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CONCILIUM

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Biotransformation potential of the *R*-(+)-limonene by endophytic fungi strain associated with Amazonian fruit *Malpighia emarginata* DC

Potencial de biotransformação do *R*-(+)-limoneno por linhagem de fungos endofíticos associados à fruta amazônica *Malpighia emarginata* DC

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ABSTRACT

This study aims to offer an account of the biotransformation of limonene through the endophytic fungi strains associated with the Amazonian fruit *Malpighia emarginata*. The fungal biotransformation was executed in flasks containing the substrate *R*-(+)-limonene in a mineral medium, which was further incubated at 28 °C on a rotary shaker (120 rpm) for 120 h. Samples of each culture were taken every 24 hours, extracted with ethyl acetate, and analyzed using gas chromatography–mass spectrometry with the National Institute of Standards and Technology database. In this study with five endophytic fungi strains, only *Penicillium* sp. AF- LAB4 was considered a potential biocatalyst found in the screening due to its capacity to use the *R*-(+)-limonene as the single carbon and energy source in a mineral medium since they eventually accumulated interesting compound as limonene-1,2-diol (128.10 µL/L) after 48 to 120 h of reaction. This achievement is important and supports the development of the production of natural aromas and demonstrates the potential of using this endophytic fungus strain from the fruit *M. emarginata*, as a new biocatalyst.

Keywords: Limoneno-1,2-diol; Acerola; *Penicillium* sp.

RESUMO

Este estudo tem como objetivo oferecer um relato da biotransformação do limoneno através de cepas de fungos endofíticos associados à fruta amazônica *Malpighia emarginata*. A biotransformação fúngica foi realizada em frascos contendo o substrato *R*-(+)-limoneno em meio mineral, que foram posteriormente incubados a 28 °C em agitador rotativo (120 rpm) por 120 h. Amostras de cada cultura foram coletadas a cada 24 horas, extraídas com acetato de etila e analisadas por cromatografia gasosa – espectrometria de massa com o banco de dados do Instituto Nacional de Padrões e Tecnologia. Neste estudo com cinco cepas de fungos endofíticos, apenas *Penicillium* sp. AF-LAB4 foi considerado o potencial biocatalisador encontrado na triagem devido à sua capacidade de utilizar o *R*-(+)-limoneno como única fonte de carbono e energia em meio mineral, uma vez que eventualmente acumulou compostos interessantes como limoneno-1,2- diol (128,10 µL/L) após 48 a 120 h de reação. Esta conquista é importante e apoia o desenvolvimento da produção de aromas naturais e demonstra o potencial da utilização desta cepa de fungo endofítico isolado da fruta *M. emarginata*, como novo biocatalisador.

Palavras-chave: Limoneno-1,2-diol; Acerola; *Penicillium* sp.

INTRODUÇÃO

The biotechnological production of aroma compounds appears as an interesting alternative to overcome the problems associated with chemical synthesis or extraction from the natural source. Biotechnology-based production of aroma compounds has emerged as an advantageous method since considered eco-friendly, occurs under mild conditions, does not use potentially toxic biocatalysts, and has fewer issues concerning waste management (Braga & Faria 2021; Singh et al. 2023).

Terpene biotransformation may be regarded as a biotechnological process aligned to sustainable development, due to the use of agro-industrial by-products as alternative raw materials, which is advantageous in terms of both ecological and economical sustainability (Paulino et al. 2021). From an economic point of view, terpenes are interesting due to their wide occurrence, some of them presenting high availability and low price. In this context, *R*-(+)-limonene (PubChem CID: 440917) is one of the most studied monocyclic monoterpenes for this purpose and can be found in abundance in several essential oils and some industrial by-products, such as those derived from the citrus industry (Bier et al. 2019; de Souza Sevalho et al. 2023a).

The fungal endophytic community, in particular, has undergone co-evolution with their host plant and has emerged as a powerful tool of genetic diversity. They can serve as a treasure trove of biocatalysts, catalyzing organic transformations of a wide range of substances into enantiopure compounds with biotechnological relevance. Endophytic fungi not only exhibit their degradation ability within the host plant but also display high degradation activity outside the plant (Santos & Silva 2019; Liu et al. 2021).

Additionally, the biocatalytic potential of endophytic fungi has traditionally been the used in the biotransformation process (Choudhary et al. 2021). In this context, the objective of this

study was to investigate the capacity of endophytic fungi strains, isolated from the fruit *Malpighia emarginata* for the biotransformation of *R*-(+)-limonene, an abundant and cheap agro-industrial precursor of many aromas with high added value.

METHODS

Chemicals

All the chemicals used for the preparation of the culture medium for the growth and maintenance of fungal cultures were purchased from Kasvi Brasil (São José dos Pinhais, Brazil), and Biotec Reagentes Analíticos (Paraná, Brazil). All the reagents used in the preparation of the mineral medium were of analytical grade and were obtained from Nuclear - CAQ Casa da Química Ltda (Diadema, Brazil). Ultrapure water (resistivity≥18.2 MΩ/cm) was purified using a Milli-Q gradient system (Millipore, Milford, USA). The standard *R*-(+)-limonene (≥93%) used as substrate was acquired from Sigma-Aldrich Brazil (São Paulo, Brazil). The ethyl acetate (HPLC/Spectrophotometric) used for sample preparation was purchased from Tedia (Rio de Janeiro, Brazil).

Collection, isolation, and inoculum preparation

Acerola fruits were collected around of the Federal Institute of Education, Science and Technology of Amazonas (IFAM) – *campus* Maués (3.3972° S, 57.6966° W). Endophytic fungi were isolated using methods proposed by Oliveira et al. (2021). Samples fruit were washed with autoclaved distilled water, and stored in sterile plastic bags at 6 ºC. For the isolation process, the leaves were sterilized by immersion in 70% ethanol for 1 min, in 3% sodium hypochlorite for 2.5 min, and in 70% ethanol again for 30 seconds, and sterile distilled water for 2 min. In this specific situation, the water was plated and incubated at 26 ºC as a control of sterilization procedure.

After the external sanitization procedure, the samples were inoculated in Petri dishes containing culture medium of potato dextrose agar yeast extract (PDAY, 200 g/L fresh potato, 20 g/L dextrose, 15 g/L agar, and 2 g/L yeast extract) supplemented with chloramphenicol 50 µg/mL, incubated at 30 °C until the appearance of colonies. Three fragments of mycelium fungi (tri-point inoculation) were sown at equidistant points and cultivated at 28 ºC for eight days to confirm the purity of the preserved samples. The pure cultures were isolated and maintained on new Petri dishes (central point) until they were well sporulated under the same conditions as used previously.

Macro- and micro morphological identification was conducted by microculture on a slide. Then, two 1 cm^2 fragments of each fungus were inoculated in Erlenmeyer flasks (125 mL) containing 50 mL of PD+ Y liquid culture medium. The conical flask was incubated at 28 ºC in a rotary shaker at 120 rpm for 72 h (de Oliveira et al., 2021).

After incubation, the humid biomass was recovered by centrifuging at 4.400 rpm, and 28 ºC for 10 min (Eppendorf Centrifuge 5702, Merck KGaA, Darmstadt, Germany) under sterile conditions. In accordance with Brazilian legislation, all fungi were registered in the Brazilian National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen) under number AC1746C.

Screening for biotransformation assays

Screening experiments were performed in accordance with de Souza Sevalho et al. (2023b) in an aqueous system to obtain a high recovery rate of both transformed products. The biomass recovered as described above (2 g wet weight) was resuspended (under aseptic conditions) in Erlenmeyer flasks (125 mL) containing 50 mL of mineral medium (ultrapure water containing 0.5 g/L $MgSO_4$, 3 g/L $NaNO_3$, 1 g/L K2HPO4, 0,5 g/L KCl, and 0.01 g/L Fe_2SO_4; pH was not adjusted). After added 0.5 % (v/v) of the substrate *R*-(+)-limonene to be tested, the flasks were incubated at 28 °C for 96 h in a rotary shaker operating at 120 rpm. Controls of the biotransformation experiments were performed with the substrate and the mineral medium, without the inoculum, and with only inoculum in the medium, without the substrate.

Gas chromatography-mass spectrometry analysis

The qualitative analysis was performed using a gas chromatograph (Trace Ultra) coupled to a mass spectrometer (ISQ Single Quadrupole, Thermo Scientific) equipped with a TR-5 capillary column (Trace) of 30 m length x 0.25 mm i.d. x 0.25 µm of film thickness. The injection was done in split mode (split ratio of 1:30) using a 1 µL sample. Helium was used as the carrier gas (flow rate 1.0 mL/min). The column temperature program was 40 °C (as the initial temperature) for 10 min, increased by 3 °C/min to 100 °C, followed by a constant ramp rate of 20 °C/min until reaching the temperature of 200 °C, which was maintained for 5 min. Temperatures of both injector and detector were maintained at 250 °C, ionization energy was 70 eV, and the scan range was *m/z* 35-400, without delay (de Souza Sevalho et al. 2023b).

Data analysis

The compounds were identified using the National Institute of Standards and Technology (NIST) library (similarities <90% were not considered) and was confirmed by comparing the retention times with retention times of the standards.

RESULTS AND DISCUSSION

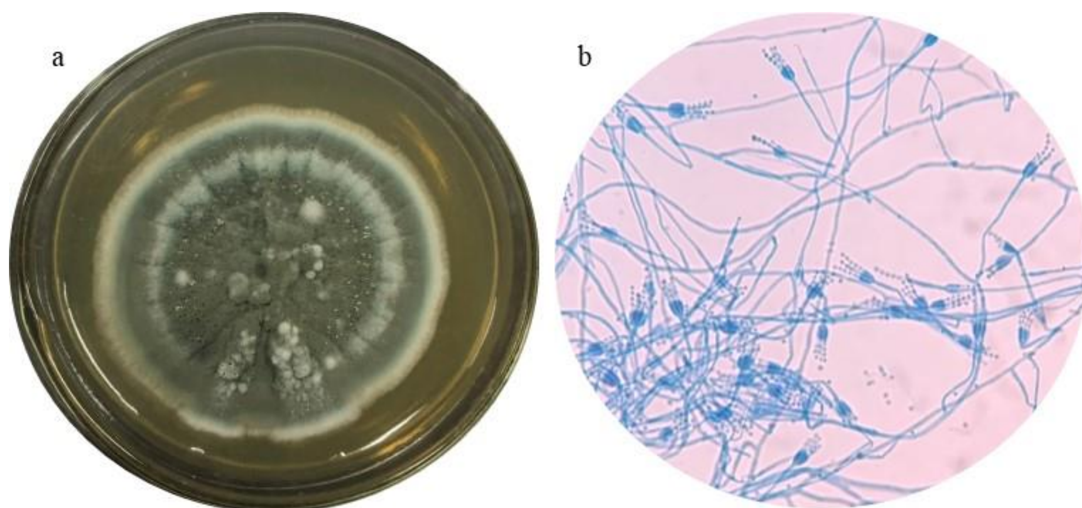
After the surface sterilization of the fruits *M. emarginata* collected from around IFAM – *campus* Maués, a total of five endophytic fungi strains appeared on the Petri dish after eight days

of incubation. Purification of the fungal outgrowth was done through subculture as a protocol to identify the morphological characteristics of each isolate. The fungal colonies exhibited diversity in colors (black, and green) and texture (velvety, powdery, cottony, and granularly). Among the isolated, two strains were associated with the *Penicillium* genus (Ascomycota). More methods for the identification of strains are required such as specific nutrient media as well as molecular biological analysis techniques. The isolates were coded as AF-LAB1, AF-LAB2, AF-LAB3, AF-LAB4, and AF- LAB5, and stored at −20°C in a glycerol solution (20%) in the strain collection of IFAM – *campus* Maués.

This investigation was focused more on the semiqualitative study of the products obtained than on your absolute earnings, to select biocatalysts with potential for application in future, more detailed and larger-scale works. Substrate consumption and formation of biotransformation products were monitored by gas chromatography–mass spectroscopy (GC–MS) analysis, which allowed us to identify some metabolic routes that were suggested in the present work and will be discussed individually in the next items.

In the screening carried in this study with five endophytic fungi strains, only *Penicillium* sp. AF- LAB4 (Figure 1) was able to biotransform the substrate R-(+)-limonene as the sole carbon and energy source in a mineral medium, which served as an indicator of biocatalytic activity and potential for producing aroma compound.

Figure 1 - Colony morphology of *Penicillium* sp. AF- LAB4 at 28° C in the PDAY medium: a) macro-morphological image after 3 days of incubation; b) micro-morphological image.



Source: Survey data (2023)

These microorganisms were capable of using *R*-(+)-limonene as the sole carbon source for growth, but not showed accumulation of metabolites, suggesting the complete degradation of

this substrate to CO_2. Even without positive results for the biotransformation, it emphasizes the potential of its use in the degradation of limonene in bioremediation processes (Birolli et al., 2019).

Among the main steps in the biotransformation process is the selection of the biocatalyst systems, which are mainly resistant and can use the precursor as the only carbon source. A huge number of biotechnological processes using whole cells have the potential of being more environmentally benign than chemical synthesis and more cost-effective as compared to isolated enzyme catalysis (Pessôa et al. 2019).

The volatile compounds accumulated in the ethyl acetate fraction via limonene biotransformation using *Penicillium* sp. AF- LAB4 after 96 h (Table 1), it demonstrates the accumulation of the limonene-1,2-diol as majority compound and limonene-1,2-epoxide as smaller amount. No auto-oxidation products as limonene-1,2-epoxide and limonene-1,2-diol were detected in the controls conducted using only the microorganism or addition of the substrate.

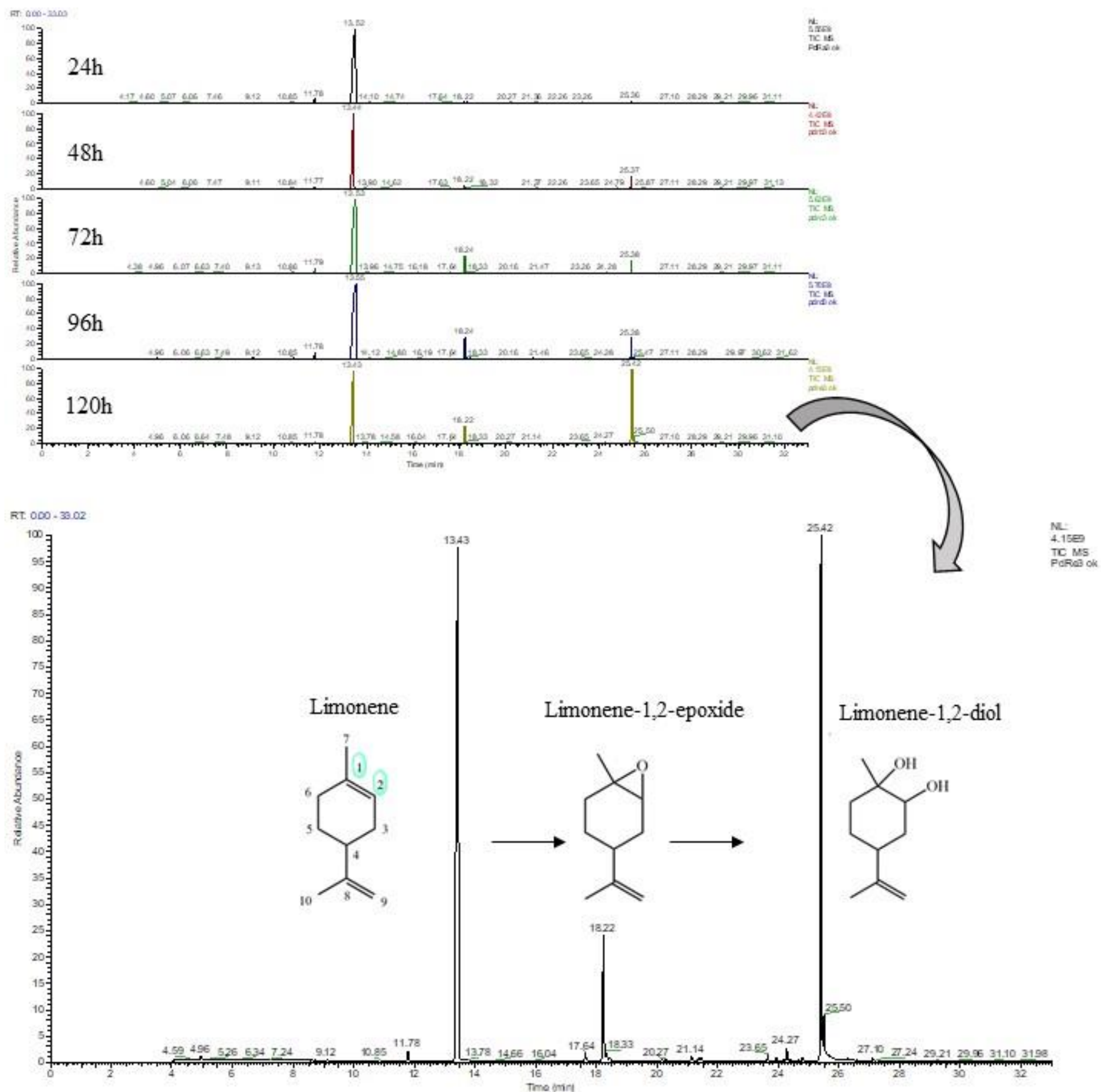
Table 1 - Composition of ethyl acetate fraction by *Penicillium* sp. AF- LAB4 in mineral medium containing 0,7 % of limonene after 120 h of reaction.

Retention time	Compounds	Concentration µL/L	Reverse search index
13.58	*R*-(+)-limonene	72.21	94.8
18.24	Limonene-1,2-epoxide	21.42	95.0
25.41	Limonene-1,2-diol	128.10	92.7

Survey data (2023)

Thus, as proven in studies performed by Sales et al. (2018b), and Sales et al. (2019a), these oxygenated derivatives are dominated by the ring double bond epoxidation of R-(+)-limonene, followed by the corresponding limonene-1,2-epoxide formation, resulting in the formation of limonene-1,2-diol. This supports the hypothesis that by *Penicillium* sp. AF- LAB4 possess a pathway of ability to recognize R-(+)-limonene as a substrate, which was then oxidized to limonene-1,2-epoxide and limonene-1,2-diol, an amount considerably produced in 48 to 120 h of reaction at temperature of 28 °C, and agitation of 120 rpm (Figure 2).

Figure 2 – GC/MS chromatograms profile and proposed pathway of compounds obtained from the biotransformation of *R*-(+)-limonene by *Penicillium* sp. AF- LAB4.



Source: Survey data (2023)

The efficiency of the biotransformation process depends on the compound employed as substrate and the specificity and selectivity of the enzymes produced by biocatalyst. In this context, the bioproduction of limonene-1,2-diol from *R*-(+)-limonene and orange residue-based media by *Phomopsis* sp. strain was described (Bier, Medeiros & Soccol, 2017). The results showed that 2.08 g/L of limonene-1,2-diol was obtained after 120 h of biotransformation using 10 g/L *R*-(+)-limonene as substrate, while that using an orange residue extract-based medium (5.36 g/L) similar concentration of limonene-1,2-diol (2.10 g/L) was obtained after 144 h of biotransformation under 120 rpm, at 30 ºC.

Considering the great potential of *Colletotrichum* species as biocatalyst for production of monoterpenoid aromas from monoterpene substrates scale-up and optimization studies about the

limonene-1,2-diol production through biotransformation of *R*-(+)-limonene by *C. nymphaeae* were investigated. A single addition of 15 g/L of *R*-(+)-limonene resulted in 4.19 g/L of limonene-1,2-diol (Sales et al., 2019a). Then, reported the limonene bioconversion mediated by *C. nymphaeae* out in a bioreactor (7.5 L) operated at 27 °C, 300 rpm, 1 vvm, and with 13.2 g/L of biomass, was observed the production of limonene-1,2-diol reached 7.1, 7.8, and 5.6 g/L after 72 h when using 20 g/L of *R*-(+)-, *S*-(-)-limonene, and citrus terpene as substrates, respectively (Sales et al. 2019b).

Recently, a study investigated the extraction and purification of limonene-1,2-diol from using *R*-(+)-limonene produced by *C. nymphaeae* CBMAI 0864 using different organic solvents. The best results were achieved by the application of ethyl acetate to the recovery of limonene-1,2-diol from the culture supernatant, being extracted around 2.14 g/L (80.8% of recovery). In addition, the use of *n*-butanol allowed the recovery of 1.8 g/L of limonene-1,2-diol, while approximately 1.6 g/L of this monoterpenoid were achieved using chloroform and dichloromethane as extraction solvent. This result can be explained due to the intermediate polarity of ethyl acetate which makes it more efficient than other solvents for extraction of limonene-1,2-diol (de Medeiros et al. 2021).

The compounds obtained, mainly the limonene-1,2-epoxide and limonene 1,2-diol are of great industrial interest to be applied as additives in food and cosmetic and also due to its potential biological activity. With this perspective it is interesting to provide further efforts in this area in order to improve product concentration and obtain higher yields, increasing the potential of natural aroma production through biotechnology (de Medeiros et al., 2021).

Among the value-added aromatic compounds, Limonene-1,2-diol (PubChem CID: 94217) is a colorless to slightly yellowish oil with a fresh mint aroma and is one of the oxyfunctionalyzed counterparts of limonene that can be obtained the through biotransformation processes. Has been associated with a significant inhibitory effect on the pro-inflammatory activities of $CD4^+$ and $CD8^+$ T lymphocytes, potential anticancer activity, besides being used as a flavoring for beverages, chewing gum, gelatins, and puddings (Sales et al. 2019a).

Economically, the advantages of biotransformation are clear when comparing the reference prices of substrates and products. In the database of Merck KGaA Brazil in the year 2022 (https://www.sigmaaldrich.com/BR/pt) the values of limonene 1,2-diol are around US$ 193.08/g and US$ 719.53 for 3 g. The reference price of limonene-1,2-epoxide is around US$ 249.50 for 100 ml and US$ 1001.81 for 500 ml, whereas the reference price of *R*-(+)-limonene is about US$ 79.10/kg. In this context, it would be a good strategy to invest efforts and resources to better understand limonene biotransformation (de Souza Sevalho et al. 2023b).

Fungal biotransformation is a relevant strategy to obtain high added-value natural compounds under controlled environmentally friendly conditions. Endophytic fungi offer great potential for the production of several groups of compounds; however, few studies have evaluated

the limonene biotransformation (de Souza Sevalho et al., 2022a). Furthermore, the use of fungal endophytes in whole-cell biotransformation is an emerging field of biotechnology that yields new modified compounds with increased (Liu et al. 2021).

CONCLUSION

Present study aimed at to isolate at least one effective strain for biotransforming *R*-(+)-limonene, a low-cost and easily available monoterpene into high-value derivatives. *Penicillium* sp. AF- LAB4, isolated from the fruit of *M. emarginata* is used as a microbial catalyst in biotransforming *R*-(+)-limonene to limonene 1,2-diol (128.10 µL/L) and limonene-1,2-epoxide (21.42 µL/L) - Acc. to peak area in GC-MS at 28 °C, 120 rpm after five days of incubation. These achievements are important to support the development of natural aroma production and to demonstrate the potential of using these wild endophytic fungi Amazon for the biotechnology. Studies for the identification of strains and production optimization and recovery of the product are already in progress.

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