Environmental Biotechnology, São Paulo State University Júlio de Mesquita Filho, Jaboticabal, São Paulo, Brazil*
[2] *Molecular Biology Laboratory, Institute for Research in Bioenergy (IPBEN), Jaboticabal, São Paulo, Brazil.*
[3] *Agricultural Microbiology Graduate Program at UNESP, Jaboticabal, São Paulo, Brazil*
** Corresponding author's email address: eliana.lemos@unesp.br*

ABSTRACT

The study focused on characterizing a newly discovered laccase, Lac_CB10, found in the genome of *Chitinophaga* sp. CB10. Lac_CB10, with a molecular mass of 100.06 kDa, was purified and investigated using guaiacol as a substrate. The enzyme displayed extremophilic traits, showing activity under alkaline (pH 10.5) and thermophilic (80–90°C) conditions. It maintained over 50% activity for 5 hours at 80°C and 90°C. Spectrally, Lac_CB10 exhibited characteristics of yellow laccases, with an absorbance peak at 300 nm (T2/T3 site) and no peak at 600 nm (T1 site). When induced with copper, it degraded 52.27% of lignin within 32 hours. These findings underscore Lac_CB10's potential as a novel yellow laccase with thermophilic, alkaline, and lignin-degrading capabilities, making it a promising candidate for biorefinery processes and environmental bioremediation, particularly in extreme conditions.

Keywords: Yellow laccase, Thermophilic, Alcaline activity, Lignin degradation, Biorefinery.

1 INTRODUCTION

Multicopper oxidases (MCOs) constitute a superfamily of oxidoreductases that play essential catalytic roles and require copper atoms as cofactors. Within the functional diversity of MCOs, distinct subclasses, such as ascorbate oxidases (EC 1.10.3.3), bilirubin oxidases (EC 1.3.3.5), ferroxidases (EC 1.16.3.1), and laccases (EC 1.10.3.2) (1).

Laccases are the largest subfamily among MCOs. The active site of these materials is composed of four copper atoms distributed across three distinct centers, exhibiting variations in their paramagnetic resonance signals. These centers are categorized as type 1 copper centers (T1), known as blue copper, with an absorption band at approximately 600 nm; type 2 copper centers (T2), corresponding to normal copper; and type 3 copper centers (T3), termed coupled binuclear centers, which absorb light at approximately 300 nm. These centers are crucial for the efficient catalytic performance of the enzyme (2). Most laccases display two absorption peaks at the copper centers T1 (600 nm) and T2/T3 (300 nm), classifying them as blue laccases (3).

However, there are specific cases where some laccases are described as having only one absorption peak at approximately 300 nm, associated with the T2/T3 centers. These laccases are referred to by some researchers as yellow laccases and by others as white laccases. Both denominations are acceptable for characterizing laccases with such spectral characteristics (4, 5). The change in the spectrum of laccases has not been well defined to date, but the absence of blue coloring may be related to low-molecular-weight phenols derived from lignin during lignocellulose degradation, a theory first proposed by Leontievsky et al. (5). Recent theories suggest that the transition from blue to yellow laccases may include changes in oxidation levels (6), structural characteristics (7), glycosylation states (8), and interactions with metal ions (6). In addition to the spectral differences between blue and yellow laccases, the latter are capable of oxidizing nonphenolic compounds in the absence of a redox mediator (5) and stand out for their high stability and functionality under extreme conditions (5, 9).

In general, laccases are widely distributed in fungi, bacteria, plants, and insects, and due to their broad substrate specificity, laccases have been extensively explored in various biotechnological applications, such as delignification, dyeing, degradation, and decolorization of textile dyes; bioremediation of xenobiotics and organic pollutants; biobleaching; biofuel production; and biosensing (6, 7).

Lignin is the most abundant component of lignocellulosic biomass, and despite its potential, the industry considers it a byproduct due to its transformation difficulties (10). Recently, publications reporting various lignin degradation compounds using whole-cell bacterial laccase strains under different pH conditions have been published (11). Recognizing the importance of finding new bacterial laccases for industrial application, the present work introduces a new yellow laccase from *Chitinophaga* sp. CB10 that has been proven to be active and stable at high temperatures and basic pH and has promising action in lignin degradation.

2 MATERIAL & METHODS

2.1. Expression, extraction, and purification of the recombinant enzyme

The enzyme was expressed in liquid LB medium supplemented with 50 µg/mL of Kanamycin for 4 hours at 28 °C. After induction, the cells were centrifuged and resuspended in a buffer. Ultrasonication was used to lyse the cells, followed by centrifugation to obtain the extracts. The soluble extract was incubated with Ni2+-NTA resin, and the protein of interest was eluted using an imidazole gradient. The recombinant protein was concentrated using an Amicon Ultra15 filter with a molecular weight

cutoff of 10 kDa and subjected to size exclusion chromatography to remove contaminants and estimate the molecular weight and oligomeric state. The protein concentration was estimated by the Bradford assay.

2.2. Determination of the optimal pH and temperature parameters

The laccase activity was determined by reading the absorbance values obtained after the oxidation of the substrate guaiacol (420 nm). The reactions were carried out in microplates with a total volume of 100 µL, consisting of 20 µL of buffer (20 mM), 1.24 µL of substrate (1 mM), and 10 µL (35,82 µg/µL) of the enzyme. The effect of pH was assessed in the range of 3.0–12.0 using the following buffers: sodium acetate (pH 3.0, 4.0, 5.0, 5.5, and 6.0); MES (pH 5.5, 6.0, and 6.5); PIPES (pH 6.0, 6.5, 7.0, and 7.5); MOPS (pH 6.5, 7.0, 7.5, and 8.5), and 8.5; Tris-HCl (pH 6.5, 7.5, and 8.0); Tris-HCl (pH 6.5, 7.0, 7.5, 8.0, and 8.5); TAPS (pH 8.0 and 9.0); Ampol (pH 9.0, 9.5, and 10.0); CAPS (9.5, 10.0, 10.5, and 11.0); glycine (pH 8.5, 9.0, 9.5, 10.0, and 10.5); sodium bicarbonate (pH 9.0, 9.5, 10.0, 10.0, 10.5, 11.0, and 12.0); and potassium phosphate (pH 11.0, 11.5, and 12.0).

The pH parameters were assessed after 16 h at 37 °C because the substrate did not immediately change in color when added to the laccase reaction, regardless of pH or temperature. Only after 16 h was it possible to observe the characteristic brown coloration resulting from substrate oxidation. The optimal temperature was determined at the optimal pH as indicated in the previous test and assessed at temperatures of 10, 15, 25, 30, 37, 40, 50, 60, 70, 80, and 90 °C for 1 h. The thermostability of the enzymes was evaluated by preincubating them for 5 h at 80°C and 90°C. After every 30 min, a 10-µL aliquot was taken and added to a mixture containing the buffer at the ideal pH and the guaiacol substrate, and the reaction was carried out at the optimal temperature, as indicated in the previous test.

2.3. Lignin degradation by Lac_CB10 whole cell and SEM analysis of lignin degradation

The assays were conducted using *E. coli* cells harboring Lac_CB10. A pre inoculum was prepared and subsequently added to flasks containing 0.4 mg/mL alkaline lignin (AL) and LB medium. The assay proceeded for 36 h under agitation at 150 rpm and a temperature of 55 °C. Two treatments were performed: one without any inducer, consisting only of the transformed cells, and another with copper (0.25 mM) as an inducer. The degradation calculation was performed following the description in (12):
Degradation (%) =Ai − Af × 100
where Ai is the initial absorbance and Af is the final absorbance.
The two lignin treatments, one without an inducer and the other with an inducer, were examined using scanning electron microscopy (SEM; ZEISS EVO MA10) to determine potential structural changes in the lignin. The samples were first lyophilized and subsequently mounted on carbon tape. Additionally, they were coated with gold to facilitate viewing and imaging.

3 RESULTS & DISCUSSION

3.1. Enzyme expression and purification

Optimized expression conditions at 28 °C and 200 rpm for 4 h resulted in an enzymatic yield of 10.92 mg/L culture. In the first purification step, recombinant laccase with a histidine tail (6× His) and nickel Ni-NTA resin (Qiagen Venlo Netherlands) were used to isolate the Lac_CB10 protein, and these fractions were analyzed via SDS–PAGE. SDS–PAGE analysis revealed a single purified band with a molecular mass of approximately 94 kDa, which was consistent with the mass predicted by the ProtParam program for the fusion protein. Molecular exclusion chromatography was used to predict the mass of the Lac_CB10 protein in its native apoenzyme configuration through linear regression analysis of the ratio of the column's void elution volume and the logarithm of the molecular mass in the same elution volume (Ve/Vo) obtained for samples of a commercial molecular weight standard. The estimated molecular mass was 100.06 kDa.

3.2. Enzymatic characterization: pH, temperature, and thermostability

pH evaluations revealed that, after 16 h of incubation at 37 °C with guaiacol, Lac_CB10 exhibited the greatest activity at relatively basic pH values, reaching 100% of its activity in CAPS buffer at pH 10.5. Guaiacol is a phenolic substrate that turns brown when oxidized by an enzyme, and it was interesting to note that as soon as the substrate was reacted with laccase, there was no change in color at any of the tested pH values at room temperature or 37 °C until after 16 h. Figure 1 shows that as the pH increases, the substrate is more oxidized by the enzyme; that is, its color becomes more brown.

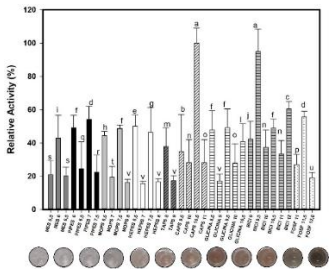

Figure 1. Effect of pH on the activity of Lac_CB10. Enzymatic activity was performed using the following 20 mM buffers.

The enzyme's extremophile condition was not limited to an optimal strongly alkaline pH profile (pH 10.5) but also revealed itself at elevated temperatures. Lac_CB10 showed low relative activity (maximum 40%) with no significant increase between 37 and 60 °C; however, at 70 °C, there was a significant increase in activity to 68%, followed by another significant increase at 80 °C (93%) and 90 °C, with the latter reaching its optimal temperature with a relative activity of 100%. Thermostability determination was carried out by incubating the enzyme for 5 h at 80 °C and 90 °C and measuring its activity on guaiacol substrate at its optimal

pH. Lac_CB10 was thermostable at both temperatures, maintaining more than 50% of its activity for 5 h and reaching a relative maximum activity of 100% at 80 °C after 270 min and at 90 °C after 210 min. The prolonged activity of the enzyme at high temperatures is considered a vital parameter for meeting commercial requirements, as laccases with good thermotolerance properties are important for industrial processes.

3.3. Enzymatic degradation and scanning electron microscopy of Lignin

The degradation of lignin by Lac_CB10 was analyzed after 32 h of treatment. The assay containing only the bacterial cell and lignin showed 16.33% degradation, while the assay containing copper as an inducer was able to degrade 52.27%. Scanning electron microscopy was used to verify the structural changes in lignin; abiotic treatments did not alter the structure of the lignin, while treatment without an inducer did not significantly change the structure (Fig. 2A and B). Treatment with copper caused the most structural changes, drastically altering the surface (Fig. 2C and D). These parameters are important since the use of inducers industrially increases the cost of the process; therefore, substituting expensive inducers like isopropyl-β-D-thiogalactopyranoside (IPTG) with copper, for more economical inducers, is advantageous, as was the case in this study. The fact that Lac_CB10 exhibits degradation capacity even in the absence of an inducer demonstrates that this enzyme indeed has the potential for lignin degradation.

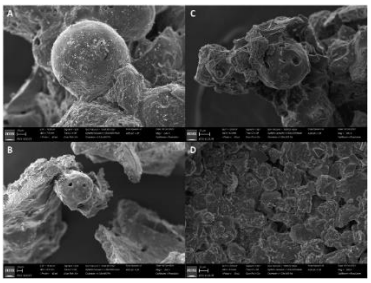

Figure 2. Scanning electron microscopy (SEM) images of alkalin lignin degradation. Treatment without inducer [(A) abiotic control and (B) treatment] and treatment with copper [(C) abiotic control and (D) treatment.

4 CONCLUSION

To the best of our knowledge, Lac_CB10 is the first yellow bacterial laccase described for the genus *Chitinophaga* that exhibits thermophilic and alkaline characteristics. These traits strongly suggest that this enzyme is highly promising for industrial processes, which are often conducted in high-temperature environments and extreme pH conditions. Lac_CB10 showed maximum activity at pH 10.5 with guaiacol as a substrate and remained stable, retaining more than 50% of its activity for 5 h at elevated temperatures (80°C and 90°C). This finding is consistent with the limited data available on yellow laccases and contributes to the establishment of this group as a promising target for biotechnological prospecting. The alkaline lignin assays indicated that Lac_CB10 is a highly promising enzyme for the degradation of this recalcitrant compound and could be effective as a biomass-degrading enzyme cocktail.

REFERENCES

1 Gräff M, Buchholz PCF, Le Roes-Hill M, Pleiss J. 2020. Multicopper oxidases: modular structure, sequence space, and evolutionary relationships. Proteins 88:1329–1339.

2 Bento I, Carrondo MA, Lindley PF. 2006. Reduction of dioxygen by enzymes containing copper. J Biol Inorg Chem 11:539–547.

3 Komori H, Higuchi Y. 2015. Structural insights into the O2 reduction mechanism of multicopper oxidase. J Biochem 158:293–298.

4 Chaoua S, Chaouche NK, Songulashvili G, Gares M, Hiligsmann S, Flahaut S. 2023. Yellow laccase produced by trametes versicolor K1 on tomato waste: a comparative study with the blue one produced on semi-synthetic medium. J Biotechnol 361:99–109.

5 Leontievsky A, Myasoedova N, Pozdnyakova N, Golovleva L. 1997. `Yellow' laccase of panus tigrinus oxidizes non-phenolic substrates without electron-transfer mediators. FEBS Lett 413:446–448.

6 Radveikienė I, Vidžiūnaitė R, Meškienė R, Meškys R, Časaitė V. 2021. Characterization of a yellow laccase from Botrytis cinerea 241. J Fungi7:143.

7 Mate DM, Garcia-Ruiz E, Camarero S, Shubin VV, Falk M, Shleev S, Ballesteros AO, Alcalde M. 2013. Switching from blue to yellow: altering the spectral properties of a high redox potential laccase by directed evolution. Biocatalysis and Biotransformation 31:8–21.

8 Mot AC, Coman C, Hadade N, Damian G, Silaghi-Dumitrescu R, Heering H. 2020. Yellow" laccase from Sclerotinia sclerotiorum is a blue laccase that enhances its substrate affinity by forming a reversible tyrosyl-product adduct. PLOS ONE 15:e0225530.

9 Pozdnyakova NN, Rodakiewicz-Nowak J, Turkovskaya OV. 2004. Catalytic properties of yellow laccase from Pleurotus ostreatus D1. Journal of Molecular Catalysis B: Enzymatic 30:19–24.

10 Pena CA, Ballesteros LF, Rodríguez H, Rodil E, Teixeira JA, Michelin M. 2023. Laccase-mediator system for the ionic liquid-assisted treatment of a technical lignin with partial dissolution. Biomass and Bioenergy 177:106928.

11 Morales M, Quintero J, Conejeros R, Aroca G. 2015. Life cycle assessment of lignocellulosic bioethanol: environmental impacts and energy balance. Renewable and Sustainable Energy Reviews 42:1349–1361.

12 Khan SI, Sahinkaya M, Colak DN, Zada NS, Uzuner U, Belduz AO, Çanakçi S, Khan AZ, Khan S, Badshah M, Shah AA. 2024. Production and characterization of novel thermostable CotA-laccase from Bacillus altitudinis SI7 and its application for lignin degradation. Enzyme Microb Technol 172:110329.

ACKNOWLEDGEMENTS

The authors would like to thank the Agricultural Microbiology Program (São Paulo State University, UNESP, Jaboticabal Campus), the Coordination for the Improvement of Higher Education Personnel (CAPES), the National Council for Scientific and Technological Development (CNPq), and the São Paulo Research Foundation for their financial support and ongoing support for this research.

This study was partially funded by the Coordination for the Improvement of Higher Education Personnel—Brazil (CAPES)—Financial Code 001, and the National Council for Scientific and Technological Development (CNPq). Partial financial support was received from the São Paulo Research Foundation (FAPESP) (2019/20825-0).