Extracellular enzymes produced by *Coriolopsis rigida* an endophytic fungus associated with *Cochlospermum regium*

Enzimas extracelulares produzidas por *Coriolospis rigida* um fungo endofítico associada a *Cochlospermum regium*

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ABSTRACT

Coriolopsis rigida was isolated as an endophytic fungus from the medicinal plant *Cochlospermum regium*. The fungus was cultivated on solid media containing rice or pineapple crown bran for analyzing the production of extracellular enzymes. The activities of lipase, β-xylosidase, pectinase, fructosyltransferase (FTase), invertase, α-amylase and total cellulolytic (FPase) were determined. The fungus showed positive activity for all enzymes with yield for FTase highest (3.59 ± 0.52 U.gss^{-1}) in pineapple crown bran medium. In general, in pineapple crown bran medium the fungus had a higher enzyme production than in rice medium. Demonstrating the biotechnological potential of *C. rigida* in the production of enzymes of industrial interest.

Keywords: Enzymatic Activity; Endophyte; fructosyltransferase;

RESUMO

Coriolopsis rigida foi isolado como um fungo endofítico da planta medicinal *Cochlospermum regium*. O fungo foi cultivado em meio sólido de arroz e farelo de abacaxi para avaliação da atividade enzimática. As atividades de lipase, β-xylosidase, pectinase, fructosiltransferase (FTase), invertase, α-amilase e total celulítico (FPase) foram determinadas. O fungo apresentou atividade enzimática para todas as enzimas avaliadas, com rendimentos maiores para a FTase ($3,59 \pm 0,52$ U.gss^{-1}) em meio de farelo de abacaxi. Em geral, no meio de farelo de abacaxi o fungo teve uma produção de enzima superior ao meio de arroz. Demonstrando o potencial biotecnológico de *C. rigida* na produção de enzimas de interesse industrial.

Keywords: Atividade enzimática; Endófito; fructosiltransferase;

INTRODUCTION

Endophytes colonize within the plant tissue without showing any symptoms, either for part or their full life cycle, and show mutualistic interaction with the plants (Keshri et al., 2021), they are perceived as an alternative source for new biologically active molecules and extracellular enzymes (Gupta et al., 2020). Endophytic fungi synthesize extracellular enzymes as a resistance mechanism against disease causing pathogens and to get nourishment from hosts. The commonly produced extracellular enzymes are pectinases, cellulase, lipase, amylase and protease (Jagannath et al., 2021; Sopalun and Iamtham 2020).

More than 700 commercial enzyme-based products are extensively used in an array of over 40 different industrial sectors, which include the detergent and textile industries, food processing, medical therapeutics, pharmaceuticals and also for agricultural purposes (Sopalun and Iamtham 2020; Raghav et al., 2022). There is a need to explore new sustainable sources for enzyme production with enriched biological applications, fungal endophytes have been demonstrated as good sources of enzymes of industrial interest (Raghav et al., 2022).

However, many endophytic fungi remain unknown and most of this fungal biodiversity is found in tropical countries, such as Brazil. Consequently, the country has significant potential to be explored for traditionally cultivated, as well as native and endemic plants from different regions (Esposito and Azevedo 2010).

The fungus *Coriolopsis rigida* (Berk. & Mont.) Murrill (Polyporales, Basidiomycota) is one of the 21 species that constitute the genus Coriolopsis (Pires et al., 2017; Saparrat et al., 2014). It is a saprotrophic organism and is mainly found on decaying wood (Saparrat et al., 2014). It belongs to a group of white-rot fungi that are considered model organisms for the study and production of laccases, with most studies on this species focused on the action of this enzyme (Saparrat et al., 2014; Saparrat et al., 2010a; Saparrat et al., 2010b; Saparrat et al., 2002; Díaz et al., 2010). Other studies have also shown that this fungus provides advantages to the plant by establishing trophic interactions with them, such as greater tolerance to Cu and Zn and an improvement in plant growth (Arriagada et al., 2010).

Cochlospermum regium (Bixaceae) is a medicinal plant, native from Brazil, the roots are used in the preparation of decoctions, infusions and bottles for the treatment of uterine, intestinal and ovarian inflammations (Camilo et al., 2016). However, few studies

from the microbiota of this plant are described (Arruda et al., 2021, Montel et al., 2021). The aim of this work was to evaluate the biotechnological potential from endophytic fungi associated with *Cochlospermum regium*, thus the plant was submitted to endophytic fungi isolation, and *Coriolopsis rigida* was obtained, the production of bioactive extracellular enzymes for commercial biotechnological values were investigated.

MATERIAL AND METHODS

Identification of fungal species

The endophytic fungus *C. regium* was acquired from the fungi collection of the Federal University of Tocantins (Gurupi Campus), and it was identified using the CTAB (cationic hexadecyl trimethyl ammonium bromide) extraction protocol adapted from Venkateswarulu *et al*. (Venkateswarulu et al., 2018; Arruda et al., 2021). The molecular analyses was performed using the genomic DNA extracted from the fungal biomass, obtained after fifteen days of cultivation in Potato Dextrose Broth (PDB). The DNA was amplified using the Internal Transcribed Spacer Region (ITS)-1 and ITS-4 primers in pre-programmed thermocycler (Techne TC-5000), and the amplified product was purified. The amplified genomic region (ITS) was sequenced using capillary electrophoresis in an ABI3730 device, with POP7 polymer and BigDye v.3.1. The nucleotide sequence was analyzed using a database deposited at National Center for Biotechnology Information (NCBI) and nucleotide BLAST. Geneious® and MEGA® were used for aligning the genetic sequences and building the phylogenetic tree.

Cultivation of *Coriolopsis rigida* and preparation of enzyme extracts

To obtain mycelium agar discs for fermentation, *C. rigida* was cultivated on PDA medium in a Petri dish for 7 days. For standardized inoculation of the fungus on solid media, three discs of the same size of the fungus grown in the PDA plate were placed in the culture media. The discs were inoculated into eight polyethylene bags containing 10 g rice and 8.9 mL sterile distilled water, or in eight polyethylene bags containing 10 g pineapple crown bran and 10 mL sterile distilled water. The fungus was incubated at 28 °C in a BOD (Bio-Oxygen Demand) chamber for different periods of time (3, 6, 10, and 16 days). After incubation, the solid cultures were transferred to an Erlenmeyer flask (250

mL) containing 50 mL of sterile distilled water for the rice media or 100 mL of sterile distilled water for the pineapple crown bran media, followed by stirring for 30 minutes at 150 rpm to ensure extraction. The solutions were then filtered and stored at 4 °C. The solutions obtained from rice and pineapple crown bran were submitted to enzyme activity.

Determining the enzyme activity for *Coriolopsis rigida* cultivated on solid media

For enzymatic assays the McIlvaine buffer (pH 5.0) was prepared with citric acid 0.1 M (Synth, Diadema, Brazil) and sodium phosphate dibasic 0.2 M (Synth, Diadema, Brazil). Enzymatic assays were carried out using Spectrophotometer UV-VIS Kazuaki IL-592 (Wuxi, China).

Determination of lipase activity — Lipase activity was determined using *p*-nitrophenyl palmitate (pNPP, Sigma-Aldrich) as a substrate. The reaction medium was prepared by solubilizing 3.8 mg pNPP in 0.5 mL dimethyl sulfoxide (DMSO, Dinâmica) and diluting in 0.1 M McIlvaine buffer (pH 5.0) containing 0.5% Triton X-100. The hydrolysis of pNPP was determined at 40 °C based upon the release of p-nitrophenol. Each test tube was pre-incubated in a water bath for approximately 5 minutes and contained 900 µL of the reaction medium. To start the reaction, 100 µL of the sample was added to the test tubes. After 5 minutes, the reaction was stopped in a boiling water bath (1 minute) and by the addition of 1 mL of saturated sodium tetraborate solution. All enzyme assays were performed in duplicates, and the absorbance was recorded at 410 nm using pNPP as the standard [molar extinction coefficient (ε): 18,677 M^{-1} cm^{-1}]. Enzyme extract was not added to the controls. The amount of enzyme required to release 1 µmol of pNPP per minute and per mL was defined as one enzyme unit (Mendes et al. 2019).

Determination of β-xylosidase activity — *β*-xylosidase activity of the enzyme extract was determined using a 0.25 % (w/v) solution of the synthetic substrate *p*-nitrophenyl *β*-D-xylopyranoside (pNPX, Sigma-Aldrich), diluted in 0.1 M McIlvaine buffer at pH 5. The assay was performed in test tubes containing 150 µL of the same buffer and 50 µL of the pNPX solution (resulting in a 200 µL solution that was then kept for 10 minutes in a water bath at 50 °C). Following this, a 200 µL diluted enzyme extract was added when necessary, to obtain 400 µL of reaction medium. After 10 minutes the reaction was stopped by adding 1 mL of saturated sodium tetraborate solution to 100 µL of the reaction medium. All enzyme assays were performed in duplicates, and the absorbance was recorded at 410 nm using pNPX as the standard (ε: 1,997 M^{-1} cm^{-1}). The

controls were prepared without adding the enzyme extract. The amount of enzyme required to release 1 μmol of *p*-nitrophenyl per minute and per mL was defined as one enzyme unit (Nascimento et al., 2019).

Determination of fructosyltransferase (FTase) activity — FTase activity was determined using a 20 % sucrose solution in 0.1 M McIlvaine buffer at pH 5 for 60 min. To start the reaction, 100 μL of the enzyme extract was added to tubes containing the sucrose solution. The reaction was stopped by boiling the mixture for 10 min in a water bath. Thereafter, 10 μL of the reacted solution was added to tubes containing 1 mL of the test reagent (Liquid Enzyme Glucose Kit, Doles). All enzyme assays were performed in duplicates, and the absorbance was measured at 510 nm, using glucose from the Liquid Enzyme Glucose Kit as a standard (ϵ: 81 M^{-1} cm^{-1}). The controls did not contain the enzyme extract. One enzyme unit was defined as the amount of enzyme required to generate 1 μmol of glucose per minute and per mL (Nascimento et al., 2019).

Determination of pectin esterase activity — Pectinolytic activity was determined using 1 % citric pectin (CP Kelco) as a substrate, dissolved in 0.1 M McIlvaine buffer at pH 5. The reaction medium contained 400 µL of the substrate. The reaction was started by adding 400 µL of the enzyme extract and was maintained at a temperature of 50 °C. It was stopped after 10 min by adding 200 µL of the reacted solution to 200 µL of 3,5-dinitrosalicylic acid (DNS, Didática). The samples were then boiled for 5 min and then cooled, before the addition of 2 mL of distilled water. The absorbance values were recorded in a spectrophotometer at 540 nm. The release of reducing sugars was determined according to Miller (1959), using galacturonic acid as a standard (ϵ: 119 M^{-1} cm^{-1}), via the DNS method. The enzyme extract was not added to the controls. One enzyme unit was defined as the amount of enzyme required to release 1 μmol of galacturonic acid per minute and per mL.

Determination of invertase activity — Invertase activity was determined using 800 μL of a 2% sucrose solution in 0.1 M McIlvaine buffer at pH 5 for 5 min. After the reaction, the absorbance values were measured using a spectrophotometer at 540 nm. The release of reducing sugars was determined according to literature (Miller 1959), using fructose as standard (ϵ: 122 M^{-1} cm^{-1}), via the DNS method (Miller 1959). One enzyme unit was defined as the amount of enzyme required to release 1 μmol of glucose per minute and per mL (Nascimento et al., 2019).

Determination of total cellulolytic activity (FPase) — FPase activity was determined by incubating 200 μL of the sample in 1 mL of 0.1 M Mcllvaine buffer at pH 5 with Whatman No. 1 filter paper at 50 °C for 60 min. After stopping the reaction, the values were recorded in a spectrophotometer at 540 nm. The release of reducing sugars was determined according to Miller (1959), via the DNS method, using glucose as a standard (ε: 108.1 M^{-1} cm^{-1}). One enzyme unit was defined as the amount of enzyme required to release 1 μmol of glucose per minute and per mL.

Determination of α-amylase activity — Amylolytic activity of α-amylase was determined using 400 μL of starch (1 %) dissolved in 0.1 M Mcllvaine buffer at pH 5. The tubes were kept at 50 °C in a water bath for approximately 5 min. The reaction was stopped using DNS solution, 10 min after addition of the enzyme extract. The tubes were then heated in a water bath at 100 °C for 5 min and then cooled, before the addition of 2 mL distilled water to each tube. The values were measured in a spectrophotometer at 540 nm. Release of reducing sugars was determined according to Miller (1959) via the DNS method, using glucose as a standard (ε: 108.1 M^{-1} cm^{-1}). One enzyme unit was defined as the amount of enzyme required to release 1 μmol of glucose per minute and per mL.

RESULTS

Identification of the endophytic fungus

Molecular identification up to the species level was based on the ITS region of the DNA, which revealed it to be the endophytic fungus *Coriolopsis rigida*. Molecular analysis showed that the endophytic fungus had 99 % genetic similarity to the species *Coriolopsis rigida*. The nucleotide sequence was deposited in the GenBank database under the accession number MN991225.

Analysis of enzyme production by the endophytic fungus *Coriolopsis rigida*

The endophyte *C. rigida* produced all the tested enzymes, when cultured in both rice and pineapple crown bran media, the enzyme yield in rice medium was lower than that in the pineapple crown bran medium (Figure 1A and 1B).

C. rigida produced considerable amounts of FTase (3.59 ± 0.52 U gss^{-1}) and invertase (2.14 ± 0.31 U gss^{-1}) after 6 days of cultivation in the pineapple crown bran medium. On the other hand, the fungus produces lipase (1.94 ± 0.07 U gss^{-1}) and

pectinase $(1.73 \pm 0.17$ U gss^{-1}) after 10 and 3 days of culturing in the pineapple crown bran medium, respectively.

Figure 1: Screening of enzyme extracts after cultivation in (A) pineapple crown bran and (B) rice medium.
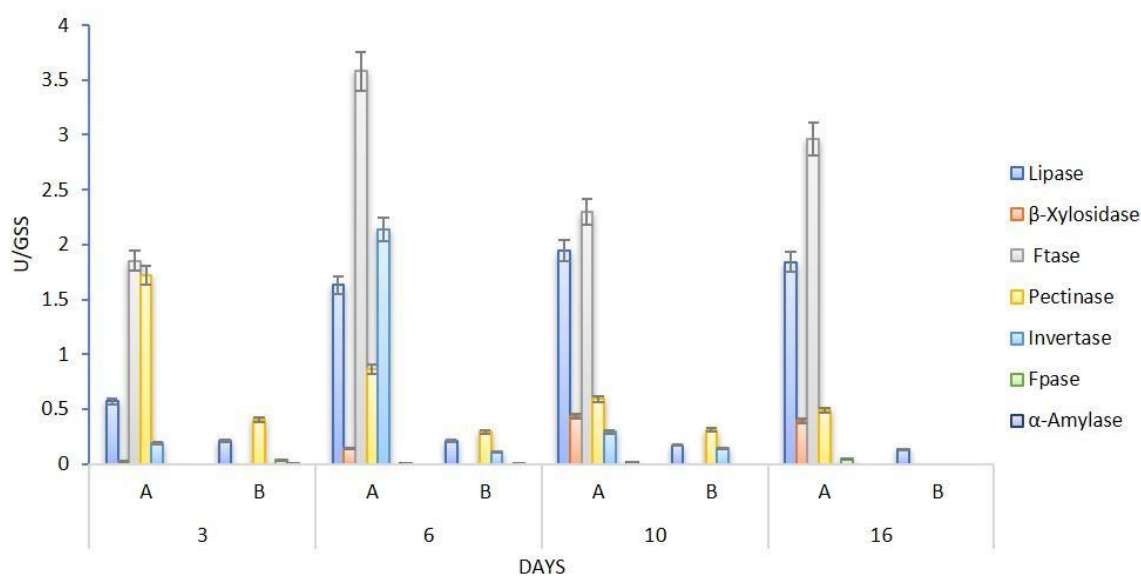
Fonte: Author

DISCUSSION

Identification of the endophytic fungus

      The genus *Coriolopsis* Murill (1905, Order Polyporales, Phylum Basidiomycota) includes species that provoke white rot of wood and are mostly restricted to tropical áreas. The taxonomic position of *Coriolopsis* is under discussion. The genus is closely related to *Trametes*, representatives of both genera are morphologically similar except for the conspicuous brown color of the context of the basidiocarp in *Coriolopsis* as well as the vegetative hyaline hyphae of *Trametes* (Saparrat et al., 2014).

      According to the Index Fungorum (http://www.indexfungorum.org) the name of *C. rigida* now has been changed to *C. floccosa* based on the priority principle (International "Melbourne" Code of Nomenclature for algae, fungi, and plants, Section 3. Priority Art. 11, Art. 12)(Saparrat et al., 2014). However, *C. rigida* is a formerly known name and have been used in several studies (Pires et al., 2017; Saparrat et al., 2014; Saparrat et al., 2010b; Saparrat et al., 2010a; Almonacid et al., 2015; Siles et al., 2014).

Analysis of enzyme production by the endophytic fungus *Coriolopsis rigida*

When endophytes inhabit a plant, they produce enzymes to hydrolyze the cell walls of the host. These enzymes can also directly suppress the activities of phytopathogens and degrade the cell walls of other microorganisms. When cultured in both rice and pineapple crown bran media the endophyte *C. rigida* have enzyme yield in rice medium lower than in the pineapple crown bran medium. This can be explained by the diverse chemical compositions of the two substrates. Moreover, pineapple crown bran is composed of 12.55 % lignin, 43.12 % cellulose, and 19.15 % hemicellulose, whereas rice is composed of 87.58 % total starch, 8.94 % protein (N × 5.95), 0.36% lipids, and 2.88% total fiber, thereby confirming that they induce the expression of different enzymes, as a consequence of their distinct chemical constituents (Walter et al., 2008; Santos 2018).

FTases and invertases were produced to the highest degree, among all other analyzed extracellular enzymes. Invertases produce inverted sugars, which are used to manufacture artificial honey and plasticizing agents. FTases have a wide range of applications, primarily in the food industry, where it is used for the production of food supplements (Sharma et al., 2020). This demonstrates the biotechnological potential of *C. rigida* for usage in the food and pharmaceutical industries.

The production of lipase and pectinase was also significant. Lipases are widely used in therapeutic industries and for the synthesis of detergents, oils, fatty acids, and triacylglycerols (Shubha et al., 2017). Pectinases are commonly expressed by endophytic and pathogenic fungi because these enzymes are important for phytopathological processes, plant-fungus symbiosis, and the decomposition of plant material (Alberto et al., 2016).

The fungus has a great versatility in the production of enzymes, demonstrating the biotechnological potential from *C. rigida*. However, an optimization in the cultivation medium may be done to increase the enzyme production. When compared to the literature the values obtained on average are lower than those reported, for example in the work developed by Marques et al. (2018) the endophytic fungus *Coniella petrakii* PM02 producers β-xylosidase reaching 13.07 $U.g^{-1}$ of activity.

In conclusion, this worth highlighting the biotechnological potential of the endophytic fungus *C. rigida* due the production of the extracellular enzymes of industrial

interest, especially FTase and invertase were obtained. The results of the present study showed the technological application of *Coriolopsis rigida*, in favor of society.

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