Taylor & Francis
Taylor & Francis Group

Preparative Biochemistry & Biotechnology

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/lpbb20

Biotransformation of monoterpenes using *Streptomyces* strains from the rhizosphere of *Inga edulis* Martius from in an Amazonian urban forest fragment

Elison de Souza Sevalho, Rafael de Souza Rodrigues, Antonia Queiroz Lima de Souza & Afonso Duarte Leão de Souza

To cite this article: Elison de Souza Sevalho, Rafael de Souza Rodrigues, Antonia Queiroz Lima de Souza & Afonso Duarte Leão de Souza (12 Mar 2024): Biotransformation of monoterpenes using *Streptomyces* strains from the rhizosphere of *Inga edulis* Martius from in an Amazonian urban forest fragment, Preparative Biochemistry & Biotechnology, DOI: 10.1080/10826068.2024.2315476

To link to this article: https://doi.org/10.1080/10826068.2024.2315476

PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2024.2315476

Taylor & Francis
Taylor & Francis Group

Biotransformation of monoterpenes using *Streptomyces* strains from the rhizosphere of *Inga edulis* Martius from in an Amazonian urban forest fragment

Elison de Souza Sevalho[a,b], Rafael de Souza Rodrigues[a,b], Antonia Queiroz Lima de Souza[a,b,c] and Afonso Duarte Leão de Souza[a,b,d]

[a]Graduate Program in Biodiversity and Biotechnology of the BIONORTE Network (PPG-BIONORTE), Amazonas State University (UEA), Manaus, Amazonas, Brazil; [b]Analytical Center - Center of Multidisciplinary Support, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil; [c]Faculty of Agricultural Sciences, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil; [d]Department of Chemistry, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil

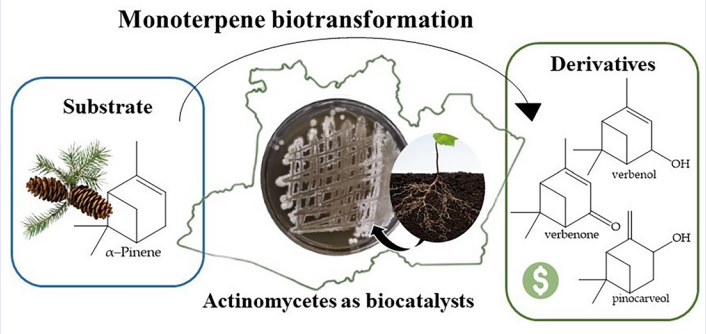
ABSTRACT

To investigate the biocatalytic potential of Amazonian actinomycetes for monoterpenes biotransformation. To carry out the present study, eleven actinomycetes of the genus *Streptomyces* isolated from inga-cipó (*Inga edulis* Mart.) rhizospheres were tested for their ability to bioconvert the substrates *R*-(+)-limonene, *S*-(-)-limonene, 1*S*-(-)-α-pinene, and (-)–β–pinene as sole carbon and energy source. According to gas chromatography-mass spectrometry analysis, three strains, LabMicra B270, LaBMicrA B310, and LaBMicrA B314, were able to biotransform 1*S*-(-)-α-pinene after 96 h of growth. However, *Streptomyces* LaBMicrA B270 was the most promising since it converted after only 72 h all the 1*S*-(-)-α-pinene mainly into *cis*-verbenol (74.9±1.24%) and verbenone (18.2±1.20%), compounds that have important biological activities and great industrial interest as additives in foods and cosmetics. These findings can stimulate the development of natural aromas using naturally abundant monoterpenes, ratify the potential of microorganisms from almost unexplored niches such as the Amazonian rhizosphere, and reinforce the importance of preserving those niches.

KEYWORDS

Amazonian actinomycetes; bioconversion; *cis*-verbenol; monoterpene hydrocarbons; rhizosphere microbiome

GRAPHICAL ABSTRACT



Introduction

Biotransformation has emerged as an attractive alternative for the derivatization of terpenes since, when compared to laboratory synthesis, it utilizes mild conditions, has an elevated regio- and enantioselectivity, does not generate toxic wastes, and generates products that can be labeled as "natural compounds." However, the most significant advantage of biotransformation processes is their ability to produce compounds that are not easily obtained by chemical methods.[1,2] Performed *in vitro* or in tanks, biotransformation with microbial cells (bacteria, yeasts, and filamentous fungi) or enzymes can be repeated indefinitely, without further damage to the environment, which gives it another advantage.[3,4]

In the last years, the biotechnological production of natural compounds has increased and has become promising for enabling the formation of aroma compounds. One reason for this rise in interest in biotechnological processes for producing aromas is the possibility of using agro-industrial by-products that are rich in monoterpene compounds, e.g., limonene and pinene, that can be used as substrates for

CONTACT Elison de Souza Sevalho elisonsevalho@hotmail.com Graduate Program in Biodiversity and Biotechnology of the BIONORTE Network (PPG-BIONORTE), Amazonas State University (UEA), Manaus, Amazonas 690065-130, Brazil.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/10826068.2024.2315476.

biotransformation.[5,6] The biotechnological importance of using monoterpenes as substrates for producing aroma compounds has been described and reviewed to a greater degree in the last few years.[2,7]

R-(+)-limonene (PubChem CID: 440917) and *S*-(−)-limonene (PubChem CID: 439250) are the most studied monoterpenes and the former is one of the most abundant in nature and a major constituent of several essential oils of mainly citrus species.[8] It is present in high concentrations in orange peel oil (approximately 90%) and can be obtained in large amounts as a by-product in the production of citrus juices and pulps.[9] The two limonene isomers are inexpensive precursors for the production of more expensive oxygenated derivatives such as carveol, carvone isomers, perillyl alcohol, menthol, *p*-cymene, limonene-1,2-diol and α-terpineol, among others. They also have a broad range of applications due to their odorant and bioactive properties.[6]

Other important monoterpenes are pinenes, which are bicyclic monoterpenes found in two isomeric forms, α-pinene (PubChem CID: 6654) and β-pinene (PubChem CID: 14896). α- and β-pinenes represent 75% to 90% of the essential oil from conifers and can be found in concentrations ranging from 50% to 70% and from 15% to 30%, respectively, in turpentine oil (crude resin from conifer trees, especially pine trees), an abundant by-product of the paper and cellulose industry.[10] As such, α–pinene and β-pinene have been employed as substrates in biotransformation processes for the formation of important aroma compounds, including verbenol, verbenone, myrtenol, and α-terpineol.[11]

Despite the various advantages of biotransformation using microorganisms, including more economical and eco-friendly procedures (green chemistry principles), it naturally has certain challenges,[4] such as achieving an efficient and feasible strain and the possible toxicity of the substrate to some of the microorganisms being screened. Therefore, many studies have focused on selecting new biocatalysts for the biotransformation of many substrates, including terpenes.[3] Fortunately, the worldwide diversity of microorganisms is enormous, which makes them an almost inexhaustible group of biocatalysts.

Brazil retains 15–20% of the world's biodiversity, especially in the Amazon, and therefore has an incalculable number of unexplored microorganisms for biotechnological applications,[12] including actinomycetes, which are highly diverse and can be found in varied habitats and can grow in a large variety of substrates.[13,14] Besides their importance to the sustainability of the Amazon Forest and as sources of antibiotics, the actinomycetes can also be explored for diverse biotechnological purposes, including the biotransformation of terpenes, which is a subject that is poorly targeted worldwide. Therefore, the objective of this study was to investigate the capacity of actinomycetes of the genus Streptomyces, isolated from the rhizosphere of inga-cipó (Inga edulis Mart.) in the Brazilian Amazon, for the biotransformation of the monoterpenes limonene and pinene, which are abundant and cheap precursors of many aromas that have high added value.

Materials and methods

Chemicals

All the chemicals for the culture media for the growth and maintenance of the 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 standards *R*–(+)–limonene (≥93%), *S*–(-)–limonene (≥95%), 1*S*-(-)-α-pinene (≥97%), and (-)–β–pinene (≥97%) used as substrates were 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).

Microorganisms

The *Streptomyces* actinomycete strains used in this study belong to the bacterial collection of the Laboratory of Bioassays and Microorganisms of the Amazon (LaBMicrA) at the Analytical Center of the Multidisciplinary Support Center of the Federal University of Amazonas (CA/CAM/UFAM). All the strains were isolated from the rhizosphere of inga-cipó plants (*Inga edulis* Mart.) collected out in three different points on the campus of the Federal University of Amazonas (UFAM) in Manaus, Amazonas state (Point 1–3°05'19.4" S 59°58'00.5" W, Point 2–3°05'20.8" S 59°57'57.5" W, and Point 3–3° 5'19.06"S 59°57'56.26"W). The actinomycete strains were identified in the *Streptomyces* genus using the genes banks NCBI and EzBioCloud (Table 1). 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. The information of all *Streptomyces* strains identified in this study is listed in Table 1.

Inoculum preparation

The strains of actinomycetes were cultivated in Petri dishes containing the International *Streptomyces* Project-2 semi-solid culture medium (ISP2) (10 g/L starch, 4 g/L yeast extract, 10 g/L malt, 4 g/L dextrose, and 20 g/L agar) at 28 °C for eight days to confirm the viability and purity of the preserved samples. Macro- and micromorphological confirmation of each strain was conducted by microculture on a slide. Then, two 1 cm^2 fragments of each strain on agar were inoculated in Erlenmeyer flasks (125 mL) containing 50 mL of ISP2 liquid culture medium. The conical flask was incubated at 28 °C in a rotary shaker at 120 rpm for 72 hr.[15]

Table 1. Similarities in two GenBanks for the *Streptomyces* strains screened for monoterpenes biotransformation in this work.

Strains[a]	Correspondent at GenBank/NCBI	Similarity (%)	Correspondent at EzBioCloud	Similarity (%)
Streptomyces sp. LaBMicrA B267	*S. pulveraceus* NBRC 3855	99.51%	*S. silvae* For3	99.34%
Streptomyces sp. LaBMicrA B270	*S. bullii* strain C2	96.41%	*S. murinus* NBRC 12799	98.36%
Streptomyces sp. LaBMicrA B271	*S. graminearus* NBRC 15420	97.53%	*S. graminearus* NBRC 15420	96.66%
Streptomyces sp. LaBMicrA B272	*S. acidicola* K1PN6	88.60%	*S. sundarbansensis* MS1/7	78.35%
Streptomyces sp. LaBMicrA B278	*S. misionensis* JCM 4497	93.78%	*S. misionensis* DSM 40306	94.51%
Streptomyces sp. LaBMicrA B301	*S. pulveraceus* NBRC 3855	96.65%	S. *durocortorensis* RHZ10	96.93 %
Streptomyces sp. LaBMicrA B310	*S. pulveraceus* sNBRC 3855	98.96%	*S. durocortorensis* RHZ10	99.07 %
Streptomyces sp. LaBMicrA B314	*S. olivaceus* NBRC 12805	99.61%	*S. olivaceus* NRRL B-3009	99.74%
Streptomyces sp. LaBMicrA B318	*S. similanensis* KC-106	95.10%	*S. similanensis* KC-106	95.45%
Streptomyces sp. LaBMicrA B303[b]	–	–		
Streptomyces sp. LaBMicrA B209[b]	–	–		

[a]The suggestions from the gene banks were on basis of the 16*S* *r*RNA sequencing.
[b]Not sequenced. Identified as *Streptomyces* by microscopic analysis.

After incubation, the humid biomass was recovered by centrifuging at 4400 rpm and 28 °C for 10 min (Eppendorf Centrifuge 5702, Merck KGaA, Darmstadt, Germany) under sterile conditions.

Screening for biotransformation assays

Screening experiments were performed in accordance with Molina et al.[16] and Bier et al.[17] 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 K_2HPO_4, 0.5 g/L KCl, and 0.01 g/L Fe_2SO_4; pH was not adjusted). After added 0.5% (v/v) of one of the substrates to be tested (*R*-(+)-limonene, *S*-(-)-limonene, 1*S*-(-)-α-pinene, and (-)–β–pinene), the flasks were incubated at 28 °C for 96 hr in a rotary shaker operating at 120 rpm. Controls of the biotransformation experiments were each substrate and the mineral medium, without any inoculum, and with only each inoculum in the medium, without any substrate. Periodically, 500 µL samples from each treatment (biotransformation and control experiments) were taken every 24 hr in order to monitor the consumption of substrate and product formation. Each sample was extracted with the same volume of ethyl acetate. After phase separation in a vortex chamber, the organic layer was separated and stored at −80 °C until analysis.

Gas chromatography-mass spectrometry analysis

The qualitative analysis was performed in accordance with Sevalho et al.[18] 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 × 0.25 mm i.d. × 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. Other conditions: both injector and detector at 250 °C, ionization energy of 70 eV, scan without delay, and mass range of *m/z* 35–400.

Data analysis

The compounds were identified using the National Institute of Standards and Technology (NIST) library (similarities <90% were not considered). The ratio between the consumption of the substrate and its derived products was determined on the basis of the chromatographic peak areas using the Xcalibur software (version 2.2) of the GCMS system used. Three independent assays were performed for each experiment. Values are presented as the mean ± standard deviation (SD) of the peak area of the chromatograms of the substrate and derived products. Statistical analyses were performed using the software GraphPad Prism (version 9.5.1) for Windows (GraphPad Software, San Diego, California, USA).

Results and discussion

Among the eleven *Streptomyces* actinomycete strains screened for the biotransformation of *R*-(+)-limonene, *S*-(-)-limonene, 1*S*-(-)-α-pinene, and (-)-β-pinene, three (27%) were able to biotransform at least or only one substrate, 1*S*-(-)-α-pinene, as the single carbon source. Eight actinomycete strains were

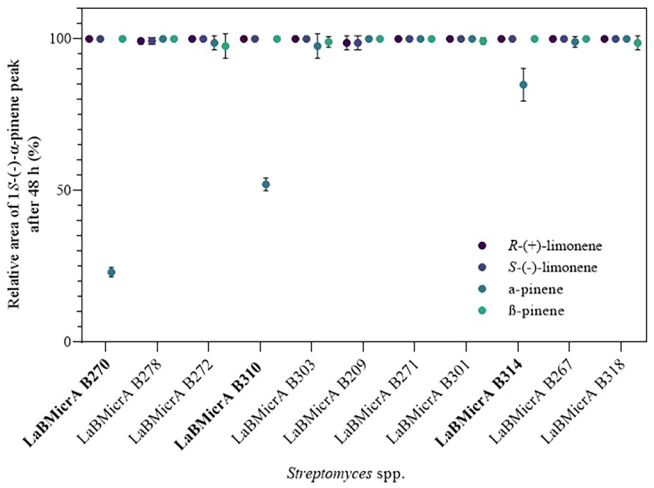


Figure 1. Diverse substrates after 48 hr of exposition for biotransformation using *Streptomyces* strains.

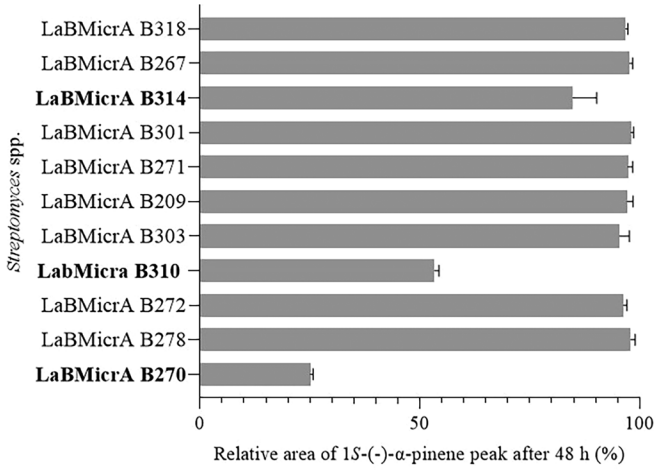


Figure 2. Relative peak areas in the GC-MS analysis showing the 1*S*-(-)-α-pinene substrate remaining after biotransformation using Streptomyces strains after 48 hr.

capable of using the hydrocarbons *R*-(+)-limonene, *S*-(-)-limonene, and (-)-β-pinene, respectively, the as the sole carbon source for growth, but not showed accumulation of metabolites (Figure 1), suggesting the complete degradation of these monoterpenes into carbon dioxide (CO_2). This problem may be related to the toxicity of monoterpene substrates that in many cases can inhibit the microorganisms and limit the maximum concentration of substrate used in the bioprocesses.[7,19] Considering the eleven strains, any decrease in the absolute area of the substrate peak combined with the absence of any detectable peak of derivatives in the gas chromatography-mass spectrometry analysis was considered as being a loss for evaporation or consumption without any biotransformation.

The hydrocarbon structure of monoterpenes provides them with high hydrophobicity and, similarly to other membrane-active organic compounds like alkanes. The toxicity derived from hydrophobic organic compounds is correlated with the logarithm of its octanol/water partition coefficient (LogP or Log K_{ow}), in which LogP values ranging from 1 to 5 usually translate into compound toxicity for whole cells. As an adaptive response, cells may change the membrane fatty acid profile to preserve membrane characteristics. Nevertheless, the mechanisms reported seem to be dependent on the genomic background of the cell and cell physiology (e.g., the growth phase), but also on the chemical properties (e.g., acyclic vs. cyclic, chain length, branching degree, and the degree and position of the oxyfunctionalization) and concentration of the stressor.[6,7,20]

For Rottava et al.[21] to reduce this effect of toxicity of these monoterpene compounds to microorganisms, the method of cell induction by the substrate has been employed, allowing the microorganism adaptation with lower amounts of substrate. In this context, slow continuous feeding (fed-batch fermentation), cell protection via immobilization or in situ flavor extraction membranes, and co-solvents are alternative approaches to overcome the problems related to substrates. Alternatively, procedures for strain adaptation or genetic engineering tools may be used to increase the substrate tolerance of fungal strains. The development of processes using engineered strains overexpressing genes related to key enzymes involved in biotransformation processes can be considered one of the most promising approaches for aroma production.[5,22,23]

The three strains that were able to biotransform 1*S*-(-)-α-pinene, *Streptomyces* spp. LaBMicrA B270, LaBMicrA B310, and LaBMicrA B314 consumed this substrate completely and accumulated interesting compounds after 96 hr of reaction. However, *Streptomyces* sp. LaBMicrA B270 was the most promising one regarding its capacity to use the substrate as the single carbon and energy source in a mineral medium and accumulate products earlier, in only 48 hr (Figure 2).

There are many reports on the biotransformation of monoterpenes using microorganisms, most fungi, but there are not many that use actinomycetes, and almost none that use Amazonian microorganisms. Generally, in screening assays, the first experiments aim to identify the most adequate and robust strain for each process, since both substrate and product may cause inhibitory effects on cell growth. Therefore, all strains to be selected are incubated with the substrate to be used in order to find which strains can use the substrate as sole the carbon source, indicating the existence of a metabolic pathway for substrate degradation and possible accumulation of interesting secondary metabolite. In this context, many researchers have been focusing on identify new microorganisms that can be used in biotechnological process, through isolation, screening and selection of the best strains capable of producing aroma compounds from different substrates. This is a promising methodology since is a natural way to obtain new products at low costs.[17]

It is interesting to notice that in this first step, the incubation conditions are usually similar in various screening works. This fact may be due to the necessity to maintain the optimum temperature, pH, and agitation for strain growth in order to have a better comparison of the results. The resistance to process conditions and ability of substrate degradation are important biocatalysts characteristics; however, it does not guarantee high concentration or specificity of products.[4,24]

This investigation was focused more on the qualitative study of the products obtained than on your absolute earnings, to

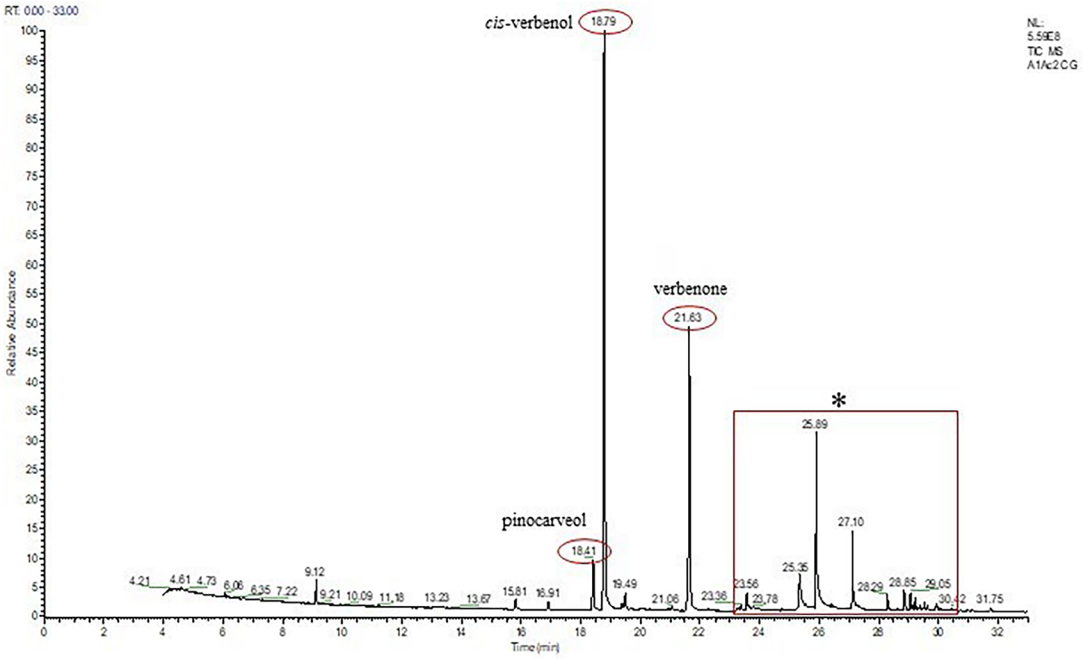
select biocatalysts with potential for application in future, more detailed and larger-scale works. Substrate consumption and formation of biotransformation products were monitored by gas chromatography–mass spectroscopy (GC–MS) analysis.

The periodic GC-MS chromatographic profiles of the biotransformation samples of 1*S*-(-)-α-pinene using *Streptomyces* sp. LaBMicrA B270 showed the complete consumption of this substrate in 72 hr (Figure S1). According to GC-MS analyses and comparisons with the NIST library, the three main products were *cis*-verbenol, verbenone, and pinocarveol, all from oxidation changes and with the same bicyclic structure of the substrate. The high rates of *cis*-verbenol and verbenone stood out among them, with chromatographic areas of 74.9 ± 1.24% and 18.2 ± 1.20% of the total peak areas, respectively (Figures 2 and 3 and Table 2).

Despite taking an additional 24 hr, in other words, 96 hr of exposure, the biotransformation of 1*S*-(-)-α-pinene by the *Streptomyces* spp. LaBMicrA B310 and LaBMicrA B314 also presented *cis*-verbenol and verbenone as the major compounds together with other minor derivatives (Figures S2 and S3, respectively). From of the Table 2 present the three *Streptomyces* strains promising able to bioconvert 1*S*-(-)-α-pinene into volatile compounds accumulated in the ethyl acetate fraction (%). Furthermore, no peak related to any hypothetic derivative from the substrates was detected in the experiment controls (Figure S4). For the above analyses, only peaks of the derivatives with similarities of >90% to the NIST proposals (Reverse Search Index) were considered.

So far, there are only a few reports on α-pinene oxidation via bacterial biotransformation. In a recent study, Saidani et al.[25] showed the biotransformation of α-pinene by cell cultures of *Paenibacillus popilliae* 1 C and *Streptomyces rochei* AB1, which resulted in the formation of main compounds such as *trans*-verbenol (59.47% and 40.40%, respectively).

Oxidative biotransformations of α-pinene led to chemical products commercially important as natural derivatives that are useful as flavors and fragrances with significant potential for industrial applications. These can take the following two competitive routes:[1] epoxidation of the double bond of the cycle leading to pinene epoxide and diverse monoterpenoid derivatives and,[2] as in the present work, allylic oxidation of α-pinene catalyzed by α-pinene monooxygenase with subsequent opening of the cyclobutene ring to form acyclic



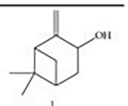
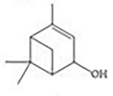
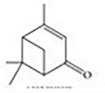
Retention time (min)	Compounds	Relative peak area (%)	RSI (%)	Structure
18.61	pinocarveol	6.81±0.05	91.8	
18.79	*cis*-verbenol	74.95±1.24	93.0	
21.64	verbenone	18.25±1.20	95.6	

Figure 3. GC-MS chromatogram showing the biotransformation derivatives of 1*S*-(-)-α-pinene using *Streptomyces* sp. LaBMicrA B270 after 72 hr. RSI: Reverse Search Index. *Chromatographic column bleed.

Table 2. Derivatives obtained via biotransformation of 1*S*-(-)-α-pinene using *Streptomyces* spp.

RT	Compounds (%)[a]	LaBMicrA B270	LaBMicrA B310	LaBMicrA B314
18.61	Pinocarveol	6.81 ± 0.05	4.82 ± 0.08	7.76 ± 0.48
18.79	*Cis*-verbenol	74.95 ± 1.24	57.39 ± 2.10	69.22 ± 1.17
21.19	Myrtenol	ND	ND	5.54 ± 0.63
21.64	Verbenone	18.25 ± 1.20	37.80 ± 2.10	17.49 ± 0.06

Note: RT: retention time; ND: not detected.
[a]Percentage of peak area in the CG-MS analysis.

aldehydes in the production of verbenol–verbenone and myrtenol–myrtenal mixtures.[26]

Among the value-added aromatic compounds, *cis*-verbenol (PubChem CID: 164888), our main finding, is a valuable food-flavoring compound and has a fresh pine odor. It is an ingredient for soft drinks, soups, meats, sausages, and ice cream. It also has biological activity such as antimicrobial and anti-fungicide properties.[11] In addition, this aroma is a natural precursor of verbenone, and possesses anti-ischemic, antioxidant, and anti-inflammatory properties that prevent neuronal cell death caused by oxygen-glucose deprivation and reduce cerebral ischemic injury.[24,27]

Verbenone (PubChem CID: 29025) is a colorless liquid with an odor typical of mint and camphor. It has been reported as having good pesticidal properties and actis as an anti-aggregation pheromone and a repellent for the pine bark beetle. It also has pharmacological properties like broncho-dilating, anti-inflammatory, and hemolytic activities.[28,29] This product is mainly used in the food industry for the formulation of bakery products and sweets, but it can also be used as a starting material in the synthesis of various organic compounds, such as bicyclic lactones. These applications and the value of this compound make the biotechnological production of verbenone of great interest.[11,30]

Economically, the advantages of the biotransformation of α-pinene are clear when comparing the reference prices of the substrate and products. For example, using the database of Merck KGaA (https://www.sigmaaldrich.com/US/en), the values of *cis*-verbenol (95%) are around US$ 126.00 for 5 g and US$ 403.00 for 25 g. The reference price of verbenone (94%) is around US$ 82.60 for 10 g and US$ 173.00 for 50 g, whereas the reference price of 1*S*-(-)-α-pinene (97%) is about US$ 76.50/kg or around US$ 0.08/g, at least 45 x more inexpensive than verbenone and 212 than *cis*-verbenol. Therefore, it seems worth making efforts and allocating resources to better understand and explore the biotransformation of pinene.

Because of the wide range of products of commercial interest that can be produced from monoterpenes, studies on the biotransformation of these compounds have been stimulated for decades. From a technological point of view, the biotransformation of monoterpenes has already shown great advances in recent years, although there are still challenges to be overcome by the aroma and fragrance industries.[11]

Conclusion

In this work, eleven *Streptomyces* strains isolated from the rhizosphere of *Inga edulis* Mart. in an urban forest fragment of the Amazon were screened for ability to biotransform *R*- and *S*-limonene, 1*S*-(-)-α-pinene, and (-)–β–pinene. While no derivative from the limonenes and (-)–β–pinene were achieved, three strains biotransformed 1*S*-(-)-α-pinene mainly into *cis*-verbenol and verbenone, which are compounds that have important biological activities and great industrial interest as additives in foods and cosmetics. Further efforts are needed to improve the concentration of these derivatives and obtain higher yields. However, work like this confirms the potential of microorganisms from yet unexplored niches such as the Amazonian rhizosphere and also the need to preserve them.

Acknowledgments

We would like to thank the Graduate Program in Biodiversity and Biotechnology of the BIONORTE for the doctorate opportunities, and also the LaBMicrA and LabCG laboratories at the CA/CAM/UFAM for the space and equipment provided, which were necessary for the development of the research.

Ethics statement

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

Author contributions

Elison de Souza Sevalho: Conceptualization, Investigation, Methodology, Data curation, Writing—original draft, and Writing—review & editing; Rafael de Souza Rodrigues: Methodology and Data curation; Antonia Queiroz Lima de Souza: Funding acquisition, Methodology, Supervision and Writing—original draft; Afonso Duarte Leão de Souza: Funding acquisition, Methodology, Supervision, and Writing—review & editing. This study is part of the doctoral theses of Elison de Souza Sevalho and Rafael de Souza Rodrigues (PPG-BIONORTE/UFAM).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was funded by the Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) via the Universal Amazonas call FAPEAM-002/2018, POSGRAD 2022/2023 call, and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The authors acknowledge FAPEAM for the PhD scholarships via the POSGRAD UEA – 003/2019 awarded to Elison de Souza Sevalho and Rafael de Souza Rodrigues. To the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for the research grant awarded to Afonso Duarte Leão de Souza.

ORCID

Elison de Souza Sevalho http://orcid.org/0000-0003-4754-3777
Rafael de Souza Rodrigues http://orcid.org/0000-0002-3890-6970
Antonia Queiroz Lima de Souza http://orcid.org/0000-0001-5602-8617
Afonso Duarte Leão de Souza http://orcid.org/0000-0001-7007-3991

References

[1] Molina, G.; Pêssoa, M. G.; Pimentel, M. R.; Pelissari, F. M.; Bicas, J. L.; Pastore, G. M. Production of natural flavor compounds us-

ing monoterpenes as substrates. In *New Developments in Terpene Research*; Hu, J., Ed.; Nova Science Publishers: New York, NY, 2014; pp cap. 1.

[2] de Oliveira Felipe, L.; de Oliveira, A. M.; Bicas, J. L. Bioaromas–perspectives for sustainable development. Trends Food Sci. *Technol.* 2017, *62*, 153. DOI: 10.1016/j.tifs.2017.02.005.

[3] Sales, A.; Paulino, B. N.; Pastore, G. M.; Bicas, J. L. Biogeneration of Aroma Compounds. *Curr. Opin. Food Sci.* 2018, *19*, 77–84. DOI: 10.1016/j.cofs.2018.03.005.

[4] Pessôa, M. G.; Vespermann, K. A.; Paulino, B. N.; Barcelos, M. C.; Pastore, G. M.; Molina, G. Newly Isolated Microorganisms with Potential Application in Biotechnology. *Biotechnol. Adv.* 2019, *37*, 319–339. DOI: 10.1016/j.biotechadv.2019.01.007.

[5] Sharma, A.; Sharma, P.; Singh, J.; Singh, S.; Nain, L. Prospecting the Potential of Agroresidues as Substrate for Microbial Flavor Production. *Front. Sustain. Food Syst.* 2020, *4*, 18. DOI: 10.3389/fsufs.2020.00018.

[6] Soares-Castro, P.; Soares, F.; Santos, P. M. Current Advances in the Bacterial Toolbox for the Biotechnological Production of Monoterpene-Based Aroma Compounds. *Molecules* 2020, *26*, 91. DOI: 10.3390/molecules26010091.

[7] de Souza Sevalho, E.; Paulino, B. N.; de Souza, A. Q. L.; de Souza, A. D. L. Fungal Biotransformation of Limonene and Pinene for Aroma Production. *Braz. J. Chem. Eng.* 2023, *40*, [1–21. DOI: 10.1016/j.tifs.2017.02.005.

[8] Ren, Y.; Liu, S.; Jin, G.; Yang, X.; Zhou, Y. J. Microbial Production of Limonene and Its Derivatives: Achievements and Perspectives. *Biotechnol. Adv.* 2020, *44*, 107628. DOI: 10.1016/j.biotechadv.2020.107628.

[9] Vieira, A. J.; Beserra, F. P.; Souza, M. C.; Totti, B. M.; Rozza, A. L. Limonene: Aroma of Innovation in Health and Disease. *Chem. Biol. Interact.* 2018, *283*, 97–106. DOI: 10.1016/j.cbi.2018.02.007.

[10] Kutyła, M.; Fiedurek, J.; Gromada, A.; Jędrzejewski, K.; Trytek, M. Mutagenesis and Adaptation of the Psychrotrophic Fungus *Chrysosporium pannorum* A-1 as a Method for Improving β-Pinene Bioconversion. *Molecules* 2020, *25*, 2589. DOI: 10.3390/molecules25112589.

[11] Vespermann, K. A.; Paulino, B. N.; Barcelos, M. C.; Pessôa, M. G.; Pastore, G. M.; Molina, G. Biotransformation of α- and β-Pinene into Flavor Compounds. *Appl. Microbiol. Biotechnol.* 2017, *101*, 1805–1817. DOI: 10.1007/s00253-016-8066-7.

[12] Borelli, T.; Hunter, D.; Padulosi, S.; Amaya, N.; Meldrum, G.; de Oliveira Beltrame, D. M.; Samarasinghe, G.; Wasike, V. W.; Güner, B.; Tan, A.; et al. Local Solutions for Sustainable Food Systems: The Contribution of Orphan Crops and Wild Edible Species. *Agronomy* 2020, *10*, 231. DOI: 10.3390/agronomy10020231.

[13] Farda, B.; Djebaili, R.; Vaccarelli, I.; Del Gallo, M.; Pellegrini, M. Actinomycetes from Caves: An Overview of Their Diversity, Biotechnological Properties, and Insights for Their Use in Soil Environments. *Microorganisms* 2022, *10*, 453. DOI: 10.3390/microorganisms10020453.

[14] Nazari, M. T.; Machado, B. S.; Marchezi, G.; Crestani, L.; Ferrari, V.; Colla, L. M.; Piccin, J. S. Use of Soil Actinomycetes for Pharmaceutical, Food, Agricultural, and Environmental Purposes. *3 Biotech* 2022, *12*, 232. DOI: 10.1007/s13205-022-03307-y.

[15] de Souza, A. Q. L.; de Souza, A. D. L.; Astolfi Filho, S.; Pinheiro, M. L. B.; Sarquis, M. I. D. M.; Pereira, J. O. Atividade Antimicrobiana De Fungos Endofíticos Isolados De Plantas Tóxicas Da Amazônia: *Palicourea longiflora* (Aubl.) Rich e Strychnos Cogens Bentham. *Acta Amaz.* 2004, *34*, 185–195. DOI: 10.1590/S0044-59672004000200006.

[16] Molina, G.; Bution, M. L.; Bicas, J. L.; Dolder, M. A. H.; Pastore, G. M. Comparative Study of the Bioconversion Process Using R-(+)- and S-(-)-Limonene as Substrates for *Fusarium oxysporum* 152B. *Food Chem.* 2015, *174*, 606–613. DOI: 10.1016/j.foodchem.2014.11.059.

[17] Bier, M. C. J.; Medeiros, A. B. P.; Soccol, C. R. Biotransformation of Limonene by an Endophytic Fungus Using Synthetic and Orange Residue-Based Media. *Fungal Biol.* 2017, *121*, 137–144. DOI: 10.1016/j.funbio.2016.11.003.

[18] Sevalho, E. d S.; dos Banhos, E. F.; de Souza, A. Q. L.; de Souza, A. D. L. Evaluation of the Biotransformation of R-(+)-Limonene to Aroma Compounds by *Pestalotiopsis versicolor* LabMicrA-478 Isolated of *Euterpe oleracea* Martius. *Rev. DELOS* 2023, *16*, 75–85. DOI: 10.55905/rdelosv16.n42-007.

[19] Birolli, W. G.; Ferreira, I. M.; Alvarenga, N.; Santos, D. D. A.; de Matos, I. L.; Comasseto, J. V.; Porto, A. L. Biocatalysis and Biotransformation in Brazil: An Overview. *Biotechnol. Adv.* 2015, *33*, 481–510. DOI: 10.1016/j.biotechadv.2015.02.001.

[20] Zielińska-Błajet, M.; Feder-Kubis, J. Monoterpenes and Their Derivatives-Recent Development in Biological and Medical Applications. *Int. J. Mol. Sci.* 2020, *21*, 7078. DOI: 10.3390/ijms21197078.

[21] Rottava, I.; Cortina, P. F.; Grando, C. E.; Colla, A. R.; Martello, E.; Cansian, R. L.; Toniazzo, G.; Treichel, H.; Antunes, O. A. C.; Oestreicher, E. G.; de Oliveira, D. Isolation and Screening of Microorganisms for R-(+)-Limonene and (−)-β-Pinene Biotransformation. *Appl. Biochem. Biotechnol.* 2010, *162*, 719–732. DOI: 10.1007/s12010-009-8872-9.

[22] Lukin, I.; Merz, J.; Schembecker, G. Techniques for the Recovery of Volatile Aroma Compounds from Biochemical Broth: A Review. *Flavour Fragrance J.* 2018, *33*, 203–216. DOI: 10.1002/ffj.3447.

[23] Hadj Saadoun, J.; Bertani, G.; Levante, A.; Vezzosi, F.; Ricci, A.; Bernini, V.; Lazzi, C. Fermentation of Agri-Food Waste: A Promising Route for the Production of Aroma Compounds. *Foods* 2021, *10*, 707. DOI: 10.3390/foods10040707.

[24] Pessôa, M. G.; Paulino, B. N.; Molina, G.; Pastore, G. M. Prospective Research and Current Technologies for Bioflavor Production. In *Bioprocessing for Biomolecules Production*; Molina, G.; Gupta, V.; Singh, B.; Gathergood, N., Ed.; JohnWiley & Sons Ltd: Hoboken, NJ, 2019; pp cap 5. DOI: 10.1002/9781119434436.ch5.

[25] Saidani, F.; Riad, N.; Beichi, M.; Ferradji, F. Z.; Zahi, M. R.; Edouaouda, K.; Mesbaiah, F. Z.; Bouzidi, N.; Badis, A.; El Hattab, M. Screening of biotransformation metabolites of α-pinene by two bacterial strains *Paenibacillus popilliae* 1C, and *Streptomyces rochei* AB1. *J. Appl. Biotechnol. Rep.* 2022, *9*, 763. DOI: 10.30491/JABR.2021.288335.1396.

[26] Gheorghita, G. R.; Sora, C.; Ion, S.; Parvulescu, V. I.; Tudorache, M. Cascade Biocatalysis Designed for the Allylic Oxidation of α-Pinene. *Catalysts* 2021, *11*, 134. DOI: 10.3390/catal11010134.

[27] Choi, I. Y.; Lim, J. H.; Hwang, S.; Lee, J. C.; Cho, G. S.; Kim, W. K. Anti-ischemic and Anti-inflammatory Activity of (S)-Cis-Verbenol. *Free Radic. Res.* 2010, *44*, 541–551. DOI: 10.3109/10715761003667562.

[28] González-Velasco, H. E.; Pérez-Gutiérrez, M. S.; Alonso-Castro, Á. J.; Zapata-Morales, J. R.; Niño-Moreno, P. D. C.; Campos-Xolalpa, N.; González-Chávez, M. M. Anti-inflammatory and Antinociceptive Activities of the Essential Oil of *Tagetes parryi* A. Gray (Asteraceae) and Verbenone. *Molecules* 2022, *27*, 2612. DOI: 10.3390/molecules27092612.

[29] Fanaro, G. B.; Fontanille, P.; MarósticaJr, M. R.; Bicas, J. L. Biotechnological aroma compounds. In *Biotechnological Production of Natural Ingredientes for Food Industry*; Bicas, J. L.; MarósticaJr, M. R.; Pastore, G. M., Eds.; Bentham Science Publishers: Bussum, Netherlands, 2022; pp cap. 6.

[30] Dionísio, A. P.; Molina, G.; de Carvalho, D. S.; dos Santos, R.; Bicas, J. L.; Pastore, G. M. Natural flavourings from biotechnology for foods and beverages. In *Natural Food Additives, Ingredients and Flavourings*; Baines, D.; Seal, R., Eds.; Woodhead Publishing: Sawston, UK, 2012; pp cap. 11. DOI: 10.1533/9780857095725.1.231.