



Cellulase and Xylanase production by *Ganoderma lucidum* in solid-state fermentation using amazonian lignocellulosic wastes

Produção de Celulase e Xilanase por *Ganoderma lucidum* em fermentação de estado sólido utilizando resíduos lignocelulósicos amazônicos

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Sérgio Dantas de Oliveira Júnior

Doutor em Engenharia Química

Institution: Instituto Nacional de Pesquisas da Amazônia (INPA)

Address: Av. André Araújo, 2936, Petrópolis, Manaus – AM, CEP: 69067-375

E-mail: sergiodantas100@hotmail.com

Paula Romenya dos Santos Gouvêa

Mestre em Agricultura no Trópico Úmido

Institution: Universidade Federal do Amazonas (UFAM)

Address: Av. General Rodrigo Octavio Jordão Ramos, 1200, Coroado I, Manaus - AM, CEP: 69067-005

E-mail: paularomenya.bio@gmail.com

Aldenora dos Santos Vasconcelos

Tecnóloga em Biotecnologia

Institution: Universidade Federal do Amazonas (UFAM)

Address: Av. General Rodrigo Octavio Jordão Ramos, 1200, Coroado I, Manaus - AM, CEP: 69067-005

E-mail: aldenora.svasconcelos@gmail.com

Larissa Batista de Brito do Nascimento

Engenheira de Bioprocessos

Institution: Universidade Federal do Amazonas (UFAM)

Address: Av. General Rodrigo Octavio Jordão Ramos, 1200, Coroado I, Manaus - AM, CEP: 69067-005

E-mail: lbbrnascimento@gmail.com

Vítor Alves Pessoa

Mestre em Biotecnologia e Recursos Naturais

Institution: Universidade Federal do Amazonas (UFAM)

Address: Av. General Rodrigo Octavio Jordão Ramos, 1200, Coroado I, Manaus - AM, CEP: 69067-005

E-mail: vitoralvespessoa10@gmail.com

**Giovanna Lima da Silva**

Tecnóloga em Biotecnologia

Institution: Universidade Federal do Amazonas (UFAM)

Address: Av. General Rodrigo Octavio Jordão Ramos, 1200, Coroado I,
Manaus - AM, CEP: 69067-005

E-mail: giovannalimafr@gmail.com

Ceci Sales-Campos

Doutora em Biotecnologia

Institution: Instituto Nacional de Pesquisas da Amazônia (INPA)

Address: Av. André Araújo, 2936, Petrópolis, Manaus – AM, CEP: 69067-375

E-mail: ceci@inpa.gov.br

Larissa Ramos Chevreuil

Doutora em Botânica

Institution: Instituto Nacional de Pesquisas da Amazônia (INPA)

Address: Av. André Araújo, 2936, Petrópolis, Manaus – AM, CEP: 69067-375

E-mail: larissachevreuil@gmail.com

ABSTRACT

Ganoderma lucidum is a basidiomycete whose production is of great interest due to its medicinal properties, and analyzing the potential of Amazonian lignocellulosic residues in the cultivation of *G. lucidum* is a way to enable the use of this material in mushroom cultivation and the production of biomolecules, including enzymes of commercial interest. The objective of this study was to evaluate the activity of cellulases and xylanases produced by *G. lucidum* when cultivated in açai seed (*Euterpe* sp.) residues and marupá (*Simarouba amara*) sawdust under supplemented and non-supplemented conditions. Solid-state cultivation was carried out in 250 mL flasks containing 50 g of lignocellulosic residues, plus 2% of CaCO₃, under supplemented (18% of rice, wheat and corn bran) and non-supplemented conditions. The flasks were incubated in a BOD incubator at 25 °C. Enzyme extraction was performed from the fungus growth substrate over 30 days of cultivation, with collections every two days. The enzymatic extracts had their endoglucanase (CMCase), total cellulase (FPase) and xylanase activities determined. The highest enzymatic activity of CMCase and xylanase were 5.97 U/g and 1.90 U/g, respectively, on the 26th day of cultivation in the supplemented marupá sawdust substrate. While the maximum FPase content was 0.24 U/g, which was observed in the 8th day for the supplemented açai substrate. Thus, the tested residues proved to be promising for the mycelial development of *G. lucidum*, with the supplementation with corn, rice and wheat bran being favorable to the production of enzymes with biotechnological interest.

Keywords: Basidiomycetes, solid-state culture, Enzymatic hydrolysis, Lignocellulolytic enzymes.

RESUMO

Ganoderma lucidum é um basidiomiceto cuja produção é de grande interesse devido às suas propriedades medicinais. Assim, analisar a potencialidade de



resíduos lignocelulósicos amazônicos no cultivo de *G. lucidum* é uma forma de viabilizar o uso destes materiais para o cultivo de cogumelos e produção de enzimas de interesse comercial. O objetivo desse estudo foi avaliar a atividade de celulases e xilanases produzidas por *G. lucidum*, quando cultivado em resíduo de sementes de açaí (*Euterpe* sp.) e serragem de marupá (*Simarouba amara*), em condição suplementada e não-suplementada. O cultivo sólido foi conduzido em frascos de 250 mL contendo 50 g dos resíduos lignocelulósicos, acrescidos de 2% de CaCO_3 , em condições suplementadas (18% de farelo de arroz, trigo e milho) e não suplementadas. Os frascos foram incubados em câmara B.O.D. a 25 °C. A extração enzimática foi realizada a partir do substrato de crescimento do fungo ao longo de 30 dias de cultivo, com coletas a cada dois dias. Os extratos enzimáticos foram submetidos à determinação das atividades de endoglucanases (CMCase), celulases totais (FPase) e xilanases. As maiores atividades enzimáticas de CMCase e xilanase foram de 5,97 U/g e 1,90 U/g, respectivamente, no 26° dia de cultivo no substrato de marupá suplementado. Ao passo que o teor máximo da FPase foi de 0,24 U/g no 8° dia de fermentação, no substrato de açaí suplementado. Diante do exposto, os resíduos testados mostraram-se promissores para o desenvolvimento micelial de *G. lucidum*, sendo a suplementação com farelos de milho, arroz e trigo favoráveis à produção de enzimas de interesse biotecnológico.

Palavras-chave: Basidiomicetos, fermentação em estado sólido, Hidrólise enzimática, Enzimas lignocelulolíticas.

1 INTRODUCTION

Mushrooms of the genus *Ganoderma*, especially *G. lucidum*, also known as the “Reishi mushroom” or the “mushroom of immortality”, are considered symbols of traditional Chinese medicine (KURD-ANJARAKI et al., 2021). In Chinese culture, when consumed in the form of infusions and capsules containing its extracts, this mushroom said to promote longevity and good quality of life, due to its wide range of bioactive compounds with antioxidant, anti-inflammatory, antimicrobial, cytotoxic and anticancer properties (BHARADWAJ et al., 2020; CHO et al., 2020).

G. lucidum has also been reported to express enzymes with hydrolytic actions, such as proteases, cellulases, xylanases and ligninases (LIU et al., 2021). Thus, this mushroom is important in the reuse of carbon since it has an enzymatic complex that is capable of hydrolyzing the cellulose and lignin that constitute plant biomass by using the products of this hydrolysis for its growth and development (HU et al., 2020).



Cellulose hydrolysis by *G. lucidum* involves the synergistic action of exoglucanases, endoglucanases and β -glucosidases, which are synthesized and excreted by the fungus in its growth substrate. These enzymes are responsible for the hydrolysis of the β -1,4-glycosidic bonds of the cellulose chain, and convert polysaccharides into oligosaccharides that will later be reduced to glucose monomers (PAUL et al., 2020).

Due to their hydrolytic action, cellulolytic enzymes are widely used in several industrial products that use plant biomass in the manufacture of high value-added products, such as biofuel industries that convert plant residues into second-generation ethanol, beverage industries that use cellulases to clarify wines and pulp and paper industries that use cellulases as a bleaching agent, and acting in the refinement of cellulosic fibers (SIQUEIRA et al., 2020; WANG et al., 2020). Moreover, it is estimated that in 2023 the annual enzyme market could reach US\$ 7 billion, with an annual growth rate of 4.9%, for which 20% corresponds to lignocellulolytic enzymes (TOUSHIK et al., 2017; SALDARRIAGA-HERNÁNDEZ et al., 2020).

Considering the wide applicability of cellulases in different industrial processes, many studies have been developed with the aim of greater production of these enzymes, and mushrooms are considered a promising alternative for the production of this class of enzyme when using lignocellulosic substrates for their solid cultivation. In this sense, the knowledge obtained regarding the regulatory mechanisms involved in the production of these enzymes indicates that the carbon and nitrogen source has a direct influence on the physiological regulation of these fungi, thus affecting their productivity (RAI et al., 2004; RASHAD et al., 2019; OKAL et al., 2020).

The açai (*Euterpe oleracea* Mart.) is a palm that is native to the Amazon and has economic prominence due to the market potential of its products, mainly represented by the palm heart and the juice extracted from the fruit (CAMPOS et al., 2020). Marupá (*Simarouba amara*) is a pioneer species that belongs to the Simaroubaceae family and is naturally distributed throughout the Amazon, and the central-west and southwest of Brazil. The wood of this species is light or beige-yellow to cream, and it is used in the furniture industry and in the manufacture of toys, linings and boxes (PAULA et al., 2020). Waste from economic activities that



involve both these species is already known to have potential for mushroom cultivation, which could help to reducing the amount of waste in landfills and could generate value-added products (AGUIAR et al., 2021; SALES-CAMPOS et al., 2021).

In this context, considering the economic importance of cellulases and the production potential of these enzymes by mushrooms, it is important to develop new methodologies that aim to increase their yield and reduce production costs. As such, the objective of this work was to evaluate the influence of açai and marupá residues from the Amazon on the induction of the synthesis of exo-, endoglucanases and xylanases by *Ganoderma lucidum* during its mycelial development, under supplemented and non-supplemented conditions.

2 MATERIAL AND METHODS

2.1 BIOLOGICAL MATERIAL

A commercial strain of *Ganoderma lucidum* was initially activated in potato dextrose agar (PDA) medium at 25 °C and kept under refrigeration (± 8 °C) until its use in the experiments. Açai seeds (*Euterpe* sp.) and marupá sawdust (*Simarouba amara*), collected from local industries in the city of Manaus, Amazonas, Brazil, were used as the substrate for cultivation.

2.3 SOLID-STATE FERMENTATION

The *G. lucidum* strain was reactivated in Petri dishes containing PDA culture medium and incubated in a BOD incubator at 25 °C. After the total colonization of the Petri dishes, the fungus grown was used as an inoculum for the spawn. The supplemented spawn was composed of lignocellulosic residues (açai or marupá) (80%), a mixture of corn, wheat and rice bran (18%) and calcium carbonate (CaCO₃) (2%). The non-supplemented spawn consisted only of lignocellulosic residue (98%) and CaCO₃ (2%). Spawns were autoclaved at 121 °C for 1 hour, cooled to room temperature, then inoculated with *G. lucidum* mycelia from PDA growth and incubated at 25 °C until complete colonization.

Solid-state cultivation substrates were prepared as described for spawns. The experiment was carried out in 250 mL glass flasks, totaling 45 culture flasks for each condition (supplemented and non-supplemented), containing 50 g of



substrate. The culture flasks with the substrates were sterilized in an autoclave at 121 °C for 1 hour and, after cooling, they were inoculated with 5% of their respective spawns. Subsequently, each flask was wrapped in a plastic bag and its end sealed with a sponge to facilitate gas exchange. The flasks were incubated for 30 days in BOD incubator at 25 °C.

2.4 PREPARATION OF ENZYMATIC EXTRACTS

During the mycelial growth of *G. lucidum*, enzyme extraction was performed every 2 days for 30 days, totaling 15 extractions. To obtain the enzymatic extracts, three culture flasks were randomly selected and homogenized. Then, 9 g of this material was homogenized with 50 mL of 1 M sodium acetate buffer (pH 5.0) at 100 rpm, 8 °C, for 1 hour. Subsequently, the material was filtered and centrifuged at 5.000 rpm, 4 °C, for 15 minutes. The supernatants were stored in a freezer (-20 °C) until the determination of enzymatic activities.

2.5 ENZYMATIC ACTIVITY DETERMINATION

For the determination of total cellulases (exoglucanases or FPases), Whatman N° 1 quantitative filter paper was used as the enzyme substrate, and the reaction mixture was composed of a disk of this paper (~50 mg), 500 µL of sodium citrate buffer (50 mM, pH 4.8) and 250 µL of the enzymatic extract. The reaction mixture was incubated in a water bath at 50 °C for 60 minutes. After this period, the reaction was stopped by cooling in an ice bath (GHOSE, 1987). Total exoglucanase activity was determined from the concentration of reducing sugars released in the reaction medium during the FPase assay, which was expressed in µmol of product released/minute.

For the determination of carboxymethylcellulases (endoglucanases or CMCase), carboxymethylcellulose - CMC (4%) in sodium citrate buffer (50 mM, pH 4.8) was used as the substrate. The reaction mixture consisted of 150 µL of CMC and 150 µL of the enzymatic extract, which was incubated in a water bath at 50 °C for 10 minutes. After this period, the reaction was stopped by cooling in an ice bath (GHOSE, 1987). Total endoglucanase activity was determined from the concentration of reducing sugars released during the CMCase assay and expressed in µmol of product released/minute.



Xylanase activity was determined using the substrate xylan (1%), dissolved in NaOH (1 M) for 30 minutes, followed by the addition of 20 mL of HCl (1 M), and the final volume (100 mL) was completed with a solution of sodium acetate (50 mM, pH 5.0). The reaction mixture consisted of 150 μ L of the xylan solution and 150 μ L of the enzymatic extract. The reaction was incubated in a water bath at 50 °C for 5 minutes. After this period, the reaction was stopped by cooling in an ice bath (GHOSE, 1987). Xylase activity was determined from the concentration of reducing sugars released during the xylanase assay and expressed in μ mol of product released/minute.

2.6 QUANTIFICATION OF REDUCING SUGARS

The quantification of reducing sugars was performed using the colorimetric assay with 3,5-dinitrosalicylic acid (DNS). In this method, 250 μ L of the reaction mixture from the enzymatic steps and 250 μ L of DNS were incubated at 100 °C for 15 minutes. Then, the reaction was stopped by cooling in an ice bath and reading of the absorbance at 540 nm. The content of reducing sugars was determined from a standard curve with glucose (MILLER, 1959).

2.7 EXPERIMENTAL DESIGN

The experiments were carried out in a completely randomized design, in a factorial scheme composed of 2 cultivation substrates (lignocellulosic residues), 2 residue supplementation conditions and 15 extraction times (2 x 2 x 15). Enzyme activity assays were performed in triplicate.

3 RESULTS AND DISCUSSION

Ganoderma lucidum did not grow on the marupá sawdust-based substrate without supplementation with corn, rice and wheat bran, which suggests that this residue may not have been favorable to fungal growth due to lack of available nutrients, such as nitrogen and carbon. Similar results were obtained by Alquati et al. (2016) when they cultivated *G. lucidum* in substrates composed of residues from the pruning of urban trees, in which they found a low C/N ratio and obtained unsatisfactory fungal development.

The C/N ratio is a determining factor for the growth of *G. lucidum* in solid



state fermentation, which present greater development with a C/N ratio in the range between 70:1 and 80:1, as described by Hsieh and Yang (2004). Sales-Campos et al. (2010) found low N content (0.28 – 0.05%) in marupá sawdust, which causes a high C/N ratio (161.39:0.8), and corroborates the low nitrogen contents (0.03 – 1%) described for woody material in the literature (CHANG; MILES, 1989). In this way, this problem can be overcome by supplementing mushroom cultivation substrates with sources of carbon and nitrogen that are easily assimilated by the fungus, which increases productivity/growth (CARRASCO et al., 2018).

During the growth of *G. lucidum* in açai residue, a maximum peak of total cellulases (FPases) was observed at the 8th day of cultivation (0.244 U/g), with values found for the supplement substrate that were around 10 times higher when compared to the non-supplemented condition. For the supplemented marupá residue, the maximum activity was observed on the 18th day of solid-state fermentation (0.123 U/g) (Figure 1). However, the values of FPases found in this study are considered low when compared to *G. lucidum* cultured in PDA (potato dextrose agar) medium, which showed FPase production from the 3rd day, with a maximum activity peak on the 8th day, reaching values of approximately 5 U/g (RODRIGUES et al., 2020).

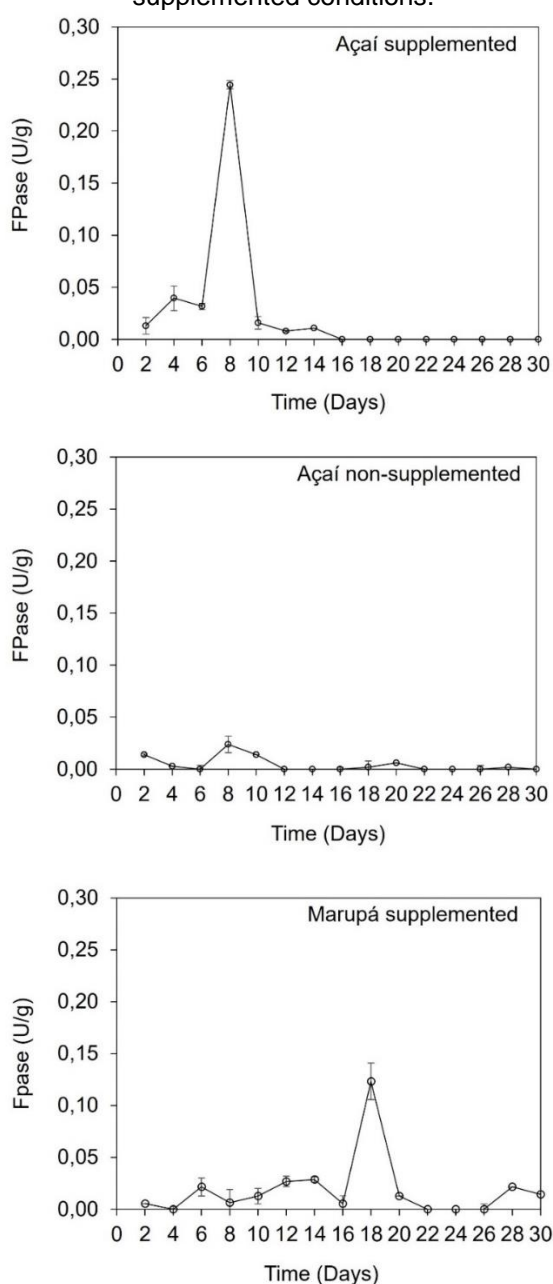
The low FPase activity found in the açai substrate without supplementation may be related to the high carbohydrate content (approximately 89%) and the low protein content (4.55%), which causes an imbalance in the carbon and nitrogen (C/N) ratio, and which is essential for the growth of the fungus and, consequently, affects the efficiency of its biological activities, such as the synthesis and excretion of cellulolytic enzymes (BELLETTINI et al., 2019; CARPINÉ et al., 2020). Maciel-Silva et al. (2019) reported a low nitrogen value in açai seeds (less than 1%) and a high concentration of carbohydrates, with a C/N ratio of greater than 35:1. These values are considered below average for good development of *G. lucidum*, since this species requires a C/N ratio of around 70:1 to 80:1 (HSIEH; YANG, 2004).

One way that was found to correct the imbalance in the C/N ratio in the substrate based on açai seed was the supplementation of the substrate, which provided a production of FPase that was 10 times higher when compared to the same substrate without supplementation. Low activity in the production of FPase was also observed when *G. lucidum* was cultivated in marupá sawdust, which may



be related to the low nitrogen content found in woody material (CHANG; MILES, 1989) since the nitrogen concentration present in the medium significantly interferes with the excretion of cellulases by white rot fungi (OKAL et al., 2020). It is importante to emphasize that supplementation with bran did not fully meet the nutritional needs of the fungus in order to induce the production of this enzyme when cultivated in marupá-based residues.

Figure 1. Activity of total cellulases (FPase) during the cultivation of *G. lucidum* in açai and marupá residues, under supplemented (addition of corn, rice and wheat bran) and non-supplemented conditions.





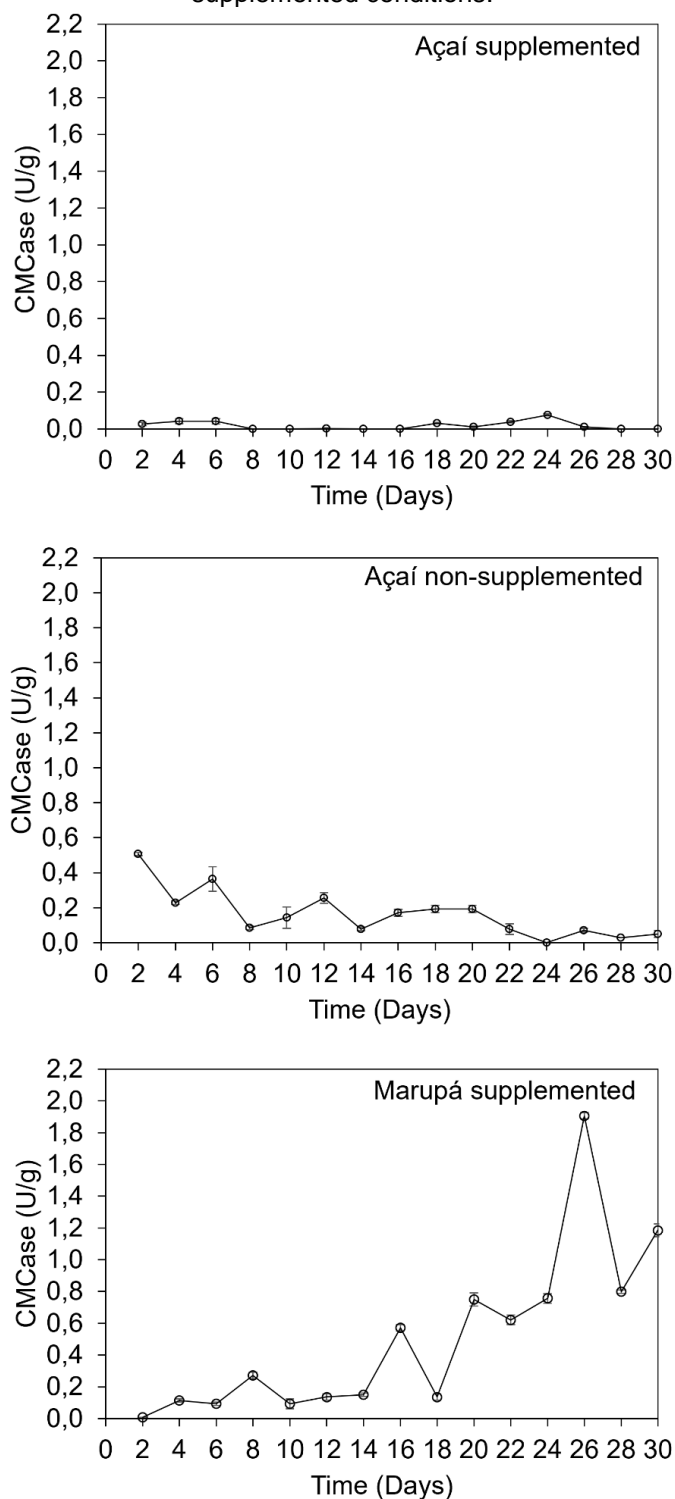
As for endoglucanases (CMCase), distinct peaks of enzymatic activity were observed throughout the growth/development of *G. lucidum*. In general, the açai-based substrates showed low CMCase activity, with maximum values of 0.5 U/g on the 2nd day of cultivation in non-supplemented açai. When cultivated in marupá-based residue with the addition of supplements, *G. lucidum* exhibited expressive CMCase activity in the most advanced stages of colonization, which correspond to the periods of formation and development of primordia, with a maximum activity of 1.9 U/g at 26 days of cultivation (Figure 2).

The colonization of lignocellulosic substrates by macrofungi can be divided into two stages: first, the initial production of mycelium biomass occurs from the consumption of the soluble sugar present in the substrate or through supplementation of the culture medium; the second stage is the degradation of the lignocellulosic matrix (cellulose, hemicellulose and lignin), when the soluble carbon source has already been exhausted. In other words, the supplementation of lignocellulosic substrates provides sufficient nutrients for fungal growth, resulting in the inhibition of the production of lignocellulolytic enzymes, synthesized late, when the source of soluble sugars is depleted and there is a need for hydrolysis of lignocellulose constituents (SCHIESSER, 1989). This fact may be responsible for the observation of higher enzymatic activity at the beginning of fermentation in the non-supplemented açai-based substrate, with a decrease in activity as lignocellulose is digested.

In the cultivation of *Ganoderma australe* over 75 days, and using a liquid inoculum and wood chips of *Eucalyptus globulus* as substrate, maximum activity of endoglucanases was observed between the 21st and 42nd day, with values in the order of 200 U/kg (corresponds to 0.2 U/g) (ELISSETCHE et al., 2007). Rodrigues et al. (2020), when cultivating *G. lucidum* in a mixture of 2.5 g of sugarcane and 2.5 g of wheat bran for 8 days, observed a maximum CMCase activity of 17.58 U/g on the 3rd day of fermentation. When grown on sugarcane bagasse, *G. lucidum* reached maximum production of endo-1,4- β -glucanases on the 20th day of cultivation (26 U/g), with a small increase between 40-45 days of fermentation (MANAVALAN et al., 2012).



Figure 2. Carboxymethylcellulases (CMCase) activity during the cultivation of *G. lucidum* in açai and marupá residues, under supplemented (addition of corn, rice and wheat bran) and non-supplemented conditions.



The maximum activity of xylanases produced by *G. lucidum* in açai residues was 3.5 U/g on the 26th day of cultivation in the supplemented substrate and on the 2nd day of cultivation in non-supplemented substrate. Whereas, in the



supplemented marupá substrate, it showed a maximum activity of 6.0 U/g on the 26th day of fermentation (Figure 3).

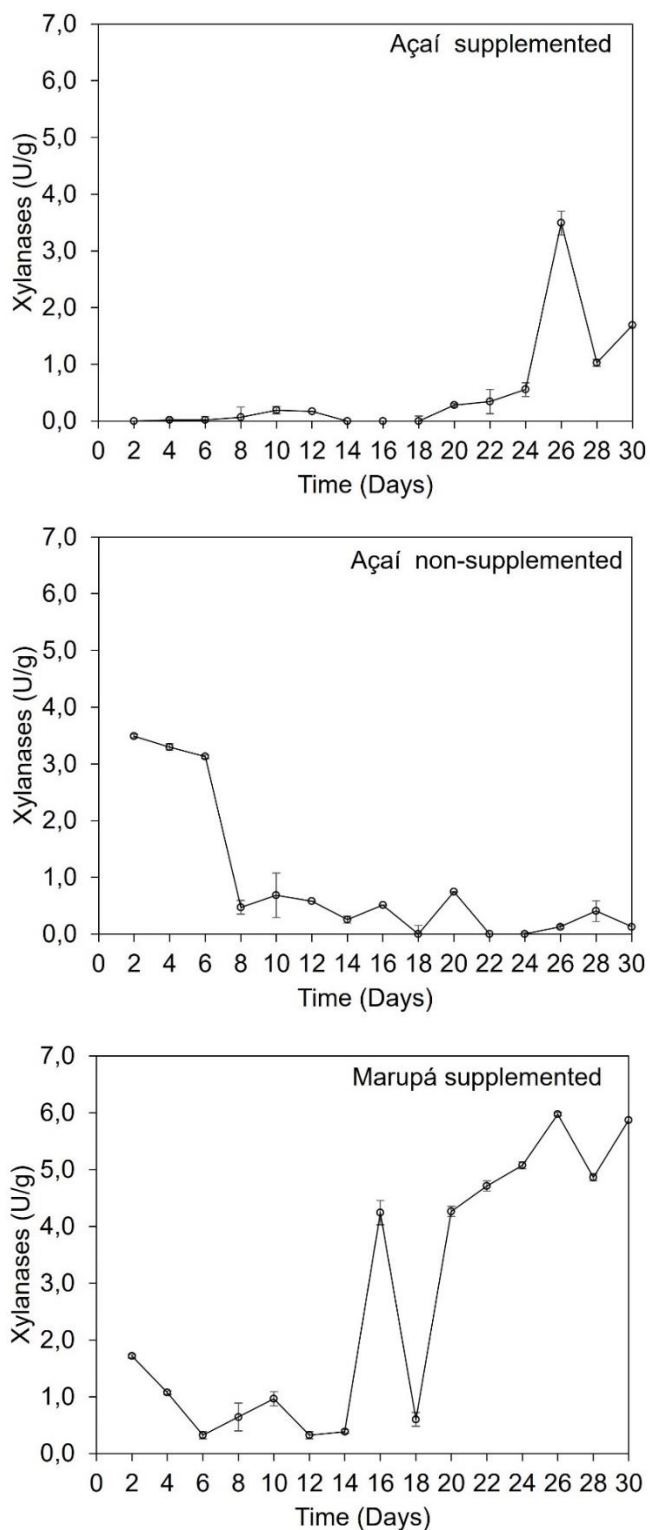
G. lucidum is considered a white rot fungus and, due to this characteristic, it is able to degrade cellulose and hemicellulose synergistically. When comparing the secretion rates of cellulases and xylanases produced by this group of fungi, a higher production of xylanase is noted, thus indicating a preference for xylan as a carbon source, when cultivated on lignocellulosic substrates (BENTIL et al., 2018). However, differences in the composition of lignocellulosic material can affect the synthesis of lignocellulolytic enzymes by the fungus (ATILA et al., 2019).

Xylanases have an added market value since they are used in the paper industry for the bleaching of cellulose pulp because they hydrolyze the xylan and facilitate the process of lignin release, thus reducing chloride consumption and, consequently, environmental damage, as well as improving fiber brightness (HUTTERER et al., 2017; SALDARRIAGA-HERNANDÉZ et al., 2020). However, cellulases acting together with xylanases can hydrolyze cellulosic fibers, which results in poor quality paper (SUBRAMANIYAN; PREMA, 2020). Therefore, there is great interest in cellulose-free xylanase formulations, so that there is a ready digestion of hemicellulose without compromising the cellulose present in the pulp. Thus, the *G. lucidum* evaluated in this study, when cultivated in marupá residue and, mainly, in açai, may be interesting for obtaining xylanases with minimal amounts of cellulases.

It is important to emphasize that the FPase activity in the supplemented açai substrate reached its maximum peak on the 8th day of cultivation (0.244 U/g), whereas the production of CMCase was not significant. However, the xylanase in the same residue was close to 4 U/g on the 26th day of cultivation, which is about 14 times higher than the cellulase activity. It is important to highlight that, on the 26th day of cultivation, there was no production of cellulases at detectable levels in the supplemented açai-based substrate, which resulted in a xylanase free of cellulose-degrading enzymes. A similar profile, however less promising, was found in the other residues, where the production of xylanase in non-supplemented açai and supplemented marupá substrates was 6 and 3 times higher than that of cellulases, respectively.



Figure 3. Xylanase activity during the cultivation of *G. lucidum* in açaí and marupá residue, under supplemented (addition of corn, rice and wheat bran) and non-supplemented conditions.





4 CONCLUSION

Ganoderma lucidum has the potential to produce xylanases when cultivated in açai seeds and marupá sawdust, and these characteristics are interesting for obtaining cellulase-free xylanase formulations, which have high added value and the potential for use in the paper industry.

INTEREST CONFLICTS

The authors declare that there are no conflicts of interest regarding the publication of this article.

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