

Lipase production from *Aniba canelilla* endophytic fungi, characterization and application of the enzymatic extract

Produção de lipase a partir de fungos endofíticos de *Aniba canelilla*, caracterização e aplicação do extrato enzimático

Producción de lipasas a partir de hongos endofíticos de *Aniba canelilla*, caracterización y aplicación del extracto enzimático

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Abstract

Endophytic fungi (EF) have a notable capacity to produce active molecules of industrial importance, such as hydrolytic enzymes. In this study we investigated the production of lipase by EFs isolated from the Amazonian species *Aniba canelilla* (Lauraceae), characterized the enzymatic extract obtained from the most promising fungus, and applied the lipolytic extract as a biocatalyst in the transesterification reaction for biodiesel production. The fungi were submitted to enzymatic screening in solid medium and in submerged fermentation to assess their lipase production. A total of 292 fungi were tested in solid media. Lipolytic activity was detected in 74% of the fungi cultivated in liquid media, 18 of which showing promising enzymatic production. The best lipase producer, *Endomelanconiopsis endophytica* QAT_7AC, was identified by sequencing of the ITS region. After adjusting the bioprocess conditions, *E. endophytica* QAT_7AC produced 2,415.5 U/mL of lipase after 72 h. The enzymatic extract showed higher lipolytic activity under pH 8.0 and 40 °C. The extract was applied as a biocatalyst in a transesterification reaction performed at 40 °C, with ethanol and waste cooking oil (3:1). The biodiesel yield was found to be 87% after 2 h reaction when the fungal enzyme was used and 89% with the commercial biocatalyst. The endophytic fungi isolated from *A. canelilla* proved themselves to be biotechnologically relevant, as they can be explored as potential producers of lipases. The lipolytic extract can be applied in the synthesis of biodiesel using waste cooking oil.

Keywords: Amazon; Endophyte; Biocatalysis; *Endomelanconiopsis endophytica*; Biodiesel.

Resumo

Os fungos endofíticos (FE) possuem notável capacidade de produzir moléculas ativas de importância industrial, como as enzimas hidrolíticas. Neste estudo foi investigada a produção de lipase por FEs isolados da espécie amazônica *Aniba canelilla* (Lauraceae), sendo caracterizado o extrato enzimático obtido a partir do fungo mais promissor e avaliada a aplicação do extrato lipolítico como biocatalisador na reação de transesterificação para a produção de biodiesel. Os fungos foram submetidos à triagem enzimática em meio sólido e por fermentação submersa, para verificar a produção de lipase. Um total de 292 fungos foram testados em meio sólido. A atividade lipolítica detectada em 74% dos fungos cultivados em meio líquido, onde 18 apresentaram atividade enzimática promissora. O melhor produtor de lipase, *Endomelanconiopsis endophytica* QAT_7AC, foi identificado pelo sequenciamento da região ITS. Após o ajuste das condições do bioprocessamento, o *E. endophytica* QAT_7AC produziu 2.415,5 U/mL de lipase em 72 h. O extrato enzimático apresentou maior atividade lipolítica em pH 8,0 e 40 °C. O extrato foi aplicado como biocatalisador em uma reação de transesterificação realizada a 40 °C, com etanol e óleo de cozinha residual (3:1). O rendimento de biodiesel foi de 87% após 2 h de reação quando a enzima fúngica foi utilizada e de 89% com o biocatalisador comercial. Os fungos endofíticos isolados de *A. canelilla* mostraram-se biotecnologicamente relevantes e podem ser explorados como potenciais produtores de lipases. O extrato lipolítico pode ser aplicado na síntese de biodiesel a partir do óleo de cozinha residual.

Palavras-chave: Amazônia; Endófito; Biocatálise; *Endomelanconiopsis endophytica*; Biodiesel.

Resumen

Los hongos endofíticos (EF) tienen una notable capacidad de producir moléculas activas de importancia industrial, como las enzimas hidrolíticas. En este estudio investigamos la producción de lipasa por hongos aislados de la especie Amazónica *Aniba canelilla* (Lauraceae), caracterizamos el extracto enzimático obtenido de lo hongo más promisor y aplicamos el extracto lipolítico como biocatalizador en la reacción de transesterificación para la producción de biodiesel. Los hongos aislados fueron sometidos a un cribado enzimático en medio sólido y fermentación sumergida para evaluar la producción de lipasa. Un total de 292 hongos fueron testados. La actividad lipolítica fue detectada en el 74% de los hongos cultivados en medio líquido, 18 de los cuales presentaron producción enzimática promissoria. El mejor productor de lipasa, *Endomelanconiopsis endophytica* QAT_7AC, fue identificado por el secuenciamento de la región ITS. Después del ajuste de las condiciones del bioproceso, *E. endophytica* QAT_7AC produjo 2,415.5 U/mL de lipasa en 72 h. El extracto enzimático presentó mayor actividad lipolítica a pH 8.0 y 40 °C. El extracto enzimático fue aplicado como biocatalizador en una reacción de transesterificación realizada a 40 °C con etanol y aceite de cocina usado (3:1). El rendimiento del biodiesel fue de 87% después de 2 h de reacción cuando la enzima fúngica fue utilizada y 89% con el biocatalizador comercial. Los hongos endofíticos aislados de *A. canelilla* fueron biotecnológicamente relevantes, y pueden ser explorados como potenciales productores de lipasas. El extracto enzimático lipolítico puede ser aplicado en la síntesis de biodiesel utilizando aceite de cocina usado.

Palabras clave: Amazonía; Endófito; Biocatálisis; *Endomelanconiopsis endophytica*; Biodiesel.

1. Introduction

Medicinal plants are widely investigated as sources of bioactive substances, which may be used in new therapies because they present close relationship with the organisms that inhabit their tissues (Palanichamy et al., 2018). A member of the Lauraceae family, *A. canelilla* is an aromatic species distributed in the northern, northeastern and midwestern regions of Brazil, and is popularly known as “casca-preciosa” (precious bark). It has been used by Amazonian traditional medicine for the treatment of pain and digestive, respiratory, inflammatory, and central nervous system disorders. Its ethanolic extracts present phenolic compounds, tannins, flavonoids, saponins, and its essential oil may contain up to 90% 1-nitro-2-phenylethane, a substance that presents several pharmaceutical properties, such as antifungal, antinociceptive, hypnotic, anticonvulsant, anxiolytic and cytotoxic activities (Barros et al., 2018; Souza-Junior et al., 2020).

Several studies that aim to elucidate the biotechnological potential of medicinal plants from the Lauraceae family have been developed in recent years, mainly concerning the applications of their essential oils (Giongo et al., 2017; Barros et al., 2018; Oliveira Filho et al., 2015; Palanichamy et al., 2018; Souza et al., 2018; Souza-Junior et al., 2020). However, despite what is known about *A. canelilla*, the biotechnological potential of the endophytic fungi associated with this medicinal plant species is still yet to be revealed.

Fungi are an important source of hydrolytic enzymes (HEs). HEs have vast catalytic versatility and represent important industrial inputs that comprise a wide range of applications (laundry detergent, textiles, cellulose and paper, leather, cosmetics,

biofuel and food industries). Of all the HEs, amylases, cellulases, xylanases, pectinases, proteases and lipases are among the most industrially used (Krishnan et al., 2016; Thapa et al., 2019; Batista et al., 2022).

Lipases (EC 3.1.1.3) are versatile and efficient biological catalysts that act on the cleavage of ester bonds, reducing triacylglycerols into smaller units (Chandra et al., 2020; Oliveira et al., 2021). Fungal lipases, as well as those of bacterial origin, are widely screened due to their versatility as biocatalysts. In addition, according to Chandra et al. (2020), the microbial lipase market is projected to reach US\$ 590.2 million by 2023, which reflects its numerous applications.

Endophytic fungi are excellent producers of lipases (Silva et al., 2014; Soldi et al., 2020; Monteiro et al., 2020; Sopalun & Iamtham, 2020; Matias et al., 2021), which can be used in various industrial processes, including the production of biodiesel (Amini et al., 2017; Guo et al., 2020; Al-Zaban et al., 2021; Cavalcante et al., 2021).

Biodiesel is a renewable fuel and has become an attractive alternative to replacing diesel of fossil origin. However, some challenges still have to be overcome, such as the cost of raw materials. Soybean oil is the main raw material used for biodiesel production in Brazil and corresponds to 70-80% of the cost of obtaining biofuel (Naylor & Higgins, 2018). One alternative for reducing this cost is the use of cooking oil (Leung & Guo, 2006). In addition, the process of conventional biodiesel production presents strategic, environmental, technological and economic challenges. On the other hand, despite still having a high cost, enzymatic transesterification has a number of advantages in this process (Kumar et al., 2021), which justifies the search for new sources of lipase.

Endophytes represent a promising alternative as a source of bioactive molecules (Fadiji & Babalola, 2020) and, therefore, it is deemed interesting to evaluate the potential of endophytic fungi associated with tropical species to produce lipase, since studies are still scarce. According to Hawksworth & Lücking (2017), there is an estimated number of 2.2 to 3.8 million species of fungi on the planet, and only around 8% of these have been isolated and identified. So far, there have been no studies regarding endophytic fungi from *A. canelilla*. Therefore, this study aimed at to assess the lipolytic potential of *A. canelilla* endophytic fungi, to improve the lipase production and characterize the enzymatic extract obtained from the best producer, as well as to apply the lipolytic extract as the biocatalyst on the synthesis of biodiesel using cooking oil.

2. Methodology

2.1 Microorganisms

The *A. canelilla* endophytic fungi used in this study are deposited in the Microbiological Collections Center at UEA, preserved by the Castellani method (Castellani, 1939). Fungi was isolated from leaves and thin branches of *A. canelilla* 5 specimens located in the Adolpho Ducke Forest Reserve (Manaus, Brazil). A total of 292 fungi were used for the screening of lipase production. The isolates were reactivated in potato-dextrose-agar (PDA) in a biochemical oxygen demand (BOD) incubator for 7 days at 28 °C.

2.2 Enzymatic Evaluation in Solid Media

To determine the lipase enzymatic index (EI), lipase agar (LA) was prepared, consisting of 6.0 g/L of peptone, 3.0 g/L of NaCl, 0.06 g/L of CaCl₂·2H₂O, 18 g/L of agar and 1% Tween 80 (autoclaved separately), which was added as an inducer of lipase production (Roy et al., 2018). The isolates were inoculated on plates containing LA and incubated for 4 days at 28 °C in a BOD incubator. Lipolytic activity was evaluated according to the formation (or not) of a clear halo around the colony, which was measured with a caliper. The EI was calculated by the ratio between the diameter of the halo and the diameter of the colony (Batista et al., 2022). Cultivation in the solid medium was carried out in triplicate.

2.3 Enzymatic Production in Submerged Fermentation

The isolates were inoculated on tomato extract agar (2% tomato juice extract and 17 g/L agar) in an inclined test tube to induce sporulation (Gomes & Pena, 2016). After 7 days of incubation at 28 °C, 4 mL of sterile distilled water was added to the tubes, which were shaken vigorously to release the spores. The inoculum was prepared at a concentration of 1×10^6 spores/mL. Liquid medium was composed of NH_2NO_3 (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), KH_2PO_4 (0.1%), peptone (2.0%) and olive oil (1.0%); 125 mL of this medium was added to Erlenmeyer flasks of 500 mL. The pH of the culture medium was adjusted to 6.0 and autoclaved at 121 °C for 15 min (Nascimento et al., 2014). After cooling, 500 μL of the spore suspension were inoculated. The experiments were carried out in triplicate for 120 h at 160 rpm and 28 °C in a shaker incubator. A control experiment (without fungal inoculation) was also carried out.

2.4 Determination of Lipase Activity

The quantification of the lipolytic activity was performed as described by Winkler & Stuckmann (1979) and used *p*-nitrophenyl palmitate (pNPP) as a substrate. To prepare the substrate solution, 1 mL of solution A (10 mL of isopropanol with 30 mg of pNPP) was mixed in 9 mL of solution B (90 mL of 0.05 M phosphate buffer, pH 8.0 containing 207 mg of deoxycholate of sodium and 100 mg of gum arabic). The substrate solution was heated to 37 °C and then 1 mL of the extract was added to 2 mL of the prepared substrate solution. After 15 min of incubation at 37 °C, a reading was made on a spectrophotometer at 410 nm. One unit (U) of enzyme activity was defined as the amount of enzyme needed to release 1.0 μmol of *p*-nitrophenol per minute. The standard curve was constructed using *p*-nitrophenol (PNP).

2.5 Improvement of Lipase Production

The most promising isolate for lipase production in submerged cultivation was further used in order to improve its lipase production. The fungal strain was inoculated in PDA and in tomato juice agar (190 g/L of tomato juice and 18 g/L of agar, TJA) to find out whether the inoculum cultivation medium was able to influence the synthesis of lipase. The fungus was grown at 30 °C for 11 days, with 65% humidity inside a BOD incubator.

After defining the best medium for inoculum production, we studied the temperature (25, 30 and 37 °C) and pH (5.0, 6.0 and 7.0) values of fungal growth that would improve the production of lipase. A mineral solution, composed of 0.3 g/L of $(\text{NH}_4)_2\text{SO}_4$; 0.9 g/L of KH_2PO_4 ; 1.8 g/L of NaH_2PO_4 ; 0.045 g/L of CaCl_2 ; and 0.15 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and supplemented with 1% soybean oil, was used as the culture medium (A'Yuni & Ilmi, 2021). Three plugs of mycelium (5 mm in diameter) were inoculated. The flasks were incubated at 140 rpm and 30 °C for 4 days in a shaker. The lipolytic extract was separated from the mycelium using sterile gauze filtration and the enzyme activity was measured as described above. The experiments were performed in triplicate.

2.6 Characterization of the Lipolytic Extract

The enzymatic extract was characterized according to the methodology described by Alabdallal et al. (2021), with modifications. To determine the optimal pH for the lipolytic reaction, the enzyme activity was measured using the following buffer solutions: 50 mM citrate buffer (pH 5.0); sodium phosphate buffer (pH 6.0, 7.0 and 8.0); and 50 mM carbonate buffer (pH 9.0, 10.0 and 10.7). The determination of the optimal temperature for the lipase reaction was evaluated after the enzymatic extract was incubated at 37, 40, 45, 50, 60, 65, 75, and 80 °C.

2.7 Enzymatic Transesterification for Biodiesel Production

The enzymatic extract was used as a biocatalyst in the transesterification reaction of the cooking oil. The cooking oil

was provided by a restaurant in the city of Manaus, Amazonas and presented 0.96% acidity, determined according to the methodology of the Adolfo Lutz Institute (IAL, 2008).

The transesterification reaction was performed using a bench reactor at 40 °C under constant agitation of 150 rpm, for 2 and 6 hours. Ethanol 96% was used in a 3:1 ratio (alcohol:oil) with approximately 3% by weight of the enzyme (Karimi, 2016). A control experiment with *Candida rugosa* commercial lipase (Sigma-Aldrich) was also performed for comparison purposes. The reactions were performed in triplicate.

At the end of the reaction, the mixture obtained was transferred to a separation funnel where it was left to rest for 24 h for the separation of the two phases. After this step, the biodiesel was heated to 80 °C and washed with 0.2 M hydrochloric acid.

The biodiesel yield was calculated as described by Al-Zaban et al. (2021). The analysis of the reaction products was performed using a gas chromatograph (Agilent Technologies, model CG-7890B) coupled to a mass spectrometer (model MS-5977A).

2.8 Identification of the Most Promising Fungus

The most promising fungus for lipase synthesis was identified via molecular analysis. The fungal DNA was extracted using phenol-chloroform (Sambrook et al., 1989) and then amplified using the following primers: ITS5f (5'-TCCTCCGCTTATTGATATGC-3') and ITS4r (5'-TCCGTAGGTGAACCTGCGC-3'). The sequencing was performed using an automatic sequencer (AB 3500 Genetic Analyzer, ACTGene), which was performed by Análises Moleculares Ltda., Biotechnology Center, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

The sequence matrix was constructed by comparing sequences present in the NCBI database with those obtained in the sequencing. The DNA sequences obtained were aligned using MAFFT software (version 7). The maximum likelihood analyses were performed on MEGA 6, using the Tamura-Nei statistical model, with a 1000-repetition bootstrap.

2.9 Statistical Analysis

The enzymatic activity data were subjected to analysis of variance (ANOVA), with a significance of 0.05, and to Tukey's comparison test, using GraphPad Prisma Software (version 8.0.1).

3. Results and Discussion

3.1 Enzymatic Screening in Solid Medium

The biodiversity of endophytic fungi associated with species of the Lauraceae family is still little known. In the present study, we sought to contribute with new information on the cultivable endophytic fungal biota associated with the species *Aniba canelilla* that produce lipase, an enzyme with numerous industrial application (Chandra et al., 2020). Of the 292 endophytic fungi isolated from *A. canelilla*, 215 (74%) were able to produce lipase in a solid medium. Of the 138 fungi isolated from leaves, 109 showed lipolytic activity, and 27 presented an EI of between 2 and 5. Of the 154 fungi isolated from branches, 101 showed lipolytic activity, and 32 had an EI of between 2 and 5. An EI lower than 2 was observed mainly in fungi isolated from leaf tissue, while the highest EI were observed in fungi isolated from thin branches, as shown in Tables 1 and 2.

Table 1. Solid media screening for lipase production of 292 endophytic fungi isolated from leaves and thin branches of *Aniba canelilla* specimens.

Specimen	Leaves				Branches			
	NIF	Total LPF	EI<2 (%)	EI>2 (%)	NIF	Total LPF	EI<2 (%)	EI>2 (%)
S1	21	16 (76.2%)	9 (56.3%)	7 (43.8%)	25	18 (72.0%)	8 (44.4%)	10 (55.6%)
S2	24	15 (62.5%)	8 (53.3%)	7 (46.7%)	20	17 (85.0%)	12 (70.6%)	5 (29.4%)
S3	39	30 (76.9%)	29 (96.7%)	1 (3.3%)	21	14 (66.7%)	13 (92.9%)	1 (7.1%)
S4	34	31 (91.2%)	27 (87.1%)	4 (12.9%)	57	26 (45.6%)	13 (50.0%)	13 (50.0%)
S5	20	17 (85.0%)	9 (52.9%)	8 (47.1%)	31	26 (83.9%)	23 (88.5%)	3 (11.3%)
Total	138	109 (79%)	82 (75.2%)	27 (24.8%)	154	101 (65.6%)	69 (68.3%)	32 (31.7%)

NIF = Number of isolated fungi. LPF = Lipase producing fungi. EI = Enzymatic index. Source: Authors.

Table 2. Lipase enzymatic index of endophytic fungi selected for submerged fermentation assay.

Fungi	EI ± SD	Fungi	EI ± SD	Fungi	EI ± SD
QAT_2AC	0	QAT_3AC	1.50 ± 0.1	QAT_21AC	2.30 ± 0.1
QAT_5AC	1.40 ± 0.1	QAT_4AC	2.30 ± 0.3	QAT_24AC	2.30 ± 0.2
QAT_6AC	3.70 ± 0.2	QAT_9AC	1.50 ± 0.1	QAT_25AC	0
QAT_7AC	1.40 ± 0.1	QAT_10AC	5.00 ± 0.4	QAT_28AC	0
QAT_8AC	1.30 ± 0.1	QAT_11AC	2.20 ± 0.1	QAT_30AC	3.90 ± 0.2
QAT_1AC	1.60 ± 0.1	QAT_13AC	2.90 ± 0.1	QAT_36AC	0

EI = Enzymatic Index. SD = Standard Deviation. Source: Authors.

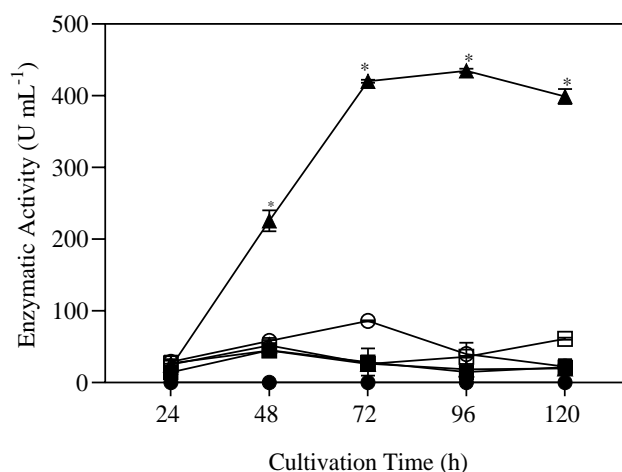
The lower lipase production capacity observed in fungi isolated from leaves when compared to that of *A. canelilla* branches, an aromatic plant that produces an aroma similar to that of cinnamon, may be correlated with the fact that its leaves have a superficial wax, which is a thin extracellular layer of lipids on the leaf blade, and which consists mainly of cutin, an insoluble polymer of fatty acids (Shepherd et al., 1995; Barros et al., 2018). The richness of leaf wax in alkanes, esters, ketones and alcohols, substances that may have a selective function in leaf tissue, it can be assumed that fungi isolated from *A. canelilla*.

The interest in new sources of fungal lipases has motivated the investigation of the catalytic potential of endophytic fungi isolated from several plant species (Carvalho Neto, 2013; Chandra et al., 2020; Putri et al., 2020; Matias et al., 2021). Zanotto et al. (2009) demonstrated the viability of using mycelium-bound lipases from Amazon fungi as a biocatalyst in esterification, hydrolysis, and racemic resolution through transesterification. The authors screened 212 fungi isolated from leaves, stems, fruits, roots, and seeds of different Amazonian hosts and observed that 64 (30%) were able to produce lipases. In the present study, a high percentage (74%) of the isolates evaluated were able to produce the enzyme. Bernardi-Wenzel et al. (2012) evaluated 28 endophytic fungi isolated from soybean leaves for their ability to synthesize lipase. Surprisingly, none of them showed lipolytic activity, although they were able to produce amylases and proteases, thus revealing an interesting fact in the biology of these fungi, since their host is an oil-rich plant.

3.2 Submerged Fermentation

Among the methods commonly used to obtain lipase, submerged fermentations have been widely used due to the availability of nutrients in the reaction medium and through the supply of oxygen through agitation. In all, eighteen endophytic fungi were selected to be evaluated in submerged fermentation (6 had EI between 2 and 5; 8 had EI<2 and 4 had EI=0). The most promising fungi in liquid medium were QAT_2AC, QAT_5AC, QAT_6AC, QAT_7AC and QAT_8AC. Of these, QAT_7AC was the isolate that stood out, since it produced higher amounts of lipase, reaching 434.89 U/mL after 72 h of cultivation. This enzymatic activity was significantly different from the lipase production observed for the other fungi (Figure 1). Interestingly, if only the solid medium assay were to be considered, this fungus would not be selected, since usually only microorganisms with EI ≥ 2 are considered potential producers of enzymes (Batista et al., 2022).

Figure 1. Lipase production in submerged fermentation of five endophytic fungi isolated from *Aniba canelilla*: QAT_5AC (■); QAT_7AC (▲); QAT_2AC (△); QAT_8AC (□); QAT_6AC (○); and negative control - NC (●). The presence of "*" indicates a significant difference in lipase production among the evaluated isolates ($p < 0.05$).



Source: Authors.

3.3 Improvement of Lipase Production by the Fungus QAT_7AC

To develop the best conditions for lipase production using *E. endophytica* QAT_7AC, it was evaluated whether the culture medium used to produce the inoculum could influence the synthesis of the enzyme. It was observed that when the tomato juice agar was used for inoculum cultivation, the enzymatic activity of QAT_7AC was significantly higher (519.3 U/mL) in submerged fermentation, when compared to the inoculum grown in potato dextrose agar (434.9 U/mL). This fact can be explained by the complex composition of tomatoes, which are a rich source of ascorbic acid, potassium, folic acid, phenolic and carotenoid compounds such as lycopene (Rattanavipanon et al., 2021; Oliveira-Bouzas et al., 2021; Vélez-Terreros et al., 2021). Therefore, the use of tomatoes in culture media used in the preparation of inoculum should be better studied, since the most commonly used culture medium is PDA (Banhos et al., 2014; Ferreira et al., 2015; Batista et al., 2018; Sopalun & Iamtham, 2020; Nascimento et al., 2020).

The fungus QAT_7AC was, therefore, cultivated in TJA and used as inoculum in the trials to evaluate the best temperature and pH values for lipase production. Lipase synthesis was 213.4 U/mL when the fungus was grown at 25 °C and pH 7.0. However, at 30 °C, lipase synthesis increased significantly at this pH, reaching a maximum activity of 2,415.5 U/mL and, at 37 °C, lipase activity dropped (197.2 U/mL). Treatment 8, therefore, appeared to be the best combination of the two factors, in which pH 7.0 and temperature of 30 °C were used (Table 4).

Table 4. Lipase production using QAT_7AC endophytic fungi from *Aniba canelilla*, cultivated under different temperature and pH values.

Run	pH	T (°C)	EA (U/mL)
1	5.0	25	103.81 ^f
2	5.0	30	200.34 ^d
3	5.0	37	3.67 ^h
4	6.0	25	127.35 ^e
5	6.0	30	930.67 ^b
6	6.0	37	76.10 ^g
7	7.0	25	213.42 ^c
8	7.0	30	2,415.5 ^a
9	7.0	37	197.17 ^d

T = culture temperature; EA = enzymatic activity. Means that do not share a letter are significantly different ($p < 0.05$). Source: Authors.

Data on the lipolytic potential of endophytic fungi isolated from Lauraceae are scarce. Carvalho Neto (2013) also identified the fungus *E. endophytica*, isolated from the stem of *A. rosaeodora*, as an efficient producer of lipase (1,821 U/mL) in seven days of submerged cultivation. In the present study, the fungus selected as the best lipase producer was identified as the same species and, after evaluating the best cultivation conditions through an experimental design, using the inoculum grown in TJA, the fungus isolated from *A. canelilla* leaves, presented high lipase activity (2,415.5 U/mL) in only 72 h of cultivation, suggesting that endophytic fungi isolated from *Aniba* species may represent an important biotechnological source for obtaining this enzyme.

The culture temperature was shown to be a determining factor for the synthesis of lipase by the fungus *E. endophytica* QAT_7AC, which is in accordance with the study of Nayana et al. (2020). The authors reported the optimization of media components, physical factors and additional additives for lignin peroxidase production by the endophytic fungus *Endomelanconiopsis* sp. under submerged fermentation, and found that at 30 °C the isolate produced higher amounts of the enzyme. On the other hand, the adequate pH value for the cultivation of QAT_7AC for lipase production was established as 7.0. Nayana et al. (2020) found pH 5.0 to be ideal for lignin peroxidase production by *Endomelanconiopsis* sp. There are still few reports about this fungus as an endophyte, and little information about the influence of pH during its enzyme production. To the best of our knowledge, this is the first report of pH assessment for lipase production by the fungus *E. endophytica*.

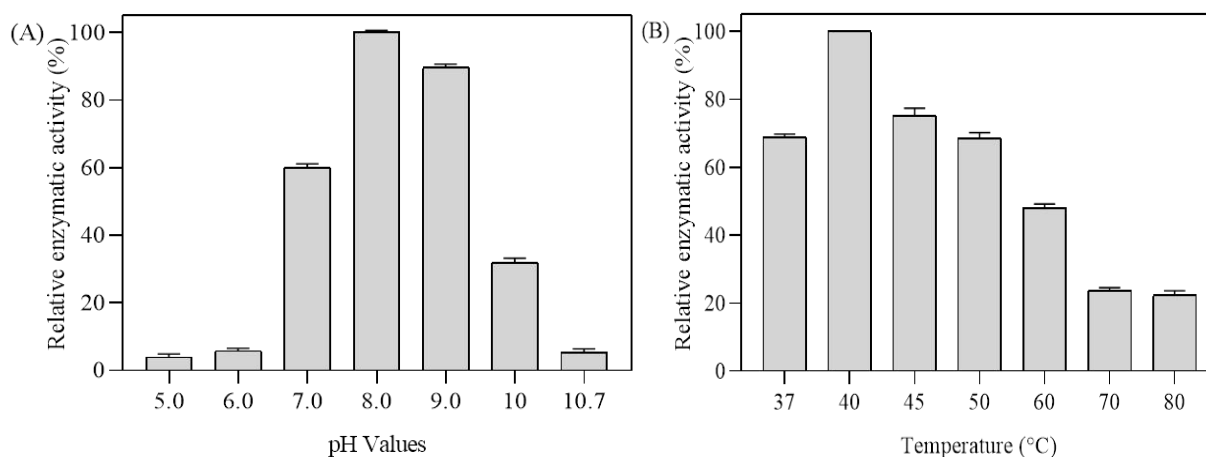
3.4 Characterization of the Lipolytic Extract Produced by the QAT_7AC Isolate

The highest enzymatic activity was found at pH 8.0. However, there was no significant difference ($p < 0.05$) between pH 8.0 and 9.0 for the enzymatic reaction when the lipolytic extract produced by the fungus QAT_7AC was used under the experimental conditions (Figure 2A). The best temperature for the enzymatic reaction was 40 °C (Figure 2B), as the highest lipolytic activity was detected at this temperature.

There are still few reports about this fungus as an endophyte, and little information about the influence of pH during its enzyme production. To the best of our knowledge, this is the first report of pH assessment for lipase production by the fungus *E. endophytica*.

The optimal pH and temperature for the reaction catalyzed by the lipolytic extract produced by *E. endophytica* QAT_7AC corroborates the findings in the literature, for which the pH of 8.0 and the temperature of 40 °C have been reported (Gama, 2012). These optimal conditions are interesting if we consider the application of the extract in industrial processes, such as the production of detergents, which requires a stable lipase activity at a high alkalinity (pH 8.0 to 11.0) and, as such, improves the ability to remove persistent stains (Huang et al., 2019). These conditions are also adequate for biodiesel production via enzymatic transesterification (Talukder et al., 2010).

Figure 2. Evaluation of optimal pH (A) and temperature (B) for the enzymatic reaction using the lipolytic extract produced by the by the endophytic fungus QAT_7AC.



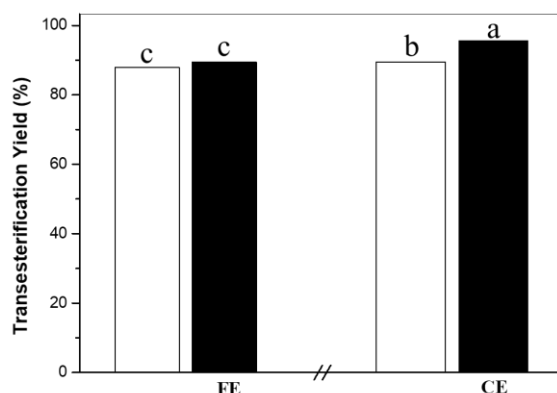
Source: Authors.

3.5 Production of Biodiesel

The yields obtained for enzymatic transesterification reactions are shown in Figure 3. It is observed that the enzymatic extract produced by the Amazonian endophytic fungus made it possible to obtain yields similar to those obtained with the commercial enzyme in 120 minutes of reaction, without statistical difference ($p = 0.051217$). In addition, it is possible to use waste oil as a raw material to obtain a biofuel from the fungal extract.

Biodiesel is composed of methyl or alkyl esters from fatty acids, usually produced through a chemically or enzymatically catalyzed transesterification reaction. Enzymatic transesterification stands out because this process has advantages over chemical catalysis: it does not form soaps and can esterify both free fatty acids and triglycerides in a single step without the need for a subsequent wash (Fan et al., 2012), which is especially interesting for raw materials with a high acidity index, such as waste oil. The raw material used to obtain biodiesel accounts for a major part of the total cost of production (Mehmood et al., 2021). Therefore, cooking oil offers significant potential as a low-cost alternative raw material for obtaining biodiesel (Leung & Guo, 2006).

Figure 2. Transesterification yield of cooking oil catalyzed by the fungal enzyme (FE) and by the commercial enzyme (CE) according to the reaction time: (□) 120 minutes and (■) 360 minutes. Means that do not share a letter are significantly different ($p < 0.05$).



Source: Authors.

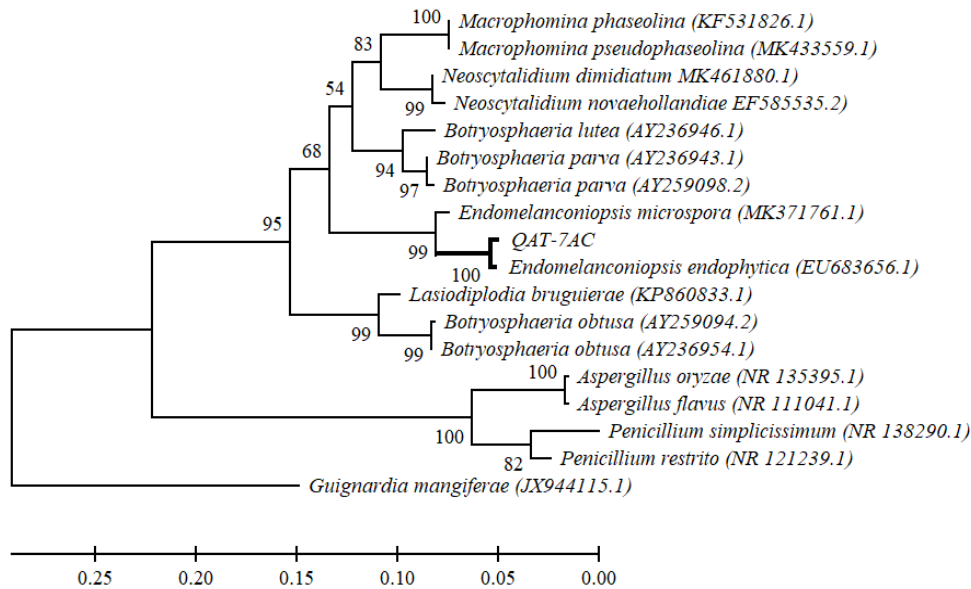
The enzyme extract, rich in lipase, produced by *E. endophytica* QAT_7AC is promising for use as a biocatalyst in the transesterification reaction of cooking oil, since the reaction yield (87.9%) was comparable to that obtained with the commercial enzyme (89.5%). Similar results have been described in the literature. Muanruksa & Kaewkannetra (2020) obtained a yield of 91.30% in a esterification reaction performed at 40 °C and 200 rpm, using an immobilised *Rhizopus oryzae* enzyme. Firdaus et al. (2016) obtained a yield of 93% using the lipase from *Thermomyces lanuginosus*. Ali et al. (2017) obtained a yield of 86% using cooking oil residue as raw material at 42 °C, with a 3:1 ratio (ethanol:oil) and 170 rpm agitation. While Rossi et al. (2018) obtained a yield of 90% using untreated waste oil for biodiesel production. Such studies demonstrate that the lipase of the Amazonian fungus QAT_7AC can be considered a potential biocatalyst for the biodiesel production reaction, when waste cooking oil is used as raw material.

3.6 Molecular Identification of Endophytic Fungi QAT_7AC from *Aniba canelilla*

The most promising isolate for lipase production was submitted to molecular analysis using the ITS intergenic spacer. The fungus QAT_7AC was identified with 100% maximum likelihood as the species *Endomelanconiopsis endophytica* (EU683656) isolated from the stem of the cocoa plant in Panama in 2008. The phylogenetic tree that illustrates the evolutionary history of the 18 sequences was obtained by the maximum likelihood method, and by the Tamura-Nei model. The tree with the highest log probability (-2999.61) is shown in Figure 4. The sequence for QAT_7AC was deposited in GeneBank under the code OL661613.

Species of this genus are complex to identify by classical taxonomy due to difficulties in the development of reproductive structures, since they are cryptic species with a dense versatility of habits (parasitic, saprophytic and endophytic) (Douanla-Meli & Scharnhorst, 2021).

Figure 4. Phylogenetic analysis of the endophytic fungus isolated from *Aniba canelilla* and sequences from GenBank (indicated by the database code). *Endomelanconiopsis endophytica*. The scale bar indicates the nucleotide substitutions per site, using the neighbor-joining method by maximum likelihood analysis. The numbers above and below each node indicate the frequency (in percentage) of each branch in 10000-repetition bootstrap analyses.



Source: Authors.

The genus *Endomelanconiopsis* was proposed as an anamorph of the Botryosphaeriaceae family by Rojas et al. (2008). The first specimens of *E. endophytica* to be identified were isolated from healthy leaves of *Theobroma cacao* (Malvaceae) and *Heisteria concinna* (Erythroplacae) in Panama, by sequencing the ITS region (ITS5 and ITS4) that has approximately 540 bp. The specimen isolated in Panama, deposited under the code EU683656 (520 bp) at GeneBank shows evolutionary proximity of 100% with the fungus QAT_7AC (574 bp), which was isolated from healthy leaves of *A. canelilla* in the Brazilian Amazon. In addition, *E. endophytica* is considered a sister species of *E. microspora*, a fact that was also verified in our phylogenetic analysis. Due to the evolutionary proximity of these species, the formation of a highly supported clade (99% bootstrap) is observed. Another interesting factor about this fungal family is the absence of interrelationships between many of the genera present and because no species of the family present a teleomorph.

In a recent study, Nagel et al. (2021) carried out a large-scale genomic analysis of the family Botryosphaeriaceae. The authors observed that this fungal family presents a richness of genes that encode for active carbohydrate enzymes (CAZymes), lipases and proteases. In addition, they observed that the genera *Botryosphaeria* present an enzymatic pattern that is consistent with necrotrophic pathogens, which tend to produce a greater number of hydrolytic enzymes than biotrophic and symbiotic fungi. In our study, the fungus *E. endophytica* QAT_7AC, also from the family Botryosphaeriaceae showed itself to be a promising lipase producer, which is in accordance with the findings of Nagel et al. (2021).

Recently, Romão et al. (2022) reported the production of amylases by *E. endophytica* isolated from the digestive tract of *Phylloicus* sp. The authors verified that the amylases were moderately tolerant to temperature variations and poorly tolerant to pH variations, when the fungus was cultivated in sweet potato starch. The same research group also verified that this fungal strain was able to produce metabolites with antioxidant activity (Romão et al., 2024).

Therefore, the hypothesis of Rojas et al. (2008) that the fungus *E. endophytica* has a wide geographical distribution and a preference for woody hosts can be confirmed, because, in Brazil, this fungus has also been isolated from another woody host of the Lauraceae family (*A. rosaeodora*) (Carvalho Neto, 2013).

4. Conclusion

The medicinal plant *Aniba canelilla* proved to be an important habitat for lipase-producing endophytic fungi, where a greater potential for lipase production was observed in isolates from branches than in leaf isolates. The fungus *Endomelanconiopsis endophytica* QAT_7AC showed the best potential for lipase synthesis.

The composition of the tomato-based inoculum production medium significantly improved enzymatic activity, and the cultivation temperature proved to be a key factor for enzymatic synthesis.

The fungal enzymatic extract was able to catalyze the transesterification reaction to obtain biodiesel using cooking oil as raw material. These results are important from a biotechnological point of view and, more importantly, to advance the environmental protection agenda of the Amazon Rainforest as a fundamentally vital source of biodiversity.

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