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Screening of ligninolytic enzymes produced by *Ganoderma lucidum* in solid residues from the Amazon

Prospecção de enzimas ligninolíticas produzidas por *Ganoderma lucidum* em resíduos sólidos da Amazônia

Detección de enzimas ligninolíticas producidas por *Ganoderma lucidum* en residuos sólidos Amazónicos

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Abstract
White rot fungi have one of the most efficient oxidative lignin degradation systems, and present an enzymatic complex that is responsible for digesting lignocellulosic matter. The aim of this study was to evaluate the production of laccase and total peroxidases by *Ganoderma lucidum* via solid-state fermentation using açaí seeds and marupá (*Simarouba amara*) sawdust unsupplemented and supplemented with wheat, corn and rice bran. *G. lucidum* was inoculated in substrates prepared with the residues and incubated at 25 ºC. Enzymatic extractions were performed on the culture substrates every 2 days for 30 days. The enzymatic activities were determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), with the addition of the enzymatic cofactor H_2O_2 for total peroxidases. The laccase activity was higher in the supplemented residues, with emphasis on the açaí-based substrate on the 16th day of cultivation, while in marupá the maximum activity was on the 6th day. In the unsupplemented açaí residue, the maximum peak of activity was on the 8th day and, in marupá, no fungal growth was observed. As for total peroxidases, *G. lucidum* cultivated in the supplemented açaí substrates showed activity peaks on the 8th, 12th, 16th and 28th day, and on 6th and 12th day under unsupplemented conditions. While, in the marupá, total peroxidase activity was

observed only on the 6th day of cultivation. Thus, *G. lucidum* showed potential for producing laccases and total peroxidases, with the substrate supplementation inducing the synthesis of these enzymes.
Keywords: Oxidative enzymes**;** Solid fermentation; Agro-industrial residues.

Resumo

Os fungos da podridão branca possuem um dos sistemas de degradação da lignina oxidativa mais eficientes, e apresentam um complexo enzimático responsável pela digestão da matéria lignocelulósica. O objetivo deste trabalho foi avaliar a produção de lacase e peroxidases totais por *Ganoderma lucidum*, via fermentação em estado sólido, utilizando sementes de açaí e serradura de marupá (*Simarouba amara*) não suplementadas e suplementadas com farelo de trigo, milho e arroz. *G. lucidum* foi inoculado em substratos preparados com resíduos e incubado a 25 ºC. Extrações enzimáticas foram realizadas nos substratos de cultura a cada 2 dias durante 30 dias. As atividades enzimáticas foram determinadas utilizando-se o sal de diamônio ABTS (2,2'-azino-bis(ácido 3-etilbenzotiazolina-6-sulfônico), com a adição do cofator enzimático H_2O_2 para as peroxidases totais. A atividade de lacase foi mais elevada nos resíduos suplementados, com destaque para o substrato à base de açaí no 16º dia de cultivo, enquanto no marupá, a atividade máxima foi no 6º dia. No resíduo não suplementado de açaí, o pico máximo de atividade foi no 8º dia e, no marupá, não foi observado qualquer crescimento de fungos. Quanto às peroxidases totais, o *G. lucidum* cultivado nos substratos suplementados de açaí apresentou picos de atividade nos dias 8, 12, 16 e 28, e nos dias 6 e 12, em condições não suplementadas. Enquanto que no marupá, a atividade total da peroxidase foi observada apenas no 6º dia de cultivo. Assim, *G. lucidum* mostrou potencial para produzir lacases e peroxidases totais, com a suplementação de substrato induzindo a síntese dessas enzimas.
Palavras-chave: Enzimas oxidativas; Fermentação sólida; Resíduos agroindustriais.

Resumen

Los hongos de podredumbre blanca tienen uno de los sistemas de degradación de lignina oxidativa más eficientes, y presentan un complejo enzimático que es responsable de digerir materia lignocelulósica. El objetivo de este estudio fue evaluar la producción de lacasa y peroxidasas totales por la *Ganoderma lucidum* a través de la fermentación de estado sólido, utilizando semillas de azaí y aserrín de aceituno (*Simarouba amara*) no suplementario y suplementado con trigo, maíz y salvado de arroz. *G. lucidum* se inoculó en sustratos preparados con residuos y se incubó a 25 ºC. Las extracciones enzimáticas se realizaron en los sustratos de cultivo cada 2 días durante 30 días. Las actividades enzimáticas se determinaron utilizando ABT (2,2′-azino-bis(3-etilbenzotiazolina-6-ácido sulfónico) sal diamonico), con la adición del cofactor enzimático H_2O_2 para peroxidasas totales. La actividad de lacasas fue mayor en los residuos suplementados, con énfasis en el sustrato a base de azaí el día 16 de cultivo, mientras que en aceituno la actividad máxima era el día 6. En el residuo de azaí no suplementado, el máximo pico de actividad fue el día 8 y, en el aceituno, no se observó un crecimiento fúngico. En cuanto a las peroxidasas totales, *G. lucidum* cultivado en los sustratos azaí suplementados mostró picos de actividad en el día 8, 12, 16 y 28, y el día 6 y 12 en condiciones no suplementadas. Mientras que en el aceituno, la actividad total de peroxidasas se observó solo el día 6 de cultivo. Por lo tanto, *G. lucidum* mostró potencial para producir lacasas y peroxidasas totales, con una suplementación con sustrato que induce la síntesis de estas enzimas.
Palabras clave: Enzimas oxidativas; Fermentación sólida; Residuos agroindustriales.

1. Introduction

As a common feature, white rot basidiomycetes have the ability to efficiently oxidize lignin as well as degrade other lignocellulose components. This is associated with its unique oxidative system, which is composed of enzyme complexes such as laccases and peroxidases (Floudas et al., 2020). *Ganoderma lucidum* (Curtis) P. Karst is a white rot basidiomycete of the class Agaricomycetes and belongs to the family Ganodermataceae. The genus *Ganoderma* has a wide distribution worldwide, with records on all continents, and about 238 described species (GBIF, 2021). Within the species of the genus, *G. lucidum* has broad pharmaceutical potential, due to its production of metabolites such as terpenoids, polysaccharides and proteins, and its biotechnological potential, through the production of lignin-modifying enzymes (Sharma et al., 2019; Bhat et al., 2021; Hu et al., 2022).

A promising way of obtaining lignin-modifying enzymes is through solid-state fermentation, in which agro-industrial residues can be used as substrates for mycelial development and as inducers of enzyme synthesis (Leite et al., 2021; Teigiserova et al, 2021; Hu et al., 2022, Melanouri et al., 2022). These enzymes are essential in the process of wood degradation by white rot

fungi and generally involve the participation of oxidative enzymes such as laccase, lignin and manganese peroxidases (Kumla et al. 2020).

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are enzymes that contain copper in their catalytic site, which are known as multicopper oxidases, and are responsible for catalyzing the oxidation of several organic and inorganic substrates, with simultaneous reduction of oxygen in water (Madhavi & Lele 2009; Arora & Sharma, 2010). Peroxidases or peroxide reductases (EC 1.11.1.x) are a group of enzymes that require H_2O_2 or other peroxides to act as an acceptor or donor of hydrogen atoms, and oxidize a wide range of polyphenols (Jaouadi et al., 2019; Agunbiade et al., 2021). In recent years, the enzymes have been applied in numerous industrial processes, such as wastewater treatment, catalysis of chemical transformations (Yadav & Yadav, 2015), dye bleaching (Schallemberger et al., 2021; Sosa–Martínez et al., 2020), bioethanol production (Devi et al., 2022), and prevention of undesirable phenolic oxidation of food components (Narnoliya et al., 2019).

As a substrate for mushroom development and obtaining enzymes for industrial application, the use of easily accessible residues that are available in the Amazon region, becomes promising, since the raw material is low cost, in addition to reducing the environmental impacts resulting from the inadequate disposal of this biomass (Sadh et al., 2018; Aguiar et al., 2021; Aguiar et al., 2022). Among the Amazonian residues, sawdust from marupá (*Simarouba amara*) has already been reported as an efficient substrate for mushroom development (Sales–Campos et al., 2010a; Sales–Campos et al., 2011; Aguiar et al., 2022).

The marupá is a tree species that is common in tropical rainforests, and is widely used in the manufacture of crates, plywood, musical instruments, wooden ceilings and matchsticks (Loureiro et al., 1979; Santos et al., 2021). Another very common residue in the Amazon comes from the açaí (*Euterpe* sp.) tree, which is a palm tree from which palm hearts and pulp from the fruit can be extracted. The seeds correspond to 93% of the fruit and are discarded without further use (Maranho & Paiva, 2012; Lima et al., 2021). Studies have already reported the use of açaí seed-based residue in biorefinery and anaerobic digestion processes, with the aim of production of mushrooms and lignocellulolytic enzymes (Santos & Cavallazzi, 2017; Lima et al., 2021; Aguiar et al., 2021; Aguiar et al., 2022).

Despite the importance of the fungal enzymes in the degradation of lignin complexes, their application is still not effective due to the high cost of production, in addition to the low yield (Wang et al., 2019). Thus, studies are needed to evaluate which species of fungi are efficient in the production of these enzymes, as well as the feasibility of using low-cost substrates as inducers of enzymatic synthesis. Therefore, the objective of this research was to evaluate the production of ligninolytic enzymes by *G. lucidum* via solid-state fermentation using Amazonian agro-industrial residues.

2. Methodology

2.1 Solid-state fermentation conditions

A commercial strain of *Ganoderma lucidum*, provided by the company Funghi Flora, was reactivated at the Laboratory of Cultivation of Edible Fungi, of the National Institute for Amazonian Research, in Petri dishes containing PDA (Potato Dextrose Agar), until complete mycelial growth. Subsequently, the fungus was cultivated on residues of açaí seeds (*Euterpe* sp.) and marupá sawdust (*Simarouba amara*), which were respectively obtained from local fairs and wood-processing industries in the city of Manaus, Amazonas state, Brazil (3° 06′ 06″ S, 60° 01′ 29″ W). For this, the spawns' substrates were prepared consisting of 78% residue (açaí and marupá), 20% supplementation with corn, wheat and rice bran, and 2% calcium carbonate, according to the methodology of Sales-Campos et al. (2010b), with modifications. For experiments without supplementation, the substrate consisted of 98% residue and 2% calcium carbonate.

The spawns were autoclaved at 121 ºC for 1 hour, and later inoculated with mycelia from the activation in PDA. These were then incubated at 25 ºC in a BOD (biochemical oxygen demand) incubator until complete colonization. After the fungal colonization of the spawns, the fermentation was carried out at a proportion of 5% spawn:cultivation substrate (w:w) in 190 mL flasks containing the different lignocellulosic residues (açaí and marupá), under the same conditions as the spawning preparation. Finally, the flasks were incubated in a BOD incubator at 25 ºC for 30 days.

2.2 Enzyme extractions

The enzymatic extracts were obtained from the growth substrates of *G. lucidum*, with collections every 2 days during a period of 30 days, thus totaling 15 extraction points. The growth substrate was subjected to extraction in cold, distilled water, under agitation at 160 rpm for 40 minutes at 10 ºC. Subsequently, the material was centrifuged at 1,500 rpm for 30 minutes at 4 ºC. The extracts were stored in a freezer (-20 ºC) until de determination of the enzymatic activities.

2.3 Quantification of enzymatic activities

Laccase activity was determined in 96-well microplates containing 135 µL of 0.2 M sodium acetate buffer pH 5, 30 µL of 2,2'-azino-bis(3-ethylbenzothializone-6-sulfonic acid) diammonium salt (ABTS, 5 mM) and 135 µL of enzyme extract. ABTS oxidation was measured at λ= 420 nm. Enzymatic activity was expressed in U/mL, where U is the amount of enzyme that oxidizes 1 µM of substrate per minute (Wolfenden & Willson, 1982).

Total peroxidase activity was determined in 96-well microplates containing 135 µL of enzyme extract, 105 µL of sodium acetate buffer (0.2 M, pH 5), 30 µL of H_2O_2 and 30 µL of ABTS (5 mM). The oxidation of ABTS was monitored at λ= 420 nm, and the results were expressed in U/mL (Heinzkill et al., 1998).

2.4 Experimental design

The experiments were arranged in a completely randomized design that consisted of two cultivation substrates (açaí and marupá), two conditions of residue supplementation (supplemented and unsupplemented) and fifteen extraction intervals (collection points along the cultivation). The assays to determine the enzymatic activities were carried out in triplicate.

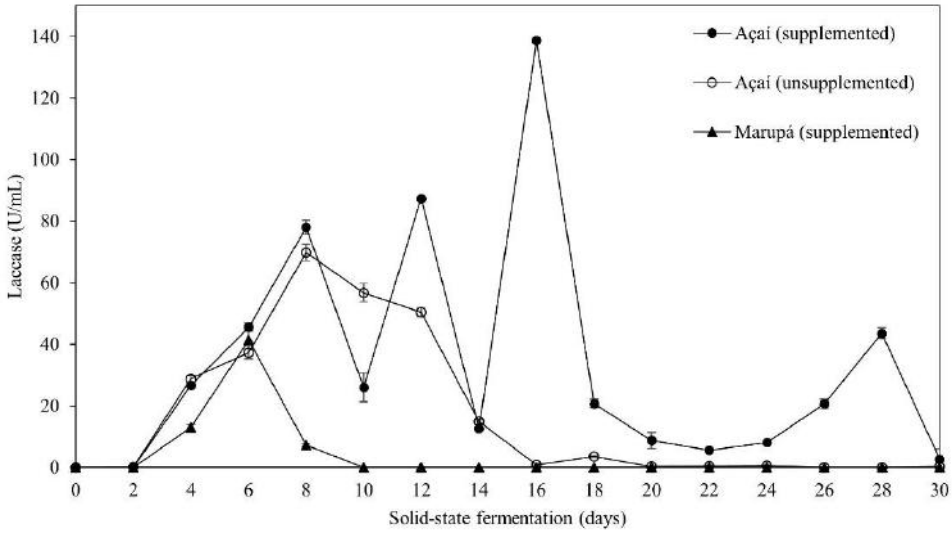
3. Results and Discussion

Ganoderma lucidum showed vigorous growth on the substrates based on açaí and marupá when supplemented with rice, wheat and corn bran, and on the açaí substrate without supplementation. On other hand, in the substrate based on marupá sawdust without supplementation, no fungal colonization was observed; therefore, there was no growth of *G. lucidum*, and it was not possible to determine the enzymatic activities for this substrate (Figure 1). The inhibition of fungal development may be associated with the low nitrogen content in the residue, which results from the low protein value that is characteristic of woody material (0.03 – 1%) and requires supplementation to achieve greater biological efficiency of the macrofungi (Chang & Miles, 1989; Sales-Campos et al., 2010a; Pedri et al., 2015; Aguiar et al., 2021; Aguiar et al., 2022).

When cultivated in açaí residue supplemented with corn, wheat and rice bran, *G. lucidum* presented four distinct peaks of laccase activity, corresponding to days 8, 12, 16 and 18 of cultivation, with emphasis on the 16th day (139 U/mL). This interval showed the highest laccase activity when compared to other peaks in this experimental condition. In the unsupplemented açaí-based substrate, the increase and decrease in laccase activity was gradual in the initial periods of cultivation, with a maximum peak on the 8th day (69.86 U/mL). As for the supplemented marupá residue, the maximum activity of the laccase was observed

on the 6th day of cultivation, with values similar to those found for the açaí residue with and without supplementation (41.36 U/mL), in the same period (Figure 1).

Figure 1 - Laccase activity during the mycelial growth of *Ganoderma lucidum* in açaí and marupá residues, in unsupplemented substrates and substrates supplemented with corn, wheat and rice bran.



Source: Authors.

Economou et al. (2017) report that the highest rate of laccase production in fungi of the genus *Ganoderma* usually occurs until the 4th week of the mycelial growth period under supplemented conditions, and reaches its maximum production in the period of 13 to 16 days. This corresponds to approximately two quarters of colonization (30 days), with a decrease in enzymatic activity at the end of the fermentation period. Makarenkova et al. (2021) found that *G. lucidum* reached higher laccase production on the 14th day of cultivation (550 U/mL) when using colza straw lignin as a substrate.

White rot fungi, such as *G. lucidum*, stand out for secreting a number of extracellular enzymes that are responsible for the degradation of lignocellulose via the breakdown of carbohydrate molecules (cellulose and hemicellulose) and aromatic constituents (lignin) (Kumla et al., 2020; Gauna et al., 2021; Hu et al., 2022). In this context, Ćilerdžić et al. (2016) evaluated the enzymatic production of *G. applanatum* in wheat straw, and observed a degradation of 13.5% of lignin, 6.4% hemicellulose and 3.5% cellulose.

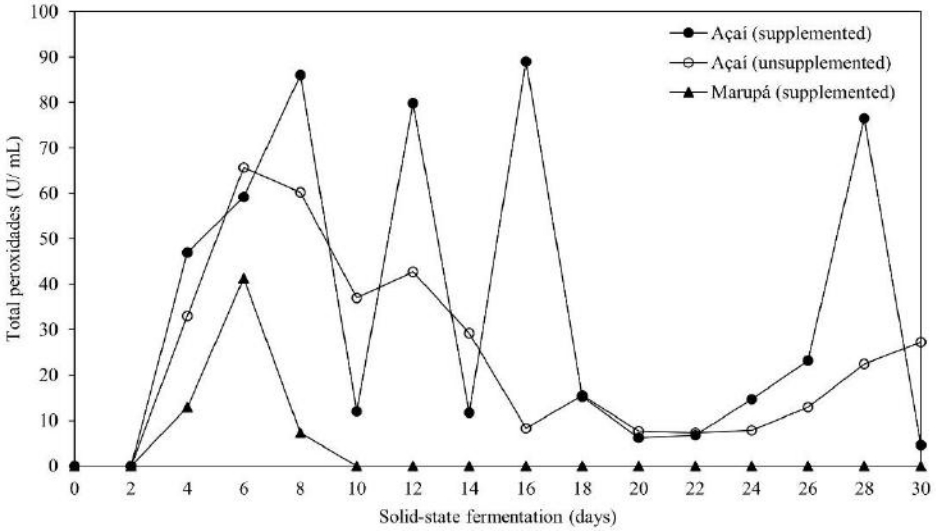
In relation to total peroxidases, the activity was similar to that observed for laccases, for which *G. lucidum*, when cultivated in the açaí-supplemented substrate, presented 4 distinct peaks, with expressive activities at the 8th, 12th, 16th and 28th days, while in under unsupplemented conditions, the maximum activity was observed on the 6th day, with a gradual reduction during the cultivation and an increase after 26 days of cultivation. As for the marupá substrate, there was a maximum peak of enzymatic activity on the 6th day of cultivation, with a significant reduction of this activity after this period (Figure 2).

During mushroom cultivation, distinct stages of development and growth of the fungus are observed, with emphasis on mycelial growth (substrate colonization), browning process, primordium formation and development of the basidiocarp (the structure known as the mushroom), and these phases are directly affected by the concentrations of lignin, cellulose and nitrogen in the fungal growth substrate (Sousa et al., 2019). Associated with mushroom development, greater laccase activity is obtained during substrate colonization when compared to basidiocarp formation (Bellettini et al., 2019).

The results observed in our study corroborate the literature, since the enzyme activity peaks occurred up to the 16th day of fermentation. Zhou et al. (2018), when evaluating the production of laccase and peroxidases by *G. lucidum* from the oxidation of ABTS, observed greater activities during the colonization phase (mycelial growth), with a decrease in enzymatic activities after the development of the primordium and a significant reduction after the formation and development of the basidiocarp.

The production of ligninolytic enzymes by these fungi is influenced by the substrate used (Leite et al., 2021), and affects the physicochemical characteristics of the mushrooms (Aguiar et al., 2022). Additionally, factors, such as type of cultivation (solid or submerged), source and concentration of carbon and nitrogen, presence of inducers, pH, temperature, and cultivation period, can also affect enzyme production (Simonić et al., 2010; Bellettini et al., 2019). Gas exchange also interferes in the production of oxidative enzymes, since the speed of fungal colonization and enzyme production can be altered as the fungus colonizes the substrate. This is due to gas exchange being limited in the lower portions of the culture bag and, consequently, the activity of these enzymes is lower in this portion (Rossi et al., 2001).

Figure 2 - Total peroxidase activity during the mycelial growth of *Ganoderma lucidum* in açaí and marupá residues in unsupplemented substrates and substrates supplemented with corn, wheat and rice bran.



Source: Authors.

Regarding the supplementation condition of the substrates, especially for the açaí-based substrate, the maximum peaks of laccase and peroxidases activity were about 50% and 25% higher, respectively, in relation to the unsupplemented experimental condition (Figures 1 and 2). In the marupá-based substrates, it was only possible to observe fungal growth in the supplemented condition, which suggests that substrate supplementation induced fungal growth and the synthesis of laccases and total peroxidases. This is because supplementation of the cultivation substrate with bran provides an additional source of carbon and nitrogen, thus favoring protein synthesis by the fungi during mycelial development (Carrasco et al., 2018).

Gurung et al. (2012), when studying sawdust from alder (*Alnus nepalensis*) trees as a substrate for fermentation of *G. lucidum*, observed an increase of up to 2-fold in the yield of basidiocarps in tests using supplementation with grass, wheat, corn and rice flour. Melanouri et al. (2022) showed that the supplementation of agro-industrial residues with bran, even in low amounts, was responsible for inducing laccase activity by *P. ostreatus*. Amiri–Sadeghan et al. (2022) report that significant increases in the biological efficiency of mushrooms, as a result of substrate supplementation, may be associated with greater induction of enzyme production, including lignocellulolytic enzymes.

Laccase activity in basidiomycetes varies according to the mushroom strain, type of cultivation substrate, nitrogen content and C:N ratio, as observed by Economou et al. (2017) when studying five different basidiomycetes cultivated in spent substrate of *Pleurotus ostreatus* supplemented with wheat bran and soybean flour. These authors observed that low C:N ratios favored an increase in the production of *Ganoderma resinaceum* biomass and, consequently, an increase in the production of enzymes necessary for fungal development, such as laccase.

Although the two enzymes evaluated showed maximum activity on the 16th day of cultivation, in the substrate supplemented with açaí, the laccase activity was about 1.5 times higher when compared to total peroxidases. The variation in the synthesis of laccases in relation to ligninases may be related to the participation of different peroxidases in the delignification process. According to Zhang et al. (2022), laccase plays an initial role in lignin degradation, but delignification only happens more efficiently when this enzyme is combined with other peroxidases. Thus, it is suggested that *G. lucidum* produced a greater amount of laccases for the initial hydrolysis of the substrate, while the peroxidases act only in a complementary way, not requiring greater production.

4. Conclusion

Ganoderma lucidum showed potential for the production of laccases and total peroxidases, with the supplementation of the substrate inducing the synthesis of these enzymes. Thus, for the cultivation of *G. lucidum*, the use of an açaí-based substrate is recommended, which should be supplemented with corn, wheat and rice bran in order to obtain these enzymes for industrial applications. However, further studies are needed to evaluate the influence of other residues on the production of lignocellulolytic enzymes by *G. lucidum*, in addition to an evaluation of parameters such as culture temperature, enzyme extraction techniques and other sources of supplementation in order to optimize the production of these enzymes.

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