REVISTA DELOS DESARROLLO LOCAL SOSTENIBLE

Evaluation of the biotransformation of *R*-(+)-limonene to aroma compounds by *Pestalotiopsis versicolor* LabMicrA-478 isolated of *Euterpe oleracea Martius*

Avaliação da biotransformação de *R*-(+)-limoneno em compostos de aroma por *Pestalotiopsis versicolor* LabMicrA-478 isolado de *Euterpe oleracea Martius*

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ABSTRACT
Fungal biotransformation is a pertinent strategy to overcome difficulties and problems arising from chemical synthesis and direct extraction from nature. This biotechnological approach is a relevant strategy to obtain high-added-value aroma compounds under environmentally friendly conditions. In order to understand the effect of an amazon endophytic fungus on the monoterpene substrate, this research work aims to investigate the biotransformation using cells cultivated of *Pestalotiopsis versicolor* LabMicrA-478 with *R*-(+)-limonene as a sole carbon and energy source. The main products of the limonene biotransformation identified by gas chromatography-mass spectrometry (Thermo Scientific™) with the NIST database were limonene-1,2-diol (74,97%) and limonene-1,2-epoxide (1.94%) in 120 hours of reaction. Finally, this is the first report to characterize the bioconversion of *R*-(+)-limonene by *P. versicolor* LabMicrA-478, as a biocatalyst.

Keywords: monoterpenes, amazon fungus, aroma compounds, biotechnological processes.

RESUMO
A biotransformação fúngica é uma estratégia pertinente para superar dificuldades e problemas decorrentes da síntese química e extração direta da natureza. Esta abordagem biotecnológica é uma estratégia relevante para obter compostos aromáticos de alto valor agregado em condições ambientalmente corretas. Para entender o perfil biocatalítico de um fungo endofítico amazônico com o substrato monoterpênico, este trabalho de pesquisa tem como objetivo avaliar a biotransformação usando células cultivadas de *Pestalotiopsis versicolor* LabMicrA-478 em *R*-(+)-limoneno, como única fonte de carbono e energia. Os principais produtos obtidos desse processo de biotransformação do limoneno identificados por cromatografia gasosa-espectrometria de massa (Thermo Scientific™) com o banco de dados NIST foram o limoneno-1,2-diol (74,97%) e limoneno-1,2-epóxido (1.94%) em 120 horas de reação. Por fim, este é o primeiro relato da bioconversão de *R*-(+)-limoneno usando *P. versicolor* LabMicrA-478, como biocatalisador.

Palavras-chave: monoterpenos, fungo amazônico, compostos aromáticos, processos biotecnológicos.

1 INTRODUCTION

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 catalysts, and has fewer issues concerning waste management (Paulino et al. 2021).

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. From an economic point of view, terpenes are interesting due to their wide occurrence, some of them presenting high availability and low price (Sharma et al. 2020). 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 (Paulino et al. 2022).

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. Among all the existing whole-cell systems, the use of fungi has traditionally been the most used in the biotransformation process (Pessôa et al. 2019; Liu et al. 2021).

The reactions catalyzed using fungi have a high degree of selectivity and attending to the reactions makes them economical and eco-friendly (green chemistry principles). Biotransformation catalyzed by fungi is considered an economically competitive technology for the modification of chemicals, leading to the structural diversification of bioactive substances (De Souza Sevalho et al. 2022). This study aimed to investigate the biotransformation of *R*-(+)-limonene by cells cultivated of *Pestalotiopsis versicolor* LabMicrA-478.

2 METHODS

2.1 CHEMICAL AND INOCULUM PREPARATION

The standard *R*-(+)-limonene (~99%) was acquired from Sigma-Aldrich®. All other reagents used in the study were of analytical grade. The endophytic fungus employed in this study was isolated from açaí core (*Euterpe oleracea* Mart.), and taxonomic identification was carried out in a previous study by Banhos (2016) using the sequencing *ITS*1 to *ITS*2 regions of the *rDNA* exhibited more than 98% identity to *Pestalotiopsis versicolor* LabMicrA-478 (Genbank access number DQ812940.1). The fungal is deposited in the work collection of the Laboratory of Bioassays and Microorganisms of the Amazon at the Federal University of Amazonas (LabMicrA/UFAM). The fungus was duly registered in the SisGen, under number AD64E07.

The fungal biomass to be used as inoculum in the biotransformation process was grown according to a procedure adapted from Souza et al. (2004). In two Erlenmeyer flasks (125 mL) containing 50 mL of the liquid medium of Potato, dextrose, and 0.2% yeast extract was inoculated 20 µl of conidial suspension, prepared as above. The conical flask was incubated at 24° C on a rotary shaker at 120 rpm for 72 h. After incubation, the humid biomass was recovered by a vacuum filtration system through a 0.45 µm Millipore membrane filter.

2.2 BIOTRANSFORMATION PROCEDURE

Following the procedure adapted previously described by Molina et al. (2015) and Sales et al. (2019), the biomass obtained was inoculated in three Erlenmeyer flasks (125 mL) containing 49,5 mL of mineral medium (0,5 g l^{-1} $MgSO_4$, 3 g l^{-1} $NaNO_3$, 1 g l^{-1} K_2HPO_4, 0,5 g l^{-1} KCl, and 0.01 g l^{-1} Fe_2SO_4, dissolved in Ultrapure water and mixed well, pH not adjusted). In these flasks, containing the cultures, 1% (v/v) of the substrate *R*-(+)-limonene to be tested was added, and incubated on a rotary shaker operating at 24° C and 120 rpm for 120 h. Two negative controls were incubated in parallel under the same conditions. The first negative control consists of a mineral medium without the addition of limonene. The second control had a fermentation medium but without inoculation.

Periodically, 500 µl samples from each treatment (the elicited and control experiments) were collected every 24 h to monitor the consumption of substrate and product formation. Each sample was extracted (1 min. in Vortex) with the same volume of ethyl acetate (1:1, v/v).

2.3 GAS CHROMATOGRAPHY ANALYSIS

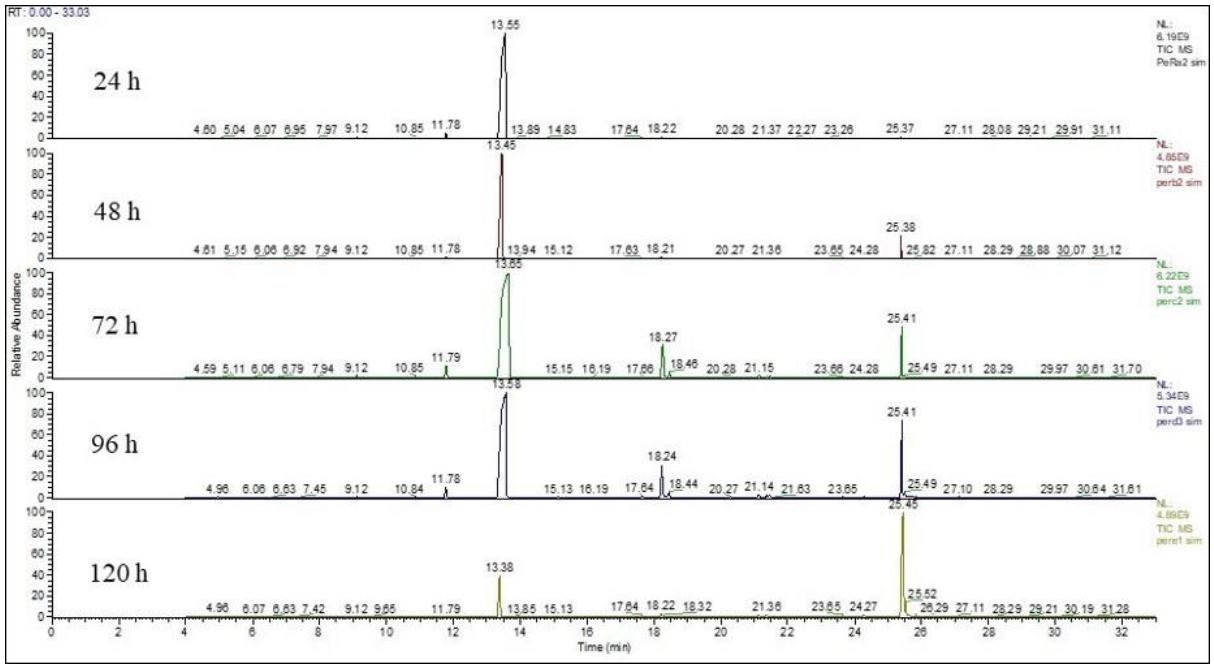
The qualitative analysis was performed by Gas chromatographic Trace Ultra coupled to mass spectrometer ISQ Single Quadrupole – GC/MS (Thermo Scientific™) equipped with a Trace™ TR-5 capillary column with 30 m length x 0.25 mm i.d. x 0.25 µm of film thickness. Helium was used as carrier gas with a flow of 1.02 mL/min. The injection was done in split mode (split ratio of 1:30) using a 1-µl sample. Helium was used as carrier gas (flow rate 1.0 ml/min). The column temperature program was 40° C as the initial temperature for 10 min, extended up to 3° C/min at an increase the rate of 100° C, followed by a constant rise at 20° C/min until reaching the temperature of 200° C, which was kept for 5 min. Temperatures of both injector and detector were kept at 250° C, ionization energy 70 eV, and the scan range *m/z* 35-400 amu,

without delay. Preliminary identifications were based on comparisons of the spectra obtained with those stored in the library of the 8th edition of Wiley (similarities <90% were discontinued).

3 RESULTS AND DISCUSSION

The fungal biotransformation of limonene is a powerful tool used to produce value-added compounds cost-effectively and selectively (De Souza Sevalho et al. 2022). The biotransformation of *R*-(+)-limonene using *P. versicolor* LabMicrA-478, resulted in the formation in 120 h of limonene-1,2-diol (74,97%), this majority compound was detected by mass spectrometry (92,7% similarity), which was confirmed by the mass spectrum. The remaining compounds are present in smaller amounts such as limonene-1,2-epoxide (1.94%). No auto-oxidation products with limonene-1,2-diol and limonene-1,2-epoxide were detected in controls conducted only by the microorganism or the substrate. The chromatograms (Figure 1) of the compound produced from the biotransformation of *R*-(+)-limonene in 24 to 120 hours of process, the temperature of 24 °C, and agitation of 120 rpm.

Figure 1 - GC-MS chromatograms profile of compounds obtained from biotransformation of *R*-(+)-limonene *P. versicolor* LabMicrA-478.

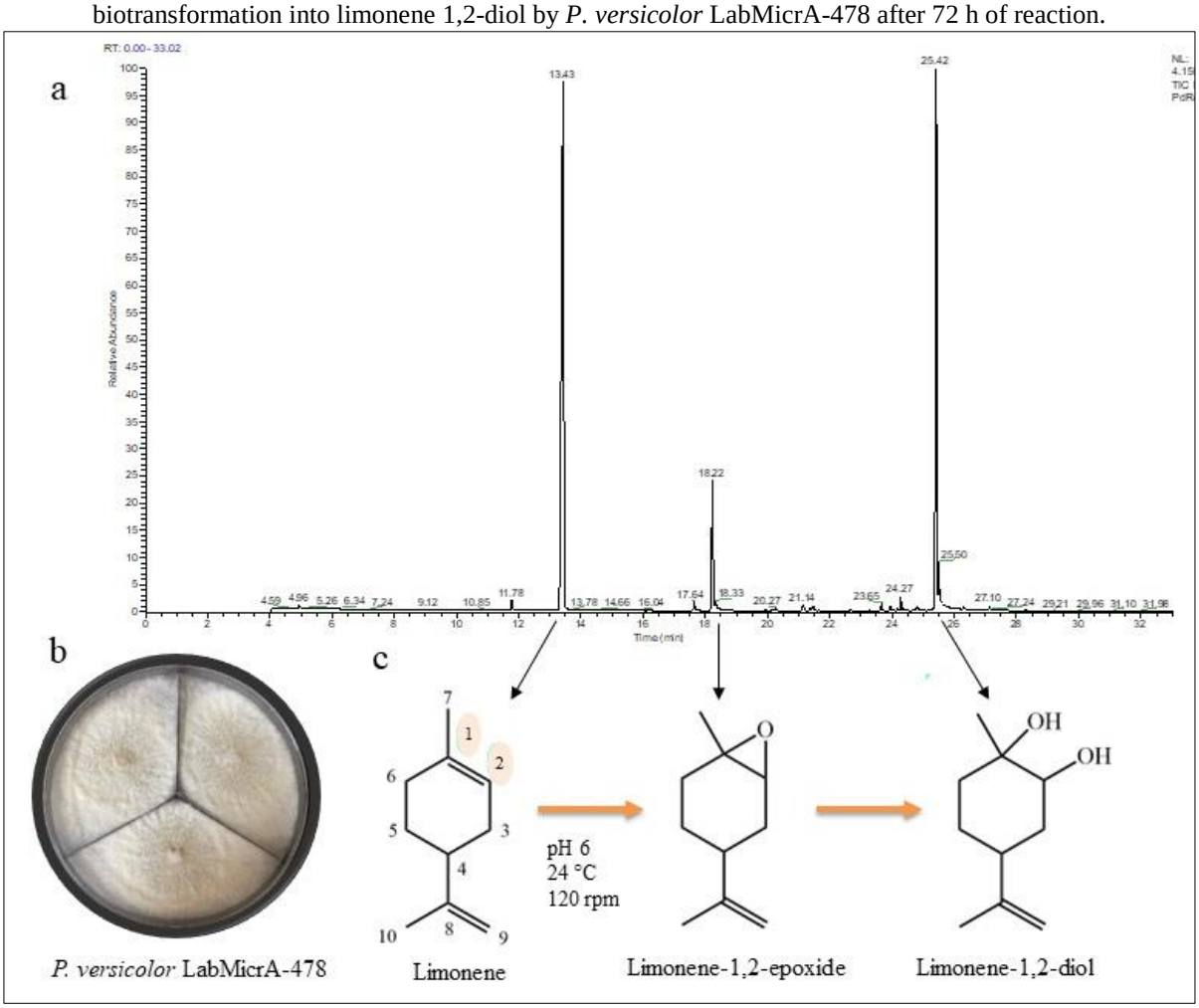


Source: Sevalho (2022)

Thus, as proven in studies performed by Sales et al. (2018), and Sales et al. (2019) the production of the intermediate limonene-1,2-epoxide was detected, indicating that the reactions

proceeded mainly due to the diol production. This supports the hypothesis that *P. versicolor* LabMicrA-478, possesses a pathway of ability to recognize *R*-(+)-limonene as a substrate, which was then oxidized to limonene-1,2-epoxide, an amount considerably high produced in 120 h of reaction. Figure 2 illustrates the proposed pathway of bioconversion, representing the consumption of substrate and product formation.

Figure 2 - a) GC-MS chromatogram; b) colony macro-morphological; c) proposed pathway of limonene biotransformation into limonene 1,2-diol by *P. versicolor* LabMicrA-478 after 72 h of reaction.



Source: Sevalho (2022)

The efficiency of the biotransformation process depends on the compound employed as the substrate and the specificity and selectivity of the enzymes produced by biocatalysts. 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 et al., 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.

Another process for bioconversion of limonene using fungi strains was reported by Cecati et al. (2018). In this study, *R*-(+)-limonene was converted by endophytic strains isolated from *Eupatorium buniifolium* Hook. & Arn. identified as *Alternaria alternata* and *Neofusicoccum* sp. EB04. Starting from 2.5 g/L of a substrate in a process carried at 28 ºC and 150 rpm for 72 h was verified that *A. alternata* and *Neofusicoccum* sp. EB04 was able to produce 1.75 g/L and 2.23 g/L of limonene-1,2-diol, respectively.

The substrate *R*-(+)-limonene is a monoterpene available in large amounts, at low cost, and can be employed in biotransformation processes as a precursor of different value-added aroma compounds. 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 cosmetics due to their potential biological activity. With this perspective, it is interesting to provide further efforts in this area to improve product concentration and obtain higher yields, increasing the potential of natural aroma production through biotechnology (Medeiros et al. 2021).

Economically, the advantages of biotransformation are clear when comparing the reference prices of substrates and products. It should be noted that limonene-1,2-epoxide and limonene 1,2-diol has a market value 16 times higher than *R*-(+)-limonene, thus it is considered that its biotransformation may be a good choice for increasing its added value. In the database of Merck KGaA Brazil (https://www.sigmaaldrich.com/BR/pt) the values of limonene 1,2-diol are around R$ 1.013,00/g and R$ 3.775,00 for 3g. The reference price of limonene-1,2-epoxide is around R$ 1.309,00 for 100 ml and R$ 5.256,00 for 500 ml whereas the reference price of *R*-(+)-limonene is about R$ 415,00/kg. In this context, it would be a good strategy to invest efforts and resources to better understand *R*-(+)-limonene biotransformation.

4 CONCLUSIONS

In this work, it was studied the biotransformation of *R*-(+)-limonene into aroma compound production. Bioaromas such as limonene-1,2-epoxide and limonene-1,2-diol were produced in a mineral medium, suggesting that *P. versicolor* LabMicrA-478 has the potential to biotransform the substrate. However, the highlight of this study was to report the first

biotransformation assay of *R*-(+)-limonene by *P. versicolor* LabMicrA-478, an endophytic fungus isolated from the Brazilian Amazonian Forest. These achievements are important to support the development of natural aroma production and to demonstrate the potential of using this wild fungus Amazon in biotechnology. Studies for the production optimization and recovery of the product are already in progress.

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No potential conflict of interest was reported by the authors.

ETHICAL STATEMENT

This article does not contain any studies with human or animal participants performed by any of the authors.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Elison de Souza Sevalho: Conceptualization, Data curation, Writing – original draft and Writing – review & editing.

Elissandro Fonseca dos Banhos: Data curation

Antonia Queiroz Lima de Souza: Supervision and Writing – original draft

Afonso Duarte Leão de Souza: Supervision and Writing – review & editing

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