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BIOTRANSFORMAÇÃO DE LIMONENO E PINENO POR MICRORGANISMOS DA AMAZÔNIA

BIOTRANSFORMATION OF LIMONENE AND PINENE BY MICROORGANISM OF THE AMAZON

ELISON DE SOUZA SEVALHO

Manaus-AM

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, na Universidade Federal do Amazonas, como requisito para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia.

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“Tudo posso naquele que me fortace”

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“Faça o teu melhor, na condição que você tem, enquanto você não tem condições melhores, para fazer melhor!”.

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RESUMO GERAL

Uma das formas promissoras para o aproveitamento de subprodutos agroindustriais é através do desenvolvimento de processos biotecnológicos, como a biotransformação de terpenos para produção de inúmeros metabólitos de interesse industrial, como, por exemplo, a produção de bioaromas. A utilização de subprodutos agroindustriais da indústria cítrica e papel/celulose possuem alto teor de substratos, como limoneno e pineno, respectivamente, e são de grande interesse para produção de aromas. Também é de grande interesse a utilização de microrganismos isolados de diferentes fontes, como biocatalizadores para a produção desses compostos. Nesse contexto, esta Tese de doutorado teve como objetivo principal prospectar novos microrganismos da Amazônia com potencial para a biotransformação de limoneno e pineno, assim como otimizar as condições de processo para a produção de compostos de aroma valorizados comercialmente nas indústrias alimentícias, farmacêuticas e de cosméticos. O Capítulo 1 apresenta um artigo de revisão atualizado sobre o potencial biotecnológico de diferentes microrganismos como potenciais biocatalizadores em processos de biotransformação do limoneno e pineno. No capítulo 2 inicia a primeira etapa do trabalho prático com a seleção de 31 espécies de fungos ascomicetos dos gêneros *Penicillium*, *Trichoderma, Pestalotiopsis* e 16 basidiomicetos (Agaricomycetes) pertencentes a coleção de trabalho do Laboratório de Bioensaios e Microrganismos da Amazônia da Central Analítica, Universidade Federal do Amazonas. O screenning resultou em 9 fungos endofíticos, 2 basidiomicetos e 1 fungo aquático considerados potenciais candidatos à biotransformação do limoneno, uma vez que acumularam eventualmente compostos interessantes após 120 h de reação. No entanto, apenas *Pestalotiopsis mangiferae* LabMicrA-505 foi o fungo endofítico mais promissor encontrado na triagem, devido à sua capacidade de utilizar todo o *R*-(+)-limoneno como única fonte de carbono e energia em meio mineral, obtendo como principais derivados o limoneno-1,2-diol (74.13±0.81%) e limoneno-1,2-epóxido (1.88±0.08%) acumulados após 72 h de reação. No capítulo 3 refere-se aos experimentos de triagem de 11 linhagens de actinomicetos do gênero *Streptomyces* isolados de solos rizosféricos de ingá (*Inga edulis* Mart.) Dessas linhagens estudadas, apenas *Streptomyces* sp. LabMicra B270 foi umas das promissoras, devido à sua capacidade de utilizar todo o α-pineno como única fonte de carbono e energia em meio mineral, obtendo como principais produtos o *cis*-verbenol (57,39±0,07%), verbenona (37,80±2,10%) e

mirtenol (4,81±2,02%) acumulados após 72 h de reação. A seguir, o capítulo 4 aborda o delineamento composto central rotacional (DCCR) empregado para otimizar os principais parâmetros do processo de produção de limoneno-1,2-diol a partir do limoneno pelo fungo *Pestalotiopsis mangiferae* LabMicrA-505. A otimização das variáveis analisadas pelo DCCR revelou que as condições ótimas para a biotransformação foram de 2.0% de substrato, 24 °C, 120 rpm e pH 6, resultando em um acúmulo de 98.34±1.53% de limoneno-1,2-diol depois de 96 h de reação. Estas pesquisas determinam o potencial destes microrganismos a serem utilizados como novos biocatalizadores. Estes resultados abrem precedentes para desenvolver a produção de aromas naturais e demonstrar o potencial do uso destes microrganismos obtidos da Amazônia em processos de biotransformação.

Palavras-chave: Biocatálise; Monterpenos; Microrganimos amazônicos; Processos biotecnológicos; Novos biocatalizadores.

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ABSTRACT

One of the promising ways to use agro-industrial by-products is through the development of biotechnological processes, such as the biotransformation of terpenes, for the production of many metabolites of industrial interest, such as bioaromas. The use of agro-industrial by-products from the citrus industry and paper/cellulose has a high content of substrates, such as limonene and pinene, respectively, and is of great interest for the production of aromas. Also of great interest is the use of microorganisms isolated from different sources as biocatalysts for the production of these compounds. In this context, this doctoral thesis had as its main objective to prospect for new microorganisms from the Amazon with potential for the biotransformation of limonene and pinene, as well as to optimize the process conditions for the production of commercially valued aroma compounds in the pharmaceutical, and cosmetic industries. Chapter 1 presents an updated review article on the biotechnological potential of different microorganisms as potential biocatalysts in limonene and pinene biotransformation processes. In chapter 2, the first stage of the practical work begins with the selection of 31 species of ascomycete fungi of the genera *Penicillium*, *Trichoderma*, *Pestalotiopsis* and 16 basidiomycetes (Agaricomycetes) belonging to the work collection of the Laboratory of Bioassays and Microorganisms of the Amazon of Central Analytical, University Federal do Amazonas. Screening resulted in 9 endophytic fungi, 2 basidiomycetes, and 1 aquatic fungus being considered potential candidates for limonene biotransformation, since they eventually accumulated interesting compounds after 120 h of reaction. However, only *Pestalotiopsis mangiferae* LabMicrA-505 was the most promising endophytic fungus found in the screening, due to its ability to use all *R*-(+)-limonene as the only source of carbon and energy in mineral medium, obtaining as main derivatives the limonene-1,2-diol (74.13±0.81%) and limonene-1,2-epoxide (1.88±0.08%) accumulated after 72 h of reaction. In chapter 3, it refers to the results obtained by the experiments that contemplate the selection of 11 actinomycete strains of the genus Streptomyces isolated from rhizosphere soils of inga-cipó (*Inga edulis* Martius). Of these strains studied, only *Streptomyces* sp. LabMicra B270 was one of the most promising due to its ability to use all α-pinene as the only source of carbon and energy in mineral medium, obtaining as main products *cis*-verbenol (57,39±0,07%), verbenone (37,80±2,10%) and myrtenol (4,81±2,02%) accumulated after 72 hours of reaction. Next, Chapter 4 discusses the rotational

central composite design (DCCR) used to optimize the main parameters of the limonene-1,2-diol production process from limonene by the fungus *Pestalotiopsis mangiferae* LabMicrA-505. The optimization of the variables analyzed by the DCCR revealed that the optimal conditions for the biotransformation were 2.0% substrate, 24 °C, 120 rpm and pH 6, resulting in an accumulation of 98.34±1.53% of limonene-1,2-diol after of 96 h of reaction. This research determines the potential of these microorganisms to be used as new biocatalysts. These results set precedents for developing the production of natural flavors and demonstrating the potential of using these Amazonian microorganisms in biotransformation processes.

Keywords: Biocatalysis; Monterpenes; Amazonian microorganisms; Biotechnological processes; New biocatalysts.

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1 **INTRODUÇÃO GERAL**

1.1 AROMAS

Os aromas sempre fizeram parte da história da humanidade, sendo que antigamente os aromas tinham a função de alertar se um alimento estava estragado ou diferenciar plantas nocivas das comestíveis. Atualmente, com o desenvolvimento tecnológico em diferentes áreas, os aromas assumem a nova função de melhorar a qualidade sensorial dos produtos. Assim, o aroma é responsável por grande parte do sabor, sendo considerado um dos aditivos mais importantes na aceitação do produto pelo consumidor (BELDA *et al*., 2017; PAULINO *et al*., 2021).

Aromas são compostos de baixo massa molecular, geralmente menores que 400 Danton, que podem ser percebidos por via nasal (odor) ou retronasal (aroma). Estes compostos orgânicos notavelmente perceptíveis pelo olfato e exercendo odores característicos muitas vezes agradáveis. Uma ampla variedade de compostos orgânicos está associada ao aroma, podendo ser hidrocarbonetos, álcoois, cetonas, aldeídos, ésteres, lactonas, ácidos carboxílicos de cadeias curta ou fenólicos, por exemplo, limoneno e pineno estão entre os hidrocarbonetos monoterpênicos mais difundidos. Além disso, apresentam grande diversidade quanto à polaridade, solubilidade, volatilidade e temperatura e pH de estabilidade. Apesar de estarem presentes em concentrações muito baixas, estes compostos são muito potentes com ampla aplicação em setores de alimentos, rações, cosméticos, químicos e farmacêuticos (FANARO *et al*., 2016; PESSÔA *et al*., 2019; YUE *et al*., 2020).

Em 2021, a indústria de aromas e fragrâncias movimentou cerca de US$ 14,66 bilhões, e deve atingir US$ 20,12 bilhões até o ano de 2028, registrando um *Compound Annual Growth Rate* (CAGR) de 4,64% durante o período de 2021-2028. As principais empresas responsáveis no mercado de aromas são: Givaudan SA (Vernier, Switzerland), International Flavors and Fragrances (New York, U.S.), Symrise AG (Holzminden, Germany), Sensient Technologies Corp (Wisconsin, U.S.), Firmenich (Geneva, Switzerland), Archer-Daniels-Midland Co. (Illinois, U.S.), Kerry Group Plc (Tarlee, Ireland), Corbion NV (Amsterdam, Netherlands), Koninklijke DSM NV (Herleen, Netherlands) e BASF SE (Ludwigshafen, Germany) (MARKET RESEARCH REPORT, 2023).

1.2 A BIOTECNOLOGIA APLICADA AOS AROMAS

A limitada disponibilidade de recursos no planeta tem estimulado as instituições de pesquisa e indústrias ao desenvolvimento da biotecnologia branca, em busca de tecnologias sustentáveis e ecológica (HEUX *et al*., 2015). Neste contexto, a produção por biotransformação microbiana de aromas tem sido apontada como a mais promissora dentre as possibilidades de obtenção dos compostos aromáticos naturais. A obtenção de compostos de aroma é obtida por três processos principais: síntese química, extração de recursos naturais ou métodos biotecnológicos (LANGE *et al*., 2015; VERMA *et al*., 2022).

Através da síntese por rotas químicas, podem ser obtidas altas quantidades do composto a baixos custos. Entretanto, os aromas não podem ser classificados como naturais e essas reações químicas podem apresentar régio- ou enantiosseletividade ao substrato, podendo formar uma mistura de isômeros indesejada. Todavia, as sínteses químicas geralmente criam altos impactos ambientais por emitirem certa carga de resíduos não-biodegradáveis. Em relação à extração de aromas a partir de fontes vegetais, os compostos são obtidos em altas concentrações, mas há instabilidade quanto a sua extração, pois, depende de fatores sazonais e climáticos (BRAGA; GUERREIRO; BELO, 2018; PESSÔA *et al*., 2019a).

Os métodos biotecnológicos para a produção de aromas incluem a síntese *de novo* através da fermentação e a biotransformação através da biocatálise de precursores de aroma. No primeiro método, os microrganismos são cultivados em meios de cultura simples e todo o arsenal metabólico é ativado para a produção de uma mistura de compostos. Por outro lado, a biotransformação ocorre devido à biocatálise de precursores adicionados ao meio de cultura e são metabolizados por microrganismos formando um produto principal por uma única reação enzimática e que possui estrutura química muito semelhante à do seu precursor (DIONÍSIO *et al*.,2012; BRAGA; GUERREIRO; BELO, 2018; PESSÔA *et al*., 2019a).

O uso de processo de biotransformação microbiana como uma alternativa a processos tradicionais (extração da natureza e síntese química) para a produção de aromas naturais, contorna problemas relacionados às transformações químicas, entre os mais importantes: (i) condições severas de operação, (ii) uso e potencial descarte volumoso de reagentes químicos, (iii) baixa especificidade de reação, (iv) alteração na percepção sensorial dos alimentos (BIROLLI *et al*.,2015, BIER *et al*., 2017; PESSÔA *et al*., 2019b).

Entre as vias microbiológicas, a biotransformação de precursores aromáticos tem se destacado no cenário mundial como uma ferramenta promissora para a obtenção de novos compostos de aromas (BHATTI *et al*., 2014). A biotransformação ocorre devido às reações dos precursores (substratos) com biocatalizador (enzimas excretadas, isoladas e imobilizadas, culturas de células ou microrganismos que também podem ser imobilizados), formando um produto principal por uma única reação enzimática e que possui estrutura química muito

semelhante à do seu precursor, mas com função diferente ou aprimorada (BIROLLI et al., 2015; CHOUDHARY et al., 2021).

Segundo a legislação brasileira da Agência Nacional de Vigilância Sanitária (ANVISA) Resolução n.º 2, de 15 de maio de 2007, define "aromas naturais" como aqueles obtidos por métodos físicos, microbiológicos ou enzimáticos a partir de matérias-primas aromatizantes (Ministério da Saúde-ANVISA, 2007), semelhante às legislações norte-americana (FDA: *Code of Federal Regulations*, 21CFR101.22) e europeia (*Council Directive* 88/388/EEC). Assim, há um grande interesse pela bioprodução e aplicação de compostos de aroma de origem (micro) biológica, resultando nos chamados bioaromas. Esses compostos são considerados substâncias naturais, e sua produção apresenta menores impactos ambientais e custos de produção (VERMA *et al*., 2022).

Além disso, faz-se necessário diferenciar a fermentação e a biodegradação da biotransformação. A fermentação acontece em algumas etapas catalíticas entre o substrato e o produto, sendo que estes não necessariamente se assemelham. A biotransformação é a catálise entre substrato e produto acontecendo em uma ou duas etapas, e o produto se assemelha ao substrato. E a biodegradação é a completa decomposição do material em estudo (BICAS *et al*., 2016; BRAGA; GUERREIRO; BELO, 2018).

1.3 LIMONENO E PINENO USADO COMO SUBSTRATO

Nos últimos dez anos, o processo de biotransformação ganhou destaque e tem sido amplamente descrito na literatura científica como método bastante versátil e promissor devido à possibilidade de utilização de subprodutos (agro)industriais ricos em monoterpenos, pois estes podem ser utilizados como substratos para a formação de novos compostos aromáticos. Mas especificamente, os produtos de biotransformação de terpenos têm uso no campo alimentício, cosmético, farmacêutico e energético, dentre outros (BORGES *et al*., 2009; HALL *et al*., 2016; BHAT *et al*., 2023).

Os monoterpenos usados na abordagem de biotransformação são comumente encontrados na natureza e adquiridos em larga escala por meio de subprodutos agroindustriais. A aplicação de monoterpenos como substrato na produção de aromas tem sido descrita e revisada nos últimos anos e é considerada uma importante abordagem em biotecnologia. Neste contexto, tanto o limoneno como o pineno são amplamente explorados como substrato/precursores para biotransformação de diferentes compostos aromáticos de valor agregado (CASERTA *et al*., 2019).

O limoneno, 1-metil-4-prop-1-en-2-ilciclohexeno (PubChem CID: 22311) é um dos monoterpenos monocíclicos mais estudados, sendo encontrado como constituinte majoritário de diversos óleos essenciais principalmente de espécies cítricas. O limoneno é um líquido incolor e existe em duas formas enantioméricas, *R*-(+)- e *S*-(-)-limoneno (Figura 1) (REN *et al.*, 2020).

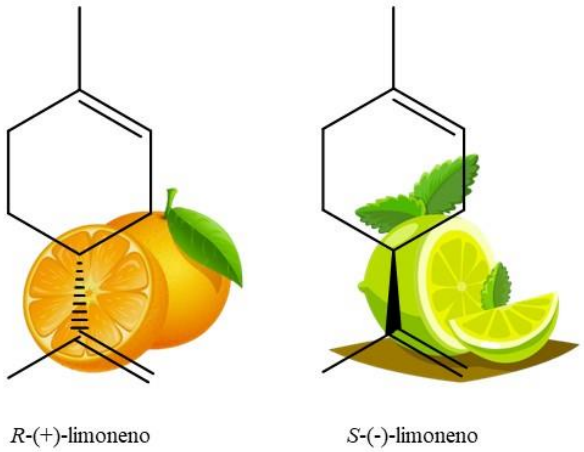

Figura 1- Fórmula estrutural dos enantiômeros de *R*-(+)- e *S*-(-)-limoneno

O *R*-(+)-limoneno (4*R*)-1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 440917), conhecido como d-limoneno é o principal composto dos óleos essenciais das cascas de frutas como a laranja (*Citrus sinensis*). Também é abundante em algumas espécies de *Lippia* sp e *Artemisia* sp. Os resultados de um estudo realizado por Ibáñez, Sanchez-Ballester e Blázquez (2020) mostram que o *R*-(+)-limoneno é mais de 90% dos terpenos na composição do óleo essencial cítrico. O *S*-(–)-limoneno, (4*S*)-1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 439250) é outra forma enatiomêrica conhecida como l-limoneno é encontrado principalmente em concentrações baixas-moderadas (20-30%) nos óleos essenciais do limão (*Cymbopogon citratus*) e menta (*Mentha spicata*) (VIEIRA *et al.*, 2018; EDDIN *et al.*, 2021).

Outro monoterpeno encontrado em abundância como subproduto (agro) industriais é o pineno, um monoterpeno bicíclico encontrado em duas formas isoméricas, α-pineno e β-pineno (Figura 2). Os diferentes teores de pinenos na terebintina podem variar de acordo com a espécie botânica (gênero *Pinus)*, mas, em geral, a quantidade de α-pineno é sempre maior que a de β-pineno nos óleos estudados. A terebintina é um subproduto agroindustrial do papel, que se destaca por ser um resíduo rico em pinenos e, devido ao preço acessível, é ideal em processos biotecnológicos para produção de bioaromas, amplamente utilizados nas indústrias farmacêutica, cosmética e alimentícia (SALEHI *et al*., 2019).

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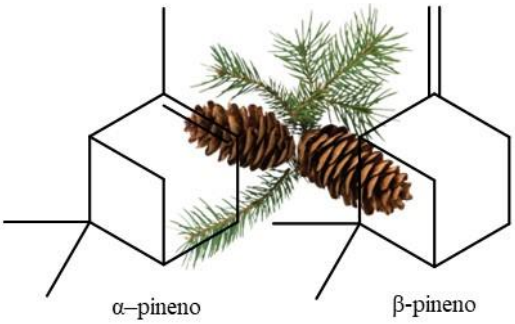

Figura 2- Fórmula estrutural dos isômeros de α e β-pineno

O α–pineno ou 2,6,6-trimethylbicyclo[3.1.1]hept-2-eno (PubChem CID: 6654) é um líquido incolor, solúvel em etanol e óleos e insolúvel em água, com ponto de ebulição de 155 °C. Possui um odor intenso e característico que lembra pinho e terebintina. Na concentração de 1%, apresenta aroma cítrico e picante, amadeirado de pinho e terebintina. O limiar de detecção é de 2,5 a 62 ppb. O limiar gustativo é de 10 ppm, e apresenta um sabor intenso, amadeirado, de pinho, com um notável sabor de cânfora e terebintina. O β-pineno ou 6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptano (PubChem CID: 14896) é um líquido incolor, solúvel em óleos e insolúvel em água e etanol, com ponto de ebulição entre 163 e 166 °C. Na concentração de 10%, as características do aroma são refrescantes, amadeiradas, pinho e terebintina, com traços de menta fresca, eucalipto e cânfora. Podendo ser detectado em um limite de 140 ppb, possuindo um sabor caracteristicamente fresco, pinho, amadeirado e resinoso em 15 a 100 ppm e uma leve nuance de picante, menta e cânfora (VESPERMANN *et al*., 2017; XU *et al*., 2019; KENSETH *et al*., 2020).

1.4 MICRORGANISMOS COMO BIOCATALIZADORES

Há na literatura alguns exemplos sobre a capacidade dos microrganismos usados como biocatalizadores em processos de biotransformação do limoneno e pineno. O fungo *Fusarium oxysporum* 152B, por exemplo, foi capaz de biotransformar o *R*-(+)-limoneno, o que resultou na obtenção de 2,4 g/L de *R*-(+)-α-terpineol (BICAS et al., 2008). Tempos depois, em novo trabalho, a mesma linhagem chegou a produzir 4 g/L de α-terpineol após 48 horas de reação (MOLINA et al., 2015). Em outro exemplo, Sales et al. (2019) relatam a produção de limoneno-1,2-diol a partir de *R*-(+)-limoneno e *S*-(-)-limoneno pelo fungo *Colletotrichum nymphaeae* CBMAI, resultando em concentrações de 7,1 e 7,8 g/L, respectivamente. Recentemente, no estudo de Bhat et al. (2023) descrevem o uso de *Aspergillus flavus* IIIMF4012 na biotransformação de limoneno em isômeros de carveol. Em um estudo, entre 400 fungos,

Aspergillus niger ATCC 16404 foi o único capaz de biotransformar o β-pineno em 2,8 g/L de α-terpineol (ROTTAVA *et al*., 2010).

Por sua vez, em um estudo com 400 fungos, *Aspergillus niger* ATCC 16404 foi o único capaz de biotransformar o β-pineno em 2,8 g/L de α-terpineol (ROTTAVA et al., 2010). Na biotransformação do α-pineno em *cis*-verbenol, um composto com valor agregado para a indústria alimentícia, pôde ser realizada pelo fungo *Chrysosporium pannorum* (A-1), com rendimento de 1,3 g/L de verbenol (TRYTEK; FIEDUREK; GROMADA, 2016). Ao usar basidiomicetos como biocatalizadores, duas espécies de *Trametes elegans* (ET-06 and EBB-046) foram capazes de biotransformar o α-pineno em *cis*-verbenol (JARAMILLO *et al*., 2020). Em um estudo recente, o actinomiceto *Streptomyces rochei* AB1, foi capaz de biotransformar o α-pinene, em *trans*-verbenol (SAIDANI *et al*., 2022).

Assim, a Amazônia brasileira se destaca, pela sua biodiversidade, sobretudo com a variedade de biomas que a compõem, quando se trata do universo microbiológico, o uso das espécies fúngicas e suas diversificadas rotas metabólicas são capazes de produzir um arsenal enzimático, usando meios de cultura de baixo custo e realizando diversas modificações estruturais eficientes nos compostos formando, por isso, os fungos podem ser amplamente aproveitados nesses processos biotecnológicos (NISA *et al*., 2015; YAN *et al*., 2018; ALCÁNTARA *et al*., 2022).

É importante perceber que os microrganismos são eficientemente adaptados aos diversos ambientes Amazônicos e por isso, são detentores de uma versatilidade bioquímica e genética que lhes garantem vantagens ecológicas fundamentais a sua sobrevivência a xenobiótico (DE SOUZA *et al.*, 2016). Essa característica associada a forma de alimentação por absorção e ao ambiente competitivo onde residem tornam os microrganismos um grupo bastante rico do ponto de vista das informações gênicas (CHAMBERGO; VALENCIA, 2016; REN *et al*., 2020). Dessa forma há um campo aberto para a pesquisa científica na região Norte do Brasil, que pode de maneira rápida responder a anseios científicos latentes, como a obtenção de novos produtos e processos advindos de microrganismos da floresta amazônica (PEREIRA *et al*., 2017).

Alguns desafios e limitações precisam ainda ser superados, entre os quais, a descoberta de novas linhagens de microrganismos adequadas. É necessário que o microrganismo seja robusto e resistente tanto ao substrato, como aos produtos formados, que podem causar efeitos inibitórios ao crescimento. Além disso, as linhagens devem conseguir metabolizar o substrato como única fonte de carbono e acumular composto de interesse com estratégias para a melhoria de rendimentos (ABRAHÃO *et al*., 2013; DE OLIVEIRA FELIPE; DE OLIVEIRA; BICAS, 2017; PESSÔA *et al*., 2019).

A seleção de novos microrganismos produtores e promissores para aplicações biotecnológicas na biocatálise de produtos naturais são de interesse, particularmente para as indústrias farmacêuticas, cosméticas e alimentícias. Neste contexto, ainda é quase totalmente desconhecido o potencial de biotransformação de limoneno e pineno por linhagens fúngicas nativas da biodiversidade amazônica. Portanto, este trabalho justifica-se pela bioprospecção de microrganismos isolados de diferentes fontes da floresta amazônica brasileira, como novos candidatos a biocatalizadores, com a perspectiva de resultados de grande potencial para biotransformação de terpenos para a pesquisa e o desenvolvimento industrial de bioaromas no estado do Amazonas.

1.5 OBJETIVOS

1.5.1 **Objetivo geral**

Avaliar o potencial de microrganismos da Amazônia para a biotransformação de limoneno e pineno para produção de aromas.

1.5.2 **Objetivos específicos**

Prospectar a capacidade de linhagens de microrganismos da Amazônia para a biotransformação de limoneno e pineno;

Investigar as condições de cultivo para a produção de limoneno-1,2-diol pela biotransformação do limoneno por uma linhagem de *Pestalotiopsis*;

Otimizar o processo da biotransformação de limoneno para produção de limoneno-1,2-diol por uma linhagem de *Pestalotiopsis*.

1.6 ORGANIZAÇÃO DA TESE

Esta Tese de Doutorado apresenta diversos experimentos desenvolvidos nos Laboratório de Bioensaios e Microrganismos da Amazônia (LABMICRA) e Laboratório de Cromatografia Gasosa (LABCG) da Central Analítica (CA), Centro de Apoio Multidisciplinar (CAM) da Universidade Federal do Amazonas (UFAM), entre os meses de maio de 2019 a janeiro de 2023, sob orientação do Prof. Dr. Dr. Afonso Duarte Leão de Souza, coorientação de Prof. Dra.

Antonia Queiroz Lima de Souza e Prof. Dr. Bruno Nicolau Paulino. A bolsa de estudo foi financiada pela Fundação de Amparo à Pesquisa do Estado do Amazonas – FAPEAM (n° 003/2019 - POSGRAD UEA).

A redação desta Tese para a para defesa está organizada conforme as resoluções da Instrução Normativa nº 04 do PPG-BIONORTE - 25 de março de 2022, que regulamenta os critérios para a defesa. Para facilitar, a Tese foi estruturado em quatro capítulos, apresentados na forma de artigos científicos, organizada conforme:

No **Capítulo 1** está apresentada uma revisão bibliográfica em formato de artigo científico publicado no periódico "*Brazilian Journal of Chemical Engineering*". A revisão aborda a produção de compostos de aroma pela biotransformação de limoneno e pineno usando diversos biocatalizadores fúngicos.

O **Capítulo 2** contém o estudo da capacidade de biotransformação de limoneno por microrganismos da Amazônia brasileira. Tal pesquisa está na etapa para submissão no periódico "*World Journal of Microbiology and Biotechnology*".

No **Capítulo 3** apresenta uma avaliação do potencial de actinomicetos do gênero *Streptomyces* na biotransformação de monoterpenos. O estudo foi preparado para submissão no periódico "*Journal Microbiology*".

O **Capítulo 4** descreve a investigação das condições de cultivo e apresenta um estudo de otimização da produção de limoneno-1,2-diol pela biotransformação de limoneno por *Pestalotiopsis mangiferae* LabMicrA-505. O estudo foi preparado para submissão no "*Biotechnology Letters*".

Foi apresentado uma Conclusão Geral a partir dos dados apresentados ao longo da Tese. Por fim, nos **Anexos** estão disponíveis o Artigo referente a pesquisa inicial do doutoramento e o Termos de Licença para o artigo publicado utilizado na Tese como primeiro capítulo.

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CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

Fungal biotransformation of limonene and pinene for aroma production[1]

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REVIEW

Fungal biotransformation of limonene and pinene for aroma production

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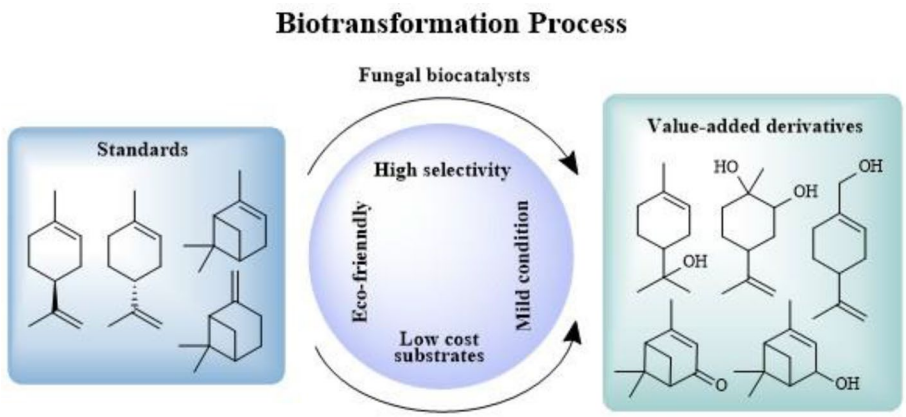
Abstract

The biotechnological production of aroma compounds may be carried out using enzymatic methodologies or microbial approaches and different substrates. In this context, the use of low-cost monoterpenes such as limonene and pinene isomers and fungal biocatalysts can be considered a promising approach for the production of oxygenated derivatives with important aroma characteristics. Considering the diversity of fungi strains employed on the biotransformation processes, this review presents the most important strategies applied to the production of monoterpene-derived aromas using limonene and pinene isomers as substrates, summarizing the main structural modifications achieved as well as strategies for the screening of novel strains, optimization of process parameters and the biological potential of these aromas.

Graphical abstract



Keywords Monoterpenes · Oxidation · Biocatalysts · Aroma compounds · Bibliometric analysis

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Introduction

Natural compounds are considered interesting alternatives for the replacement of traditional chemicals, and can be employed in the food, cosmetic, and pharmaceutical industries. In the last years, many chemical companies have increased their portfolios with the inclusion of natural compounds produced by biotechnology-based approaches (Oliveira Felipe et al. 2017). In this sense, great efforts between scientists, and industry have been directed towards the development of processes for the generation of these compounds, with aroma compounds being one of the main targets (Paulino et al. 2021).

Conceptually, aroma or odoriferous compounds are organic molecules with low molecular weight, commonly less than 400 Da, that exhibits remarkably and perceptible odors characteristic that is often pleasant and applicable in cosmetic, pharmaceutical, and food formulations and products (Sales et al. 2018a; Pessôa et al. 2019a, b; Yue et al. 2020). Traditionally, aromas compounds are obtained from chemical processes or extraction of plant matrices, and many companies increasing efforts to adapt their processes to recent global demand related to environmental concerns where biotechnological approaches (e.g. microbial and enzymatic biotransformations) emerge as being sustainable and innovative alternatives (Molina et al. 2014; Bicas et al. 2016; Pessôa et al. 2019a, b). Moreover, the global Food Flavors market in 2018 was estimated at around 13.3 billion USD and is expected to reach 19.7 billion USD by the year 2026, resulting in a Compound Annual Growth Rate (CAGR) close to 5% (Food Flavors Market To Reach USD 19.72 Billion By 2026 2019).

Compared with chemical synthesis and direct extraction from nature biotechnological processes (biotransformation, and de novo synthesis) presents two advantages: (i) the obtained products are considered natural as the extracted ones, (ii) the production processes are aligned with the best practices in environmental preservation and "green chemistry" concepts (Dionísio et al. 2012; Oliveira Felipe et al. 2017).

Biotransformation is defined as the biotechnological process that uses biological systems to catalyze specific chemical modifications of a defined substrate to obtain the desired substance (Carvalho 2016; Fanaro et al. 2016). Generally, biotransformation processes are carried out under mild conditions and with high selectivity, which includes regio-, stereo-, and enantioselective modifications based on single or multistep reactions. Moreover, this process can be carried out using isolated enzymes or whole cells (bacteria, yeasts, and fungi) and the selection of the biocatalytic system is considered crucial for the process to be efficient and produce the desired compound (Molina et al. 2014; Sales et al. 2018a).

Many studies have been reported the use of whole-cells or isolated enzymes on biotransformation of terpenes for the breeding of oxygenated derivatives that exhibit important odor characteristics (Sales et al. 2018a). In this context, the application of fungi strains as biocatalysts on monoterpene biotransformation has been proposed as a promising approach, along with the use of statistical process optimization strategies and genetic engineering tools (Molina et al., 2014, 2015, 2019; Sales et al., 2019a, 2019b). These microorganisms occur in different ecosystems and can adapt to extreme environmental conditions such as the presence of metals, high concentrations of organic/inorganic compounds, and adverse temperature, pH, and salinity conditions (Borges et al. 2009; Carvalho 2016). Moreover, the diversity of enzymes produced by fungal strains allows the catalysis of various reactions such as hydroxylation, oxidation, epoxidation, dealkylation, dehydration, deamination, desaturation, dehalogenation, and demethylation (Bicas et al. 2009; Janocha, Schmitz and Bernhardt 2015; Zhang et al. 2016; Puentes-Cala et al. 2018).

Thus, the biotransformation processes of monoterpenes have been very well established using fungal strains allowing promising results that show it is potential for industrial scale-up (Braga et al. 2018). In this context, limonene, and pinenes are the most applied monoterpenes for this purpose, considering that they can be found in abundance in several essential oils and some industrial by-products, such as those derived from the citrus industry (Vespermann et al., 2017; Molina et al. 2014; Sharma et al. 2020). From an economic point of view, limonene has a weighted average price of US$ 34/L, while its oxygenated derivatives as carveol, perillyl alcohol, and carvone present reference prices of US$ 529/L, US$ 405/L, and US$ 350/L, respectively. In its turn, α-pinene has an average price of US$ 64/L, much less than its oxygenated derivatives, such as myrtenol (US$ 1939/L), verbenol (US$ 1926/L), myrtenal (US$ 913/L), and verbenone (US$ 906/L). These monoterpenoids are aroma compounds widely used in different industrial Fields and are found in low concentrations in natural sources, being the biotechnological approaches are an important alternative for obtaining on large scale. Thus, the use of limonene and α-pinene as a substrate for the breeding of value-added derivatives through biotransformation can be considered economically interesting.

Therefore, this review presents the advances in the use the potential of fungal strains for aroma production through biotransformation of limonene and pinene isomers focusing on the description of the main characteristics of the biotechnological processes including process parameters employed and approaches for optimization. Moreover, we described the biological the potential of the main compounds produced

from bioconversion of limonene and pinene isomers as well as discussed the limitations and future perspectives concerning the use of biotransformation as a biotechnological strategy for aroma production.

Literature research methodology, and bibliometric analysis

A mixed methodology is used to carry out this bibliographic review—a bibliometric network, followed by a review of the literature, a method also used by Melo et al. (2021). For data collection, a search was carried out in the Web of Science (WoS) Core Collection electronic database (Clarivate Analytics, Philadelphia, USA) during January 2022. The literature data (research articles, reviews, and book chapter) were filtered using terms: "Biotransformation of limonene" OR "Biotransformation of pinene" OR "Biotransformations using fungi" OR "Production of perillyl derivatives" OR "production of α-terpineol" OR "Production of limonene-1,2-diol" OR "Potential biological aroma compounds" in the topic item that includes the title, summary, and keywords plus. We restricted our search to reports published from January 1, 2000, to December 31, 2021.

The materials collected in the database were then processed using VOSviewer© software version 1.6.17 (www.vosviewer.com) to gather information concerning cooperation among countries, organizations, and authors. The data were evaluated through a co-authorship analysis that considered the strength of the link established by the program, whereas the most frequently quoted documents and journals, quotation analysis, and trends in the field were consulted via the co-occurrence of the keywords of the authors, with no consideration given to the strength of the link established by the program to rank the results. The data presented in this study were organized in tables and then analyzed, highlighted, and discussed. The chemical structures of the selected compounds were drawn using ChemDraw© software version 19.1.

Bibliometric network and overlay visualization

Study of publications from year, the field of publication, organizations, and cooperation among countries

Up to the time that the data were collected for the writing of this review, 85 articles had been published in the database WOS involving fungal biotransformation of limonene and pinene. The largest concentration of articles occurred in 2009, during which 10 studies (Fig. 1), nevertheless,

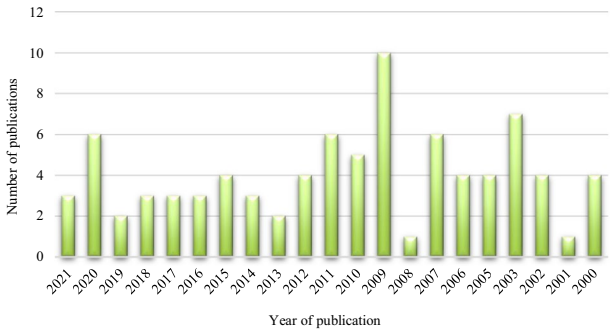

Fig. 1 Publication trends per year related to the subject (Research done in the Web of Science © database during January 2022)

subsequent years see a reduction in the number of articles published. In other words, according to the literature, this was the year in which the subject was most studied.

Despite the vast description of current methodologies in the field of biotransformation, since 2000, only 30% of the biocatalytic processes involved fungi. In general, the various publications on this topic were related to 12 fields of knowledge according to the Web of Science Categories. The distribution of the central theme of this review, in which the fields "Biotechnology Applied Microbiology" possessed the highest percentage (35.3%), followed by "Chemistry" (27%), making it the largest field of study output on the topic. These journals account for 57.21% of all highly cited articles used in this study. The 12 fields of WoS have played a relevant role in the development of the subject and evidence that this topic is in continuous ascension over the last years. Ren et al. (2020) show that the numbers might indicate a growing interest in research from fungal biotransformation, as it offers an alternative approach for sustainable production of high-value natural products. This ranking of the 10 fields with the greatest number of publications is also shown in Table 1.

The publication number provides insight for researchers to identify the global trends and increase collaboration in their respective fields of study. The 27 countries with publications on the biotransformation of limonene and pinene by fungi in the last 21 years were grouped and classified according to the most expressive intervals of the number of publications. Although Brazil is the country with the greatest number of researchers who have published studies on the subject, being present in 32.9% of the 85 studies, other countries such as Poland, China, Germany, India, and Japan, respectively, also had the greatest scientific output. The ranking of the 10 most productive countries in terms of the number of publications is also listed in Table 1.

The analysis of the publications evaluated showed that 91 organizations between universities, and institutions were responsible for the published research related to the subject,

Table 1 Publication areas, organizations and countries that have published the most

Ranking	Name	Number	Percentage (%)*
Publication areas			
1	Biotechnology Applied Microbiology	30	35.294
2	Chemistry	23	27.059
3	Biochemistry Molecular Biology	18	21.176
4	Food Science Technology	17	20.000
5	Microbiology	10	11.765
6	Pharmacology Pharmacy	9	10.588
7	Engineering	7	8.235
8	Life Sciences Biomedicine Other Topics	4	4.706
9	Agriculture	3	3.529
10	Mycology	2	2.353
Countries			
1	Brazil	28	32.941
2	Poland	11	12.941
3	China	7	8.235
4	Germany	6	7.059
5	India	5	5.882
6	Japan	4	4.706
7	Spain	4	4.706
8	Portugal	3	3.529
9	Canada	2	2.353
10	Colombia	2	2.353
Organizations			
1	State University of Campinas	16	11,679
2	Maria Curie-Skłodowska University	8	5,839
3	Federal University of Rio de Janeiro	5	3,650
4	Central Food Technological Research Institute	3	2,190
5	Huazhong Agricultural University	3	2,190
6	Regional Integrated University of Upper Uruguai and Missions	3	2,190
7	Technical University of Lisbon	3	2,190
8	Chinese Academy of Sciences	2	1,460
9	Chemische Technik und Biotechnologie	2	1,460
10	ETH Zurich Hönggerberg	2	1,460

*(%): percentage of 85 publications

of which, 13 are Brazilian. In the last years, the Brazilian universities (The State University of Campinas, and Federal University of Rio de Janeiro) ranked among the top 3 institutions with the most publications and citations on the subject. This fact may be related to the increase in the use of fungal biotransformation, over the years. Research organizations are likely to provide their support by conducting studies on the topic. Among the organizations, 4 institutions have published at least 3 articles each involving the subject, whereas another 12 institutions have published 2 articles each, and the remaining 72 have published only 1 article each. The top 10 performing organizations based on the number of papers contributed are shown in Table 1. The interaction among the institutions can be seen in Fig. 2, which shows a relationship between Brazilian universities and major organizations that interconnect.

Journals, articles, and author keywords analysis

In addition to the above study methods, a quotation analysis is used to identify the most frequently quoted journals and articles. The 85 publications in the dataset have been published in 52 different journals. The results reveal that the three most quoted journals are Applied Microbiology and Biotechnology, Journal of Industrial Microbiology Biotechnology, and Process Biochemistry, both with 4 articles. Table 2 shows the 10 most listed journals in the WoS under the subject category of "Biotechnology Applied

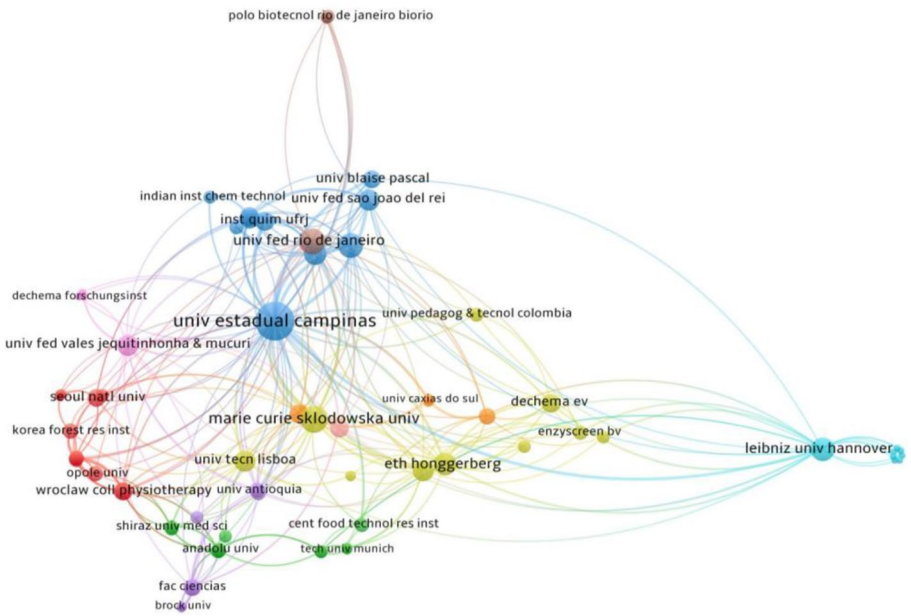

Fig. 2 Network visualization map of collaboration among organizations according to co-authorship

Table 2 Ranking of the most cited journals and documents

Ranking	Name	Number	Percentage (%)*	Impact Factor**
Publication areas				
1	Applied Microbiology and Biotechnology	4	4.706	4.81
2	Journal of Industrial Microbiology Biotechnology	4	4.706	3.34
3	Process Biochemistry	4	4.706	3.75
4	Applied Biochemistry and Biotechnology	3	3.529	2.92
5	Brazilian Archives of Biology and Technology	3	3.529	0.57
6	Food Science and Biotechnology	3	3.529	2.39
7	Tetrahedron Asymmetry	3	3.529	2.12
8	Advanced Synthesis Catalysis	2	2.353	5.83
9	Applied and Environmental Microbiology	2	2.353	4.57
10	Biotechnology Letters	2	2.353	2.46

*(%): percentage of 85 publications; **Impact factor in the year 2020

Microbiology" and "Chemistry". The common element of all the subject categories in those 10 top journals was "biotransformation", which reflected the close nexus between biotechnology and the microbiology field. All of these journals publish research in the English language.

Citation analysis was conducted to select the most frequently cited articles out of the WoS, which shows the number of times a specific article is cited in all journals in the database. The total citation count (TC) does not necessarily reflect the quality of the article but rather, it may indicate the scholarly impact thereof. The most cited article was "Biotransformation of limonene by bacteria, fungi, yeasts, and plants", which was published by Duetz in 2003 in Applied Microbiology and Biotechnology. This is also the first article that officially and systematically touched upon the concept of limonene biotransformation, especially about the regiospecificity of microbial biocatalysts. This article was cited 171 times during 2003–2021 with an average citation count of 8.55 times/year. Characteristics of publications including their subject categories provide useful information for the examination of bibliographic trends of journal publications, citations, and performances of most frequently cited journals and papers.

In the past 20 years, enzyme immobilization has attracted great interest among researchers worldwide (Fig. 3), a total of 284 authors had their articles published during the period from 2000 to 2021. Glaucia Maria Pastore was the most cited and influential Brazilian author, having published 13 papers and received 414 citations. The author is a researcher at the Bioflavors and Bioactive Compounds Laboratory, Department of Food Science, School of Food Engineering, University of Campinas, and leader of a research group focused on the

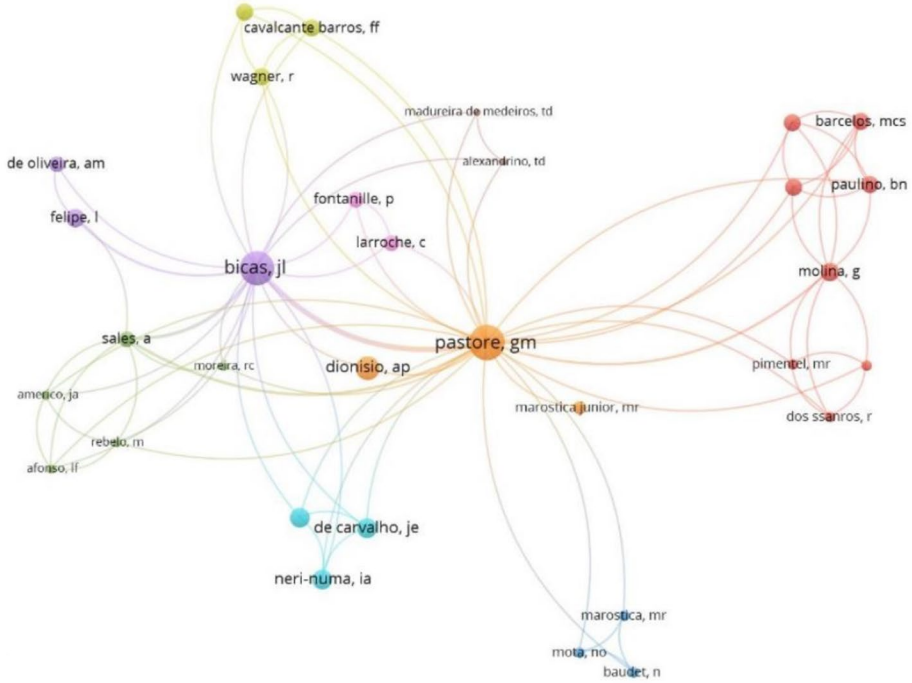

Fig. 3 Collaboration Networks between top authors according to co-authorship

microbial production of aroma esters by terpene biotransformation. Juliano Lemos Bicas, the second most cited author (11 papers and received 380 citations), is part of the same research group as Pastore. Both coauthored experimental studies and reviews on terpene biotransformation. Other noticeable names in this list were Fiedurek (8 articles), Trytek (8 articles), and Antunes (5 articles). Nevertheless, bias could arise as the names of two or more researchers were the same, or in some cases, authors adopt different names in their publications (for instance, due to a change of name after marriage).

Author-provided keywords illustrate information about research trends that capture researchers' ultimate concern. We have thus identified a list of 228 author keywords that have most constantly recurred in articles from 2000 to 2021. The most frequent searching term is "biotransformation" (56 times), followed by "bioconversion" (28), "limonene" (27), "alfa-terpineol" (20), "terpenes" (18), "alfa-pinene" (17) and "*R*-(+)-limonene" (16). Index keywords define the content of scholarly publications, but the word is chosen from pre-determined material known as a data structure used in bibliographic repositories. As shown in Fig. 4, 21 words clusters were found according to the cooccurrence keywords network, thus, index keywords detain the heart of an article's theme.

Biotransformation of enantiomers *R*-(+)-, and *S*-(-)-limonene

Limonene, 1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 22,311) is the most studied monoterpene and one the most abundant in nature, being found as the major constituent of several essential oils mainly citrus species. Limonene is a colorless liquid and it exists as two enantiomeric forms, named *R*-(+)- and *S*-(-)-limonene (Vieira et al. 2018).

The *R*-(+)-limonene, (4*R*)-1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 440,917), also known as d-limonene is the main compound in the essential oils of the peels of *Citrus* spp. and *R*-(+)-limonene is a cheap by-product of orange (Vieira et al. 2018). It is also abundant in some *Lippia* and *Artemisia* species (Aggarwal et al. 2001). The results of a study performed by Ibáñez et al. (2020) show that *R*-(+)-limonene is more than 90% of the terpenes in the composition of citrus essential oil.

Nevertheless, *S*-(–)-limonene, (4*S*)-1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 439,250), also known as l-limonene is mainly found in low to moderate

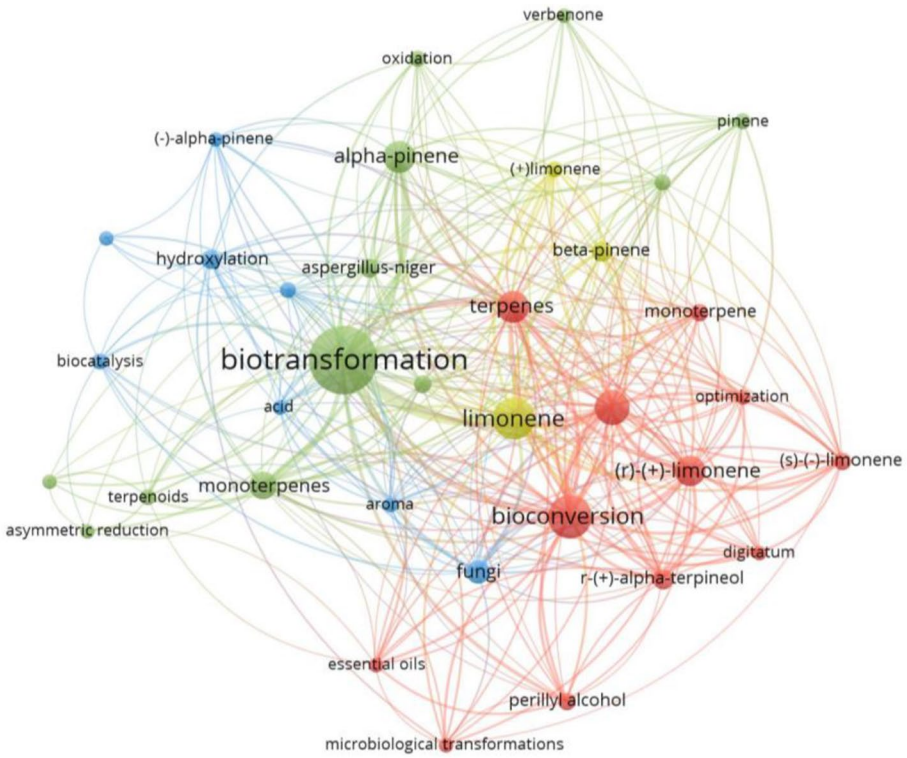

Fig. 4 The co-occurrence keywords network

concentrations (20–30%) in the essential oils of *Pinus* (e.g. pine needle oils) and *Mentha* (e.g. spearmint) species (Ren et al. 2020). Limonene also occurs as a racemic mixture known as dipentene (Aggarwal et al. 2001).

Limonene, as isolated isomers, or mixture is one of the most frequent and inexpensive fragrances used in cosmetics products including soaps, perfumes, shampoos, hair conditioners, as well as in foodstuffs, cleaning products, and eco-friendly pesticides (Sales et al. 2018b; Vieira et al. 2018). In addition, it is considered a safe food preservative and could be used as a green solvent for the extraction of natural products (Kumar et al. 2020).

The oxidation of limonene mediated by fungi strains can promote selective hydroxylation and enantioselective epoxidation reactions (Lerin et al. 2010). These structural modifications produce different acyclic, monocyclic, and bicyclic derivatives with interesting aroma characteristics (Maróstica and Pastore 2007; Duetz et al. 2003). Thus, many studies reported the application of novel fungi strains isolated from natural sources for selective biotransformation of limonene, and the next sections will be described the main finds in this context.

Oxidation in position 7 for production of perillyl derivatives

The hydroxylation reaction of natural compounds, including terpenes, has been reported as a strategy for the generation of bioactive analogs with improved bioactivity and solubility profiles (Janocha, Schmitz and Bernhardt 2015; Puentes-Cala et al. 2018; Souza et al. 2019). Specifically, the methyl allylic oxidation of *R*-(+)-limonene by fungal biocatalysts (hydroxylation at position 7) can produce an important aroma compound named perillyl alcohol, (4-prop-1-en-2-yl-cyclohexen-1-yl)methanol (PubChem CID:10,819) which can be successively oxidized to form perillyl aldehyde, 4-prop-1-en-2-ylcyclohexene-1-carbaldehyde (PubChem CID: 16,441), and perillyl acid, 4-prop-1-en-2-ylcyclohexene-1-carboxylate (PubChem CID: 9,543,109) (Ferrara et al. 2013; Carvalho et al. 2017).

Among these oxygenated monoterpenes, perillyl alcohol is recognized by its anticancer properties, being pointed as a potential alternative for the treatment of different tumors including glioblastoma, liver, breast, and lung cancer (Castellanos, Villamil and López 2007; Bicas, Fontanille and Larroche 2008a). In addition, this monoterpenoid is highly valued commercially, with average prices around US$300–600/kg (Duetz et al. 2003; Carvalho et al. 2017).

There are many examples of the production of perillyl derivatives through the biotransformation process employing limonene enantiomers as substrate and by fungi strains as biocatalysts (Cornelissen et al. 2013; Ferrara et al. 2013). In an experiment using *Mortierella minutissima*, Trytek and Fiedurek (2005) reported a recovery of over 120 mg/L of perillyl alcohol in 5 days at 15 °C and 150 rpm. The fungus produced mainly the perillyl derivatives, *R*-(+)-perillyl

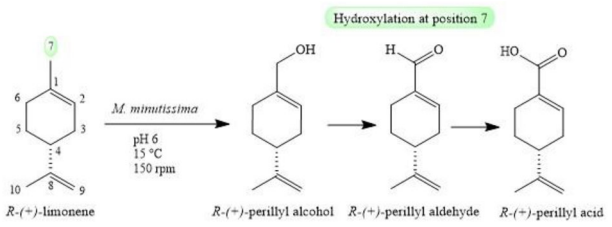

Fig. 5 Perillyl derivatives obtained by biotransformation of *R*-(+)-limonene by *M. minutissima* (Trytek and Fiedurek, 2005)

alcohol (83.5%), *R*-(+)-perillyl acid (13.4%), and *R*-(+)-perillyl aldehyde (2.7%), while other minor derivatives were verified in trace levels (Fig. 5).

Moreover, *M. minutissima* strain was used under new optimized conditions, in an integrated array of micro bioreactors (250 mL) for biotransformation of *R*-(+)-limonene in presence of hydrogen peroxide (H_2O_2). The best results were achieved using 0.5% (v/v) of *R*-(+)-limonene, 1% hydrogen peroxide, at 15 °C and 150 rpm after 48 h of cultivation. These conditions resulted in a twofold increase in productivity, being obtained 258.1 mg/L of perillyl alcohol (Trytek, Fiedurek and Skowronek 2009). The composition of the culture medium is considered another important variable in the production of perillyl alcohol. In this context, a study carried out with *Aspergillus niger* DSM 821 and *R*-(+)-limonene resulted in the production of different amounts of perillyl alcohol depending on the culture media applied after 72 h of the process (Castellanos, Villamil and López 2007). Using YMPG as culture media 14.7% of perillyl alcohol was produced, while using PDB and PDA media 14.6% and 4.9% of perillyl alcohol were achieved, respectively.

Another biotransformation process of *R*-(+)-limonene using an endophytic strain identified as *Penicillium* sp. starts from 840 mg/L of the substrate, being produced 39.9 mg/L of perillyl alcohol, together with a trace of perillyl aldehyde, *p*-menth-1-en-9-ol (2-(4-methylcyclohex-3-en-1-yl)propan-1-ol) (PubChem CID: 86,753), and *p*-mentha-1,3,8-triene (1-methyl-4-prop-1-en-2-ylcyclohexa-1,3-diene) (PubChem CID: 176,983) (Tai et al. 2016).

The conversion of *R*-(+)-limonene by a yeast *Yarrowia lipolytica* ATCC 18,942 was evaluated by varying the pH (3–8) and the temperature (25–30 °C) in a malt medium (YMB). The best results showed that 562 mg/L of perillyl acid was produced after 48 h in buffered medium supplemented with one-step addition of 0.5% v/v limonene at pH 7.1 at 25 ºC. The yield was improved to 855 mg/L by the addition of 0.5% limonene distributed in 6 dosages using the same conditions (Ferrara et al. 2013).

Using this same yeast an optimization study was carried out applying two statistical methods (factorial design and central composite design) to maximize the formation of perillyl acid using limonene and orange essential oil as substrates. The results showed that under optimized process conditions 0.368 g/L of perillyl acid was produced using 0.16% (v/v) of limonene at pH 6.9 after 24 h and subsequent addition of substrate under the same conditions resulted in 0.793 g/L of perillyl acid. Using orange essential oil under optimized conditions the production of 0.872 g/L was achieved. These studies corroborate with current knowledge about the robustness of the application of *Yarrowia lipolytica* in biotechnological processes for the generation of value-added chemicals of industrial interest as aromas compounds (Worland et al. 2020; Muhammad et al. 2020).

Epoxidation to the 1,2-double bond for production of limonene-1,2-diol

The biotechnological production of limonene-1,2-diol involves the action of two enzymes, the first is limonene 1,2-monooxygenase, which catalyzes the epoxidation of the limonene to generate limonene-1,2-epoxide through attack to the 1,2-double bond, then the limonene-1,2-epoxide hydrolase catalyzes the hydrolysis of limonene-1,2-epoxide to limonene-1,2-diol (Maróstica and Pastore 2007; Bicas, Fontanille and Larroche 2008a; Carvalho et al. 2017).

Limonene-1,2-diol, 1-methyl-4-prop-1-en-2-ylcyclohexane-1,2-diol (PubChem CID: 94,217) is a colorless to slightly yellowish oil with a fresh mint aroma. The conversion of limonene to limonene-1,2- diol may also be economically attractive due to its potential application in the food industry as an aroma additive. It also has insect-attractant properties, besides being used as a flavoring for beverages, chewing gum, gelatins, and puddings. To have an idea, this product is marketed at US$ 8500/kg (www.molbase.com), while the substrate costs US$ 34/L (Sales 2019). Limonene-1,2-diol has been associated with a significant inhibitory effect on the pro-inflammatory activities of $CD4^+$ and $CD8^+$ T lymphocytes; a potential anticancer activity (Molina et al. 2015; Sales et al. 2018a, b; Sales 2019).

The efficiency of the biotransformation process depends on the enantiomer of the compound employed as substrate and specificity and selectivity of the enzymes produced by biocatalyst. In this context, the potential use of limonene *R*-(+)- and *S*-(-)-limonene isomers for production of limonene-1,2-diol using *Fusarium oxysporum* 152B as biocatalyst was reported by Molina et al. (2015). The results showed that starting from 5 g/L of *S*-(-)-limonene the concentration of limonene-1,2-diol produced was close to 3.7 g/L after 72 h of reaction. However, limonene-1,2-diol was not detected in the biotransformation of *R*-(+)-limonene. Differently, 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 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.

Biotransformation of enantiomers *R*-(+)-, and *S*-(−)-limonene by *Colletotrichum nymphaeae* CBMAI 0864 and *C. acutatum* TQ058A showed that both enantiomers are converted into *R*-(+)- and *S*-(−)-limonene-1,2-diol under conditions at 30 ºC and 150 rpm after 192 h using 15 g/L of isomers limonene as substrates. *C. acutatum* accumulated up to 3 g/L of *R*-(+)-limonene-1,2-diol when *R*-(+)-limonene was used as substrate. A similar profile was observed when the substrate was the *S*-(-)-limonene. As for *C. nymphaeae*, higher amounts of isomers limonene-1,2-diol were produced: 4.01 and 4.06 g/L was accumulated for both *R*-(+)- and *S*-(−)-limonene, respectively. Traces of 1-methyl-4-prop-1-en-2-yl-7-oxabicyclo[4.1.0]heptane or limonene-1,2-epoxide (PubChem CID: 91,496) also was verified as minor product (Sales et al. 2018b).

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 (Fig. 6) (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

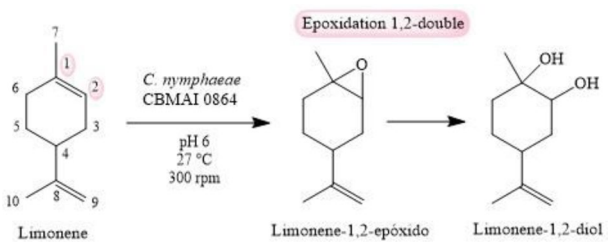

Fig. 6 Limonene-1,2-diol obtained by biotransformation of limonene by *C. nymphaeae* CBMAI 0864 (Sales et al. 2019a, b)

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 (Medeiros et al. 2021). The processes characteristics of recent screening studies for limonene-1,2-diol production by fungal biotransformation of limonene enantiomers were summarized in Table 3.

Dehydration in the isoprenyl unit for production of α-terpineol

The biotransformation processes using limonene as a substrate in the production of aroma compounds have been described and reviewed in the last few years and are considered an important approach in biotechnology. Microbial production of α-terpineol is also important and various authors have reported the epoxidation reaction of the double bond in the isoprenyl unit from limonene biotransformation (Maróstica and Pastore 2007; Tai et al. 2016). In such conditions, other products may be produced, such as terpin hydrate, but this compound may be easily converted into the desired product (α-terpineol) by partial dehydration (Bnina et al. 2020).

α-terpineol, 2-(4-methylcyclohex-3-en-1-yl)propan-2-ol (PubChem CID: 17,100), has an odor that is typical of lilac (*Syringa vulgaris*) and a sweet smell reminiscent of peach, with an aroma threshold of 280–350 ppb. Its annual consumption is estimated to be approximately 9.2 tons, which represents an individual intake of US$ 15,100/kg (www.molbase.com), while the substrate costs US$ 34/L (Tai et al. 2016; Molina et al. 2019). The traditional commercial uses of α-terpineol include household products and food technology, including baked goods, chewing gum, condiments, dairy products, candies, and beverages. Due to the in vitro antioxidant and anti-inflammatory activities that have already been associated with this alcohol, it is normally used in the formulation of soaps, cosmetics, and flavor preparations (Bicas et al. 2010; Sousa et al. 2020a, b; Sales et al. 2020).

In the work of Adams et al. (2003), the bioconversion of *R*-(+)- and *S*-(−)-limonene into α-terpineol was performed

Table 3 Process conditions for limonene-1,2-diol production through fungal biotransformation of limonene

Biocatalyst	System	pH	°C	Agitation	Substrate	Substrate concentration (g/L)	Biomass (g/L)	Product (g/L)	Maximum time (h)	References
F. oxysporum 152B	flasks	6.5	28	250	*S-(-)*-limonene	5.0	3.0	3.7	72	Molina et al. (2015)
Phomopsis sp.	flasks	n.e*	30	120	*R-(+)*-limonene	10.0	3.0	2.08	120	Bier et al. (2017)
Phomopsis sp.	flasks	n.e*	30	120	Essential oil	5.36	13.0	2.10	144	Bier et al. (2017)
C. nymphaeae	flasks	7.0	30	150	*R-(+)*-limonene	15.0	6.2	4.01	192	Sales et al. (2018b)
C. acutatum	flasks	7.0	30	150	*R-(+)*-limonene	15.0	6.2	3.0	192	Sales et al. (2018b)
C. nymphaeae	flasks	7.0	30	150	*S-(-)*-limonene	15.0	6.4	4.06	192	Sales et al. (2018b)
C. acutatum	flasks	7.0	30	150	*S-(-)*-limonene	15.0	6.4	3.08	192	Sales et al. (2018b)
A. alternata Eb03 (KY968699)	flasks	7.0	28	150	*R-(+)*-limonene	2.5	2.0	1.75	72	Cecati et al. (2018)
Neofusicoccum sp. EB04	flasks	7.0	28	150	*R-(+)*-limonene	2.5	2.0	2.23	72	Cecati et al. (2018)
C. nymphaeae CBMAI 0864	flasks	7.0	30	150	*R-(+)*-limonene	15.0	6.6	4.2	192	Sales et al. (2019a)
C. nymphaeae CBMAI 0864	bioreactor	6.0	27	300	*R-(+)*-limonene	20.0	13.2	7.1	72	Sales et al. (2019b)
C. nymphaeae CBMAI 0864	bioreactor	6.0	27	300	*S-(-)*-limonene	20.0	13.2	7.8	72	Sales et al. (2019b)
C. nymphaeae CBMAI 0864	bioreactor	6.0	27	300	Essential oil	20.0	13.2	5.6	72	Sales et al. (2019b)
C. nymphaeae CBMAI 0864	flasks	7.0	27	200	*R-(+)*-limonene	20.0	4.8	2.14	192	Medeiros et al. (2021)

*Not evaluated

Table 4 Process conditions for α-terpineol production through fungal biotransformation of limonene

Biocatalyst	System	pH	°C	Agitation	Substrate	Substrate concentration (g/L)	Biomass (g/L)	Product (g/L)	Maximum time (h)	References
P. digitatum ATCC 201,167	flasks	3.5	26	150	*R*-(+)-limonene	5.7	n.e*	0.9	8	Adams et al. (2003)
F. oxysporum 152b	flasks	5.2	30	270	*R*-(+)-limonene	2.1	3.75	2.4	72	Bicas et al. (2008b)
F. oxysporum 152b	flasks	6.7	30	200	*S*-(-)-limonene	15.0	3.75	2.0	48	Bicas et al. (2010)
Aspergillus sp. 05.01.35	flasks	n.e*	30	175	*R*-(+)-limonene	1.7	2.0	1.7	n.e*	Rottava et al. (2011)
P. digitatum DSM 62,840	flasks	3.5	27	150	*R*-(+)-limonene	2.0	n.e*	1.8	48	Prieto et al. (2011)
P. digitatum DSM 62,840	flasks	6.0	24	150	*R*-(+)-limonene	0.9	n.e*	0.8	12	Tai et al. (2016)
Phomopsis sp.	flasks	n.e*	30	120	*R*-(+)-limonene	10.0	3.0	0.2	120	Bier et al. (2017)
Phomopsis sp.	flasks	n.e*	30	120	Essential oil	5.0	3.0	0.3	144	Bier et al. (2017)

*Not evaluated

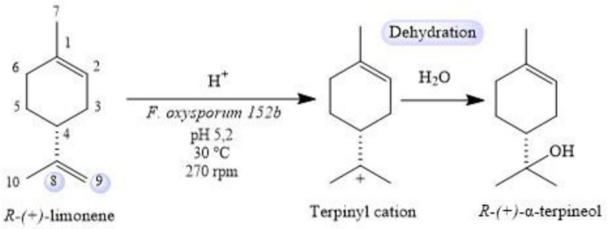

Fig. 7 *R*-(+)-α-terpineol obtained from biotransformation of *R*-(+)-limonene by *F. oxysporum* 152b (Bicas et al. 2010)

under optimized conditions using five *Penicillium digitatum* strains. The *P. digitatum* ATCC 201,167 proved to be an efficient biocatalyst, producing 0.93 g/L of α-terpineol from 5.7 g/L of *R*-(+)-limonene in a process carried at 26 °C, pH 3, and 150 rpm after 8 h. However, when this a strain of fungus was applied in biotransformation experiments using *S*-(−)-limonene as substrate, the capacity to produce α-terpineol was not observed.

Based on the use of variable screening (Plackett–Burman methodology), followed by a central composite experimental design, the optimization of the main parameters involved in the biotransformation was described. These techniques have been very useful for a full understanding of the process, and presented the best conditions for the breeding of *R*-(+)-α-terpineol, which were a 72 h-reaction in pure distilled water as the culture medium, a temperature between 24 and 28 °C, agitation of 200–310 rpm, 0.5% *R*-(+)-limonene concentration, and an inoculum/culture medium ratio of 0.25 (m/m). This is a the simple and low-cost process from which concentrations up to 2.4 g/L of the product was obtained using *Fusarium oxysporum* 152b (Fig. 7) (Bicas et al. 2008b).

An integrated biotechnological process for the production of various natural products, with a co-production process and optimized conditions using *F. oxysporum* 152b was evaluated for the breeding of *R*-(+)-α-terpineol and alkaline lipase. In this study, 15 g/L of *R*-(+)-limonene in a process carried at 30 ºC and 200 rpm for 72 h was verified that the production of alkaline lipase was close to 14 U/mL, while that 4 g/L of *R*-(+)-α-terpineol, demonstrating it to be one of the highest already described for a fungal process. This analogous study is interesting for enzyme-based industries that might use the results obtained to the benefit of their production practices. This resulted in the generation of two valuable bioproducts in parallel, and it was demonstrated that the biomass stored in frozen or lyophilized forms could be used for α-terpineol production, for which the reaction rate was significantly increased in the first case (Bicas et al. 2010).

Rottava et al. (2011) used a central composite design and response surface methodology to evaluate the process

parameters in the biotransformation of *R*-(+)-limonene into α-terpineol employing two unidentified yeast strains (coded as 03.03.03 and 05.01.35) isolated from citrus wastes. The production of α-terpineol by 03.03.03 and 05.01.35 strains ranged from 16 mg/L to 1.3 g/L and 2.5 mg/L to 1.74 g/L depending on the substrate concentration, substrate/ethanol ration, and inoculum mass used, respectively.

The biotransformation of *R*-(+)-limonene using *P. digitatum* (DSM 62,840) gave an α-terpineol concentration of 1.8 g/L after 48 h, and this production could be increased to 2 g/L of substrate, inoculum/media ratio of 0.25 (w/w), in pH 3.5, 27 °C, and 150 rpm (Prieto et al. 2011). Similarly, *P. digitatum* (DSM 62,840) was able to produce 0.8 g/L of α-terpineol when the pre-culture medium was in medium log-phase by adding 0.9 g/L of *R*-(+)-limonene dissolved in ethanol, and cultivation was performed at 24 °C, 150 rpm, and pH 6.0 after 12 h of incubation. The addition of small amounts of *R*-(+)-limonene (84 mg/L) at the start of the fungal log-phase growth yielded a 1.5-fold yield of α-terpineol, indicating that the enzyme was inducible (Tai et al. 2016).

Recently, a study investigated the production of α-terpineol from biotransformation of *R*-(+)-limonene and orange residue-based media by an endophytic fungi strain identified as *Phomopsis* sp. Results showed that α-terpineol concentrations followed a similar profile to that observed for both incubated 10 g/L of *R*-(+)-limonene, with an accumulated concentration of 0.2 g/L of α-terpineol that was obtained after 120 h of biotransformation. A high concentration of α-terpineol was obtained from biotransformation using 5 g/L orange-residue-based extracts, which reached 0.3 g/L after 144 h of biotransformation with no significant increase for longer periods (Bier, Medeiros and Soccol, 2017a). Table 4) summarizes the screening studies for α-terpineol production by fungal biotransformation of limonene processes.

Other oxidation of limonene

Different oxidations in different positions can result in the production of a variety of other aroma compounds, which can be used in the food, perfumery, cosmetic, pharmaceutical, and fuel industries (Maróstica and Pastore 2007). Two examples important limonene derivatives obtained by biotransformation are the 2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-ol or Carveol (PubChem CID: 7438), and 2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-one or Carvone (PubChem CID: 7439). Among other applications, these compounds can be used as food additives, antimicrobial/antifungal agents, and flavor pharmaceutical products (Bouyahya et al. 2021).

Carveol, and carvone are monoterpene ketone responsible for the typical odor of spearmint and are used aroma additives in baked goods, chewing gum, frozen dairy products, gelatins, puddings, beverages, and candies, in a concentration close to 220 ppm. Its annual consumption is around 1.2 tons, representing an individual intake of US$ 529/L (www.molbase.com) while the substrate costs US$ 34/L (Sousa et al. 2020a, b).

Trytek and Fiedurek (2005) reported the biotransformation of *R*-(+)-limonene using psychrotrophic fungus *M. minutissima.* This study described the hydroxylation at position 6 of *R*-(+)-limonene resulted in the production of 4.3% of carvone and trace amounts of carveol (0.18%), and 0.05% of limonene 1,2-epoxide after 120 h in process carried at 30 °C employing 0.8% (v/m) of *R*-(+)-limonene as substrate (Fig. 8a).

Subsequently, this study followed a central composite design that was described to optimize the main parameters involved in the biotransformation using *P. digitatum* (DSM 62,840). For this process, the experimental condition used was pH levels of 4.5 and 6.0, a temperature of 27 °C, and rotation at 150 rpm, which represented the best conditions of monitored oxidation of C1, and C6 of *R*-(+)-limonene for the formation of the oxygenated derivatives, such as 27.8 -28.6 mg/L (*R*)-(–)-linalool or (3*R*)-3,7-dimethylocta-1,6-dien-3-ol (PubChem CID: 91,496), isomers *cis* and *trans*-carveol (20.1—66.9 mg/L), *cis* and *trans-p*-menth-2,8-dien-1-ol (19.5—42.9 mg/L), and carvone (23.0—27.3 mg/L). These conditions implicated an intermediate (limonene-8,9-epoxide), which is formed via epoxidation for an attack at the 8, 9-double bond catalyzed by cytochrome *P*-450-dependent monooxygenase (Fig. 8b) (Prieto et al. 2011).

The allylic oxidation at C3, C6, and C9 of *R*-(+)-limonene was reported in a biotransformation process carried using *F. oxysporum* CICC41029 at 24 °C, 150 rpm, and pH 6.0 (Fig. 8c) (Tai et al. 2016). The results showed that 19.53 mg/L of 2-methyl-5-prop-1-en-2-ylcyclohexan-1-one (PubChem CID: 24,473), 46.6 mg/L of carvone were produced after 72 h, while 8.74 mg/L of 3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-one (PubChem CID: 79,036), 3 mg/L of 3-methyl-6-propan-2-ylidenecyclohex-2-en-1-one (PubChem CID: 381,152), 6.25 mg/L of *p*-menth-1-en-9-al, and 3.35 mg/L of carveol were produced after 48 h process.

Another bioconversion process was based on the use of the *Phomopsis* sp. strain for the biotransformation of *R*-(+)-limonene and presented some compounds of interest, such as trace amounts of *trans-p*-mentha-2,8-dien-1ol, *cis-p*-mentha-2,8-dien-1ol, menthol, 1,6-dihydrocarveol, *cis* and *trans*-carveol, and (*R*)-(−)-carvone as its principal compound, which reached a maximum concentration of 536 mg/L. Biotransformation was performed at 30 °C, 120 rpm for 120 h (Fig. 8d). However, the chemical diversification of orange-residue-based media produced only minor quantities of carvone, obtaining only 12.56 mg/L after 144 h (Bier, Medeiros, and Soccol, 2017a).

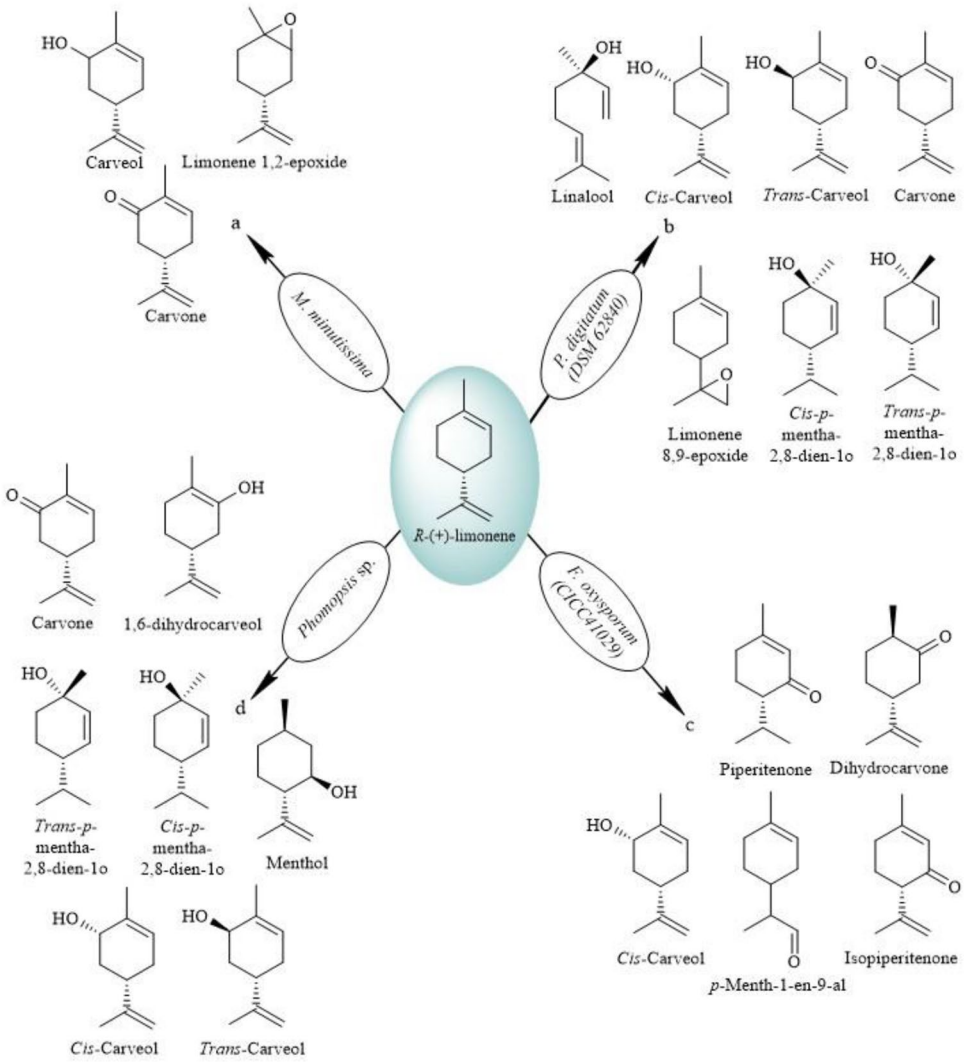

Fig. 8 Different derivatives obtained from biotransformation of *R*-(+)-limonene by different fungi (**a**—Trytek and Fiedurek 2005; **b**—Prieto et al. 2011; **c**—Tai et al. 2016; **d**—Bier et al. 2017)

Biotransformation of pinene isomers

The reason for this is that monoterpenes are abundantly found in industrial wastes—for example, pinene is a bicyclic monoterpene and can be found in two isomeric forms, α-pinene, and β-pinene. The monoterpene α–pinene or 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (PubChem CID: 6654) is a colorless liquid that is soluble in ethanol and oils, and insoluble in water, with a boiling point of 155 °C. This compound exhibit citrus, spicy, woody pine, and turpentine-like aroma and is marketed at US$ 66.00/kg (www.molbase.com). The gustative the threshold is 10 ppm, a threshold of detection is 2.5–62 ppb and it presents an intense, woody, piney taste with a notable camphor-like and turpentine taste (Vespermann et al. 2017; Xu et al. 2019; Kenseth et al. 2020).

Similarly, β-pinene or 6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptane (PubChem CID: 14,896) is a colorless liquid soluble in oils and insoluble in water and ethanol, with a boiling point between 163 and 166 °C. In addition, this monoterpene is marketed at US$ 66.00/kg (www.molbase.com), and exhibit aroma characteristics such as cooling, woody, piney, and turpentine, with traces of fresh mint, eucalyptus, and camphor (Vespermann et al. 2017; Xu et al. 2019; Kenseth et al. 2020). These bicyclic monoterpenes are abundantly found in industrial products as turpentine produced through the distillation of resins of certain pine trees and essential oils obtained from conifers and other plants (Salehi et al. 2019).

The different levels of pinenes in turpentine may vary according to the botanical species, but in general, the amount of α-pinene is always higher than that of β-pinene in the studied oils. This secondary product is rich in pinenes, and due to the low cost has been considered as a promising substrate for biotechnological purposes, including the aroma production of cosmetic, and food interest (Molina et al. 2014; Paulino et al. 2020).

Therefore, 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 (Sales et al. 2018a). Thus, different approaches have been successfully implemented at laboratory scale such as the use of biphasic systems, which provide a better dissolution of a substrate and increase the permeability of the cells to organic solvents to facilitate the diffusion of the product to the extracellular media (Vespermann et al. 2017; Kutyła et al. 2020).

Fungal biotransformation β-pinene for the production of aroma compounds

Screening studies can be carried out for the selection of wild fungal strains with the potential to produce α-terpineol from β-pinene through biotransformation. In this context, a screening study carried out with 400 microorganisms showed that some stains showed interesting potential for bioconversion of β-pinene (Rottava et al., 2010). Among them, *A. Niger* ATCC 16,404 was able to produce around 2.8 g/L of α-terpineol and traces of pinocamphone or (1*S*,2*R*,5*R*)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-one (PubChem CID: 6,427,105), fenchol or 1,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (PubChem CID: 15,406), *trans*-pinocarveol or (1*S*,3*R*,5*S*)-6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptan-3-ol (PubChem CID: 88,302), borneol or (1*S*,2*R*,4*S*)-1,7,7-trimethylbicyclo [2.2.1]heptan-2-ol (PubChem CID: 1,201,518), and cycle-hexanemethanol or (1-methyl-4-propan-2-ylcyclohexyl)methanol (PubChem CID: 44,511,635) at 25 °C and 150 rpm after 72 h of process Fig. 9a. Applying different experimental conditions (35 °C and 150 rpm without addition of a vitamin solution) the concentration of α-terpineol produced was close to 15.5 g/L after 6 days. Furthermore, as shown in Fig. 9a. The fungal *A. Niger* ATCC 9642, and *A. Niger* ATCC 1004 were able to biotransform β-pinene to α-terpineol reaching the concentrations up to 688.13 mg/L and 172.07 mg/L after 192 h, respectively Fig. 9a (Rottava et al. 2010).

In addition, the potential of *β*-pinene as a substrate for α-terpineol production by *Aspergillus* sp. strains (coded as 04.05.08 and 01.04.03) using the central composite design was evaluated. The results showed that high amounts were achieved using a substrate the concentration of 1.75%, 2 g of inoculum, and substrate/ethanol ratio of 1:1, where 761 mg/L and 763 mg/L of α-terpineol using produced using *Aspergillus* sp. (04.05.08), and (01.04.03), respectively (Rottava et al. 2011) (Fig. 9b).

Another approach for improving the biotransformation capacity of microorganisms consists in the use of genetic engineering methods and classical mutagenesis to obtain highly efficient strains able to produce or convert specific substrates to value-added compounds, including monoterpene-derived aromas (Schewe et al. 2009; Kallscheuer et al. 2019; Kutyła et al. 2020). Recently, the induced mutagenesis applying UV irradiation and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG), as well as the exposure of microbial cells of the psychrotrophic fungus *C. Pannorum*

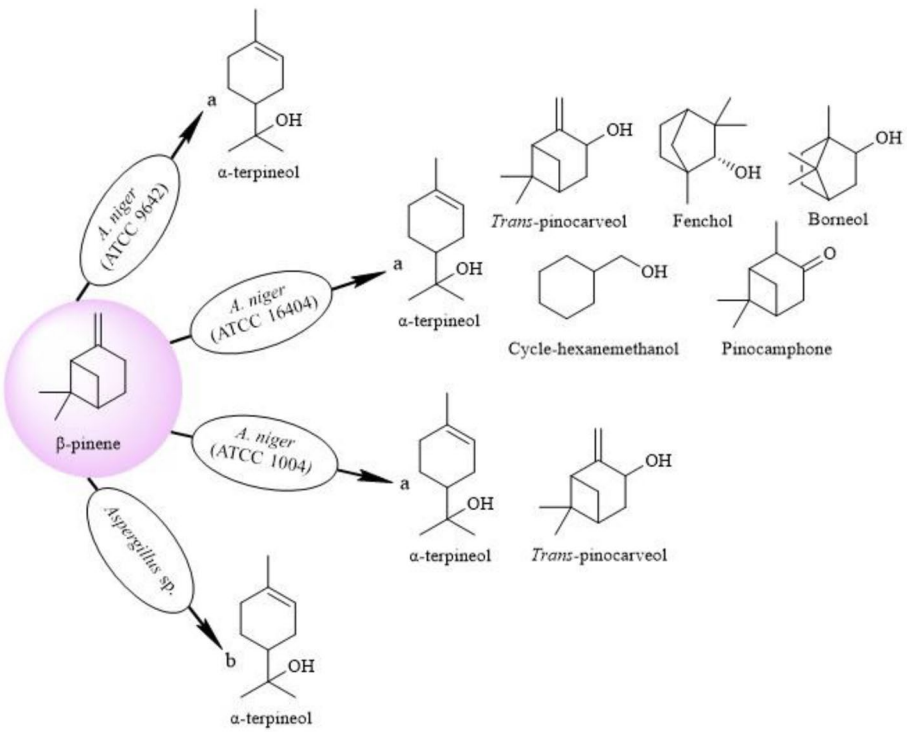

Fig. 9 Different derivatives obtained by biotransformation of β-pinene by different *Aspergillus* strains (**a**—Rottava et al. 2010; **b**—Rottava et al. 2011)

A-1 against high concentrations of substrate (β-pinene), was used to select mutant strains with improving on the efficiency for biotransformation of β-pinene to *trans*-pinocarveol (Kutyła et al. 2020).

A total of 137 mutant strains were obtained from mutagenesis experiments, being twelve of them selected due to their better biotransformation capacity. The results showed that mutant strains adapted to 1% β-pinene produced higher yields of the *trans*-pinocarveol (on average 1.7-fold higher) when compared with non-adapted mutant strains. Moreover, the maximum *trans*-pinocarveol concentrations achieved in this study ranged from 147.2 to 314.7 mg/g d.w. × L showing that the use of classical mutagenesis strategies and the adaptation of microbial cells by exposure of high concentration of substrate for production and selection of novel mutant strains with better biocatalytic characteristics can be considered an interesting approach for application in biotransformation processes aiming the production of aroma compounds.

Fungal biotransformation α-pinene for the production of aroma compounds

Other pathways that lead to the formation of oxygenated derivatives occur through the biotransformation of α-pinene. Several oxygenated bioproducts were obtained from a comparative study of the biotransformation of α-pinene isomers by cultures of *Fusarium solani* (Eb01) and *Neofusicoccum* sp. (Eb04). In Fig. 10a, the potential biotransformations of α-(−)-pinene and α-(+)-pinene by culture of *F. solani* were obtained several oxygenated monoterpenes such as the concentrations 33%, and 50.3% of terpinen-4-ol or 4-methyl-1-propan-2-ylcyclohex-3-en-1-ol (PubChem CID: 11,230); 3%–2.4% of α-terpineol; 1.1%–0.5% of *cis*-sabinene hydrate or [(2*R*,5*S*)-2-methyl-5-propan-2-yl-2-bicyclo[3.1.0]hexanyl]acetate (PubChem CID: 6,427,493) respectively, while, the biotransformations of α-(−)-pinene and α-(+)-pinene to 39.7%–34.6% of limone-1,2-diol, 7.6%–8.7 of borneol, 2.4%–6.0% of *p*-menth-1-em-7-al, and 3.1%-0.6% of fenchol respectively, were performed using the strain *Neofusicoccum* sp. (EB04) visualized in Fig. 10-g. These compounds

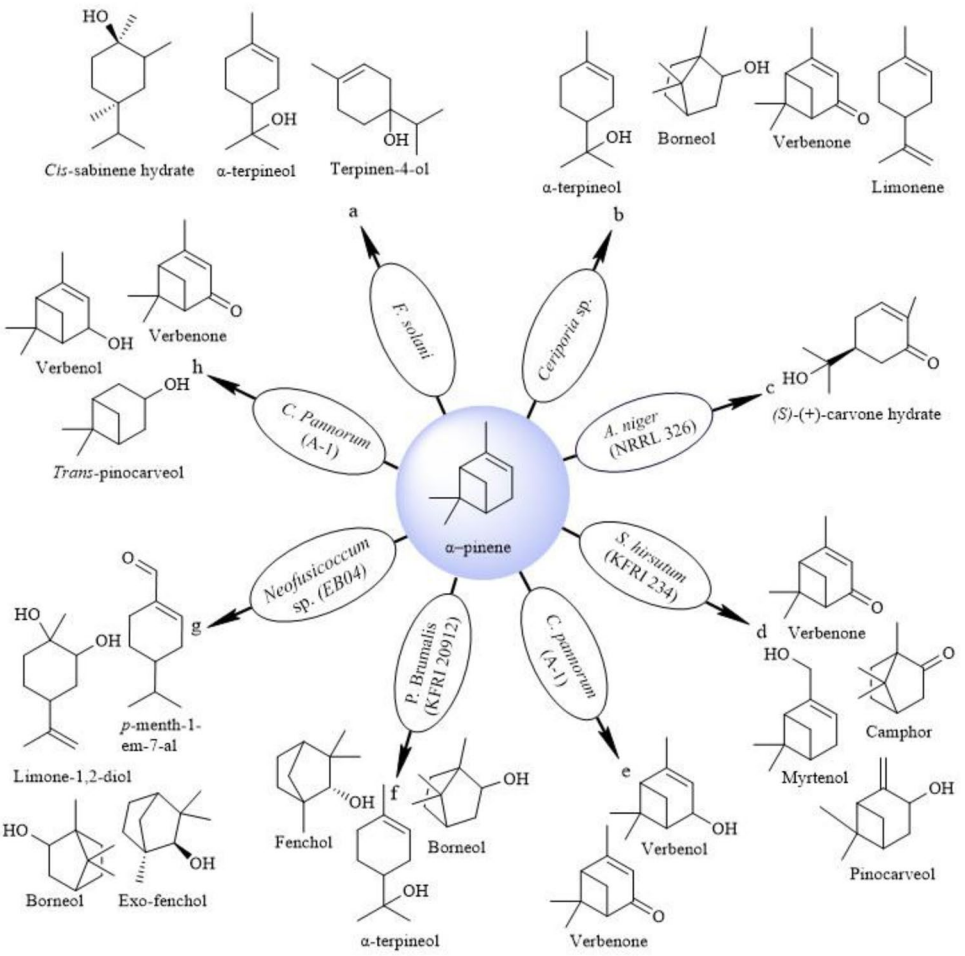

Fig. 10 Different derivatives obtained by biotransformation of α-pinene by different fungi (**a**—Cecati et al. 2018; **b**—Lee et al. 2015a; **c**– Çorbacı, 2020; **d**—Lee et al. 2015a; **e**—Trytek et al. 2015; **f**—Lee et al. 2015b; **g**—Cecati et al. 2018; **h**—Trytek et al. 2016)

were identified after 72 h of incubation, characterized using GC–MS, and quantified using GC-FID (Cecati et al. 2018).

As shown in Fig. 10b, the main oxygenated monoterpenes obtained by biotransformation of α-pinene using *Ceriporia* sp were α-terpineol (30.38%) as the majority compound, at a concentration of 0.05 g/L, with minor products, such as fenchol (17.78%), borneol (7.19%), limonene (3.90%), and verbenone (3.03%) also identified. Another study, which used the strain *Stereum hirsutum* (KFRI 234), suggested that the biotransformation of α-pinene has to undergo a dehydrogenation reaction in order to form verbenone (27.64%) and myrtenol or (1*R*,5*S*)-6,6-dimethyl-2-bicyclo[3.1.1]hept-2-enyl]methanol (PubChem CID: 88,301) (17.75%), with minor products, such as the 8.49% of camphor or 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (PubChem CID: 2537), and 3.10% of pinocarveol or 6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptan-3-ol (PubChem CID: 102,667) after 96 h (Fig. 10d) (Lee et al. 2015a).

The fungus *Polyporus brumalis* (KFRI 20,912) initiates the metabolism of α-pinene through the oxidation of the double bond at carbons 8–9, forming an epoxide and the hydration. This reaction is catalyzed by *P. brumalis* and results in the formation of α-terpineol (35.85% − 39.05%), with minor products, such as borneol (8.59%) and fenchol (5.54%), also identified, after five days of reaction (Fig. 10f) (Lee et al. 2015a, b).

Cultures of the psychrotrophic fungus *Chrysosporium pannorum* (A-1) show promise for allylic hydroxylation in the C-3-position of α-pinene. The metabolites obtained from this process were verbenol or 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol (PubChem CID: 61,126), and verbenone or 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one (PubChem CID: 29,025) (Fig. 10e). The highest concentrations of verbenol, about 722 mg/L, were reached using 1.5% (v/v) of the substrate and 72-hold mycelium, whereas the best result for about 176 mg/L of verbenone was obtained using 1% (v/v) of the substrate, and 48-hold mycelium. The sequential addition of substrate proved to be a very efficient strategy to increase the yield because it was threefold higher than that obtained with a single addition of substrate after 72 h and reaching a concentration of 1.3 g/L when verbenol and verbenone were added together (Trytek et al. 2015).

Trytek et al. (2016) studied the effect of cell induction with the monoterpene substrate (1% v/v) and the use of dioxane, chloroform, and ethanol as the co-solvent in the process of bioconversion of α-pinene to verbenol, verbenone, and *trans*-pinocarveol (1*R*,3*S*,5*R*)-6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptan-3-ol (PubChem CID: 88,302) (Fig. 10h) using the psychrotrophic *C. pannorum*. The best results were obtained with cells that were not precultivated with the substrate (non-inducted), in the presence of ethanol, and with the gradual addition of substrate. Accordingly, it was proposed that the addition of substrate should be gradual to minimize the eventual toxicity and subsequent inhibition of biocatalyst cells, and thereby, facilitate the biotransformation of α-pinene.

In a recent study, the biotransformation of α-pinene by cell cultures of *A. niger* (NRRL 326) resulted in the formation of mainly 4.28% of (*S*)-(+)-carvone hydrate or (5*S*)-5-(2-hydroxypropan-2-yl)-2-methylcyclohex-2-en-1-one (PubChem CID: 134,688,181), according to the GC–MS analysis, after 4 days, when all the α-pinene had been consumed (Fig. 10c) (Çorbacı 2020). Many authors have proposed schematic representations of the main metabolic pathways of interest that produce these respective derivatives and these are shown in Fig. 10c.

Because of the wide range of products of commercial interest that can be produced from α- and β-pinene, studies on the biotransformation of these compounds have been stimulated for decades. From a technological point of view, the biotransformation of α- and β-pinene has already shown great advances in recent years, although there are still challenges to be overcome by the aroma and fragrance industries (Vespermann et al. 2017).

Biological activities of aroma compounds derived from limonene and pinene biotransformation

Many monoterpenoids that can be produced through fungal biotransformation of limonene and α- and β-pinenes are recognized as potential bioactive compounds since that several biological activities have been reported by the use of in vitro, and in vivo studies (Silva et al. 2021; Pina et al. 2021; Ni et al. 2021). However, only some studies assess the biological activities of aroma products from biotechnological processes, being that the most available data come from specific studies focused on the pharmacology of these compounds. In this section, the main biological potential of monoterpenoids derived from limonene and pinenes will be presented, comprising mainly studies of compounds produced by biotechnological processes.

The results of a study performed by Junior et al. (2009) show that limonene biotransformation extract had free radical-scavenging activity (EC_{50} = 2.09) and inhibited lipid peroxidation (EC_{50} = 0.13%). The extract, perillyl alcohol, and α-terpineol inhibited lipid peroxidation by ~ 80% at a concentration of 0.02% (v/v). Perillyl alcohol, and α-terpineol also reduced the release of superoxide anions by cultured leukemic cells, by 3- and tenfold, respectively, at concentrations of < 0.02% (v/v). The biotransformation extract inhibited the conversion of nitrophenyl acetate top-nitrophenol in the glutathione assay by ~ 50%. Bicas et al. (2011) reported the antioxidant potential of carvone, perillyl alcohol, and α-terpineol using the DPPH· and ORAC antioxidant assays.

In addition, the evaluation of the antiproliferative capacity of this alcohol against nine cancerous cell lines was performed and compared to limonene and doxorubicin. The results of this study showed that all the samples tested had very low antioxidant activity in the DPPH· assay, but α-terpineol (2.72 µmol TE/µmol) could be compared to commercial antioxidants in the ORAC assay. The antiproliferative results obtained encourage future in vivo studies for α-terpineol, since this monoterpenoid presented cytostatic effect against six cell lines, especially for breast adenocarcinoma and chronic myeloid leukemia with effect concentration ranging from 181–588 µM.

Recently, the evaluation of the antioxidant capacity of the product from *R*-(+)-limonene the biotransformation was performed by DPPH·, ORAC, and CUPRAC methods (Bier et al. 2019). According to DPPH· results, limonene biotransformation extract presented 20.17% antioxidant activity compared to 12.1% from the orange waste extract, being that this trend also was observed using ORAC and CUPRAC methods.

Recently, the effect of supplementation of limonene-1,2-diol, *R*-(+)- and *S*-(−)-α-terpineol produced through biotransformation of limonene isomers were evaluated in animal models of obesity and TNBS-induced colitis (Sousa et al. 2020a, b; Alexandrino et al. 2020). These studies showed that *R*-(+)- and (−)-α-terpineol enantiomers exhibited anti-inflammatory activities, being that in diet-induced obese rats the diet supplemented with (−)-α-terpineol resulted in a more pronounced effect on the levels of TNF-α than those with R-(+)-α-terpineol. Likewise, anti-inflammatory effects were observed in rats with TNBS-induced colitis using diets supplemented with oxygenated limonene derivatives from biotransformation processes, being that *S*-(−)-α-terpineol, and limonene-1,2-diol were able to increase the gene expression of the anti-inflammatory cytokine IL-10 and reduce the TNF-α levels.

Limitations of biotransformation processes for the production of monoterpene-based aromas

In biotechnological processes, including biotransformation procedures, product recovery is considered an important and expensive step. In the context of the production of aromas from the biotransformation of monoterpenes, the recovery steps should be further studied because of the volatility and low solubility of substrates and products. Thus, the recovery processes of aromas produced by biotechnological processes are challenging and demand the development or improvement of new technologies to ensure the recovery and purification of these compounds. Nowadays, several methods for aroma extraction from the fermentation media have been used technologies based in absorption on resins, extraction in two-phase systems, membrane permeation, and pervaporation (a combination of membrane permeation and evaporation) (Hadj Saadoun et al. 2021; Zhuk et al. 2021). Therefore, the development of efficient and inexpensive extraction methods has aroused the interest of researchers and industries in recent decades, making this process decisive for the industrial application of a biotransformation process (Lukin et al. 2018).

Another challenge is related to the toxicity of monoterpene substrates that in many cases can inhibit the fungal strains used as biocatalysts and limit the maximum concentration of substrate used in the bioprocesses. 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 (Lukin et al. 2018; Sharma et al. 2020). Alternatively, procedures for strain adaptation or genetic engineering tools maybe used to increase the substrate tolerance by 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 (Hadj Saadoun et al. 2021). Moreover, the use of statistical tools to optimize the media composition as well as the process parameters is another alternative that can make the processes for the formation of monoterpene-based aroma viable for scale-up. Finally, it should be highlighted that the use of alternative substrates rich in limonene and pinene isomers (e.g. orange essential oil, and turpentine) can also, be considered for cost reduction in the aroma production process.

Concluding remarks

This review summarized the main findings concerning the fungal biotransformation of limonene, and pinene isomers. The use of these substrates can be considered an eco-friendly and cost-effective alternative and the employing of fungal biocatalysts showed several advantages when compared to bacteria strains due to high selectivity, resistance to substrates, and other abiotic stress as temperature and pH. The most important fungi species applied to limonene and pinene biotransformation including traditional species belonging to *Fusarium, Aspergillus,* and *Penicillium* genus as well as species of unconventional fungi as *Colletotrichum, Chrysosporium, Ceriporia, Polyporus,* and others, which led to the production of a wide range of oxygenated derivatives with interesting aroma characteristics. Screening studies also are considered an important approach for the selection of new fungi strains that can be exhibit better biocatalytic potential.

Moreover, further studies must be carried out to improve the recovery of aroma compounds from biotransformation processes that use limonene and pinene isomers. This step is crucial for the application of these products as industrial additives or as bioactive agents in several fields including food, cosmetics, biofuel, and pharmaceutical. Therefore, the use of fungi in biotechnological processes have always been important to produce diferente compounds and, in the context of the biotransformation of limonene and pinenes, they are versatile and promising microorganisms that provide numerously innovative possibilities.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

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

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CAPÍTULO II

Limonene biotransformation mediated by filamentous fungi from the Brazilian Amazon[1]

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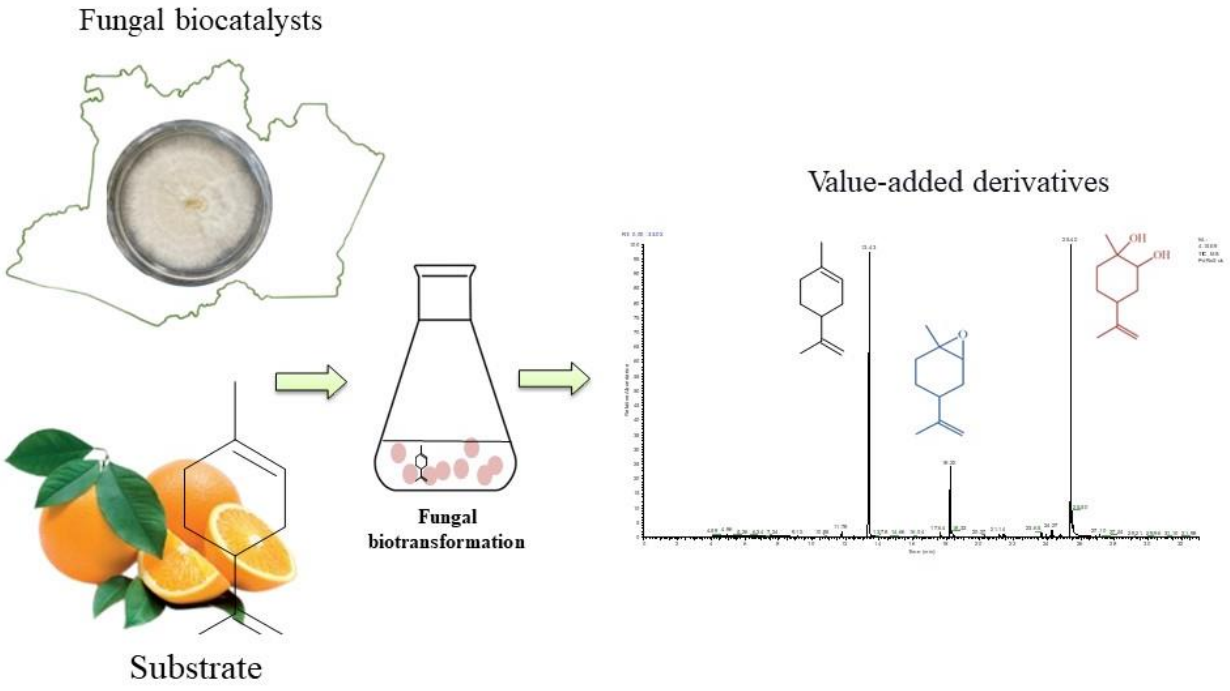
Highlights

- Bioconversion of limonene into limonene-1,2-diol.
- First report on biotransformation using these ascomycetes and basidiomycetes.
- *Pestalotiopsis mangiferae* LabMicrA-505 was the most promising endophytic fungus found in the screening.
- Biotransformations mediated by endophytic fungi pave the way towards green chemistry.

Graphical abstract



Abstract

The goal of the study was to evaluate the ability of filamentous fungi from the Brazilian Amazon to biotransform *R*-(+)-limonene. The fungal biotransformation was screened using forty-seven fungi: thirty-one ascomycetes of the genera *Penicillium*, *Trichoderma*, *Pestalotiopsis*, and sixteen basidiomycetes (Agaricomycetes, Basidiomycota), which belong to the working collection of the

Laboratory of Bioassays and Microorganisms of the Amazon at the Federal University of Amazonas. The biotransformation assays were executed in flasks containing the substrate *R*-(+)-limonene in mineral medium for 120 h. Samples of each culture were taken every 24 h, extracted with ethyl acetate, analyzed using gas chromatography–mass spectrometry and then compared with the National Institute of Standards and Technology database. In this study, nine endophytic fungi, two macrofungi, and one aquatic fungus were considered potential candidates for limonene biotransformation, since they eventually accumulated interesting compounds after 120 h of reaction. However, only *Pestalotiopsis mangiferae* LabMicrA-505 was the most promising endophytic fungi found in the screening, due to its capacity to use all the *R*-(+)-limonene as the single carbon and energy source in a mineral medium, with the main oxygenated derivatives being limonene-1,2-diol (74.13±0.81%) and limonene-1,2-epoxide (1.88±0.08%), which were accumulated after 72 h of reaction. These achievements are important, and support the development of the production of natural aromas and also demonstrate the potential of using these wild Amazonian fungi as new biocatalysts.

Keywords: Bioaroma; Limonene oxidation; Biotechnological process; New biocatalysts; Amazon.

Introduction

In recent years, many studies have changed the methods for aroma production that involve chemical processes, such as direct extraction from plants and classical chemical synthesis by biotechnological transformations via microbial and enzymatic biotransformations. When compared with chemical synthesis and direct extraction from nature, biotransformation is attractive and can be used to identify pathways that are useful for the production of biotechnologically relevant compounds (Paulino et al., 2021). These new approaches allow them to compete with and, in many cases, overcome classical synthetic methods (Otles & Özyurt, 2021).

The fact that most of the industrial chemical reactions involve the release of a large mass of waste products turns biotransformation into a rational choice from the ecological point of view when planning a particular process (Çorbacı, 2020). In the literature, the word “biotransformation” can be defined as the use of biological systems to catalyze chemical changes in substances that do not constitute their common precursor (substrate) (Braga, Guerreiro & Belo, 2018).

Among the most targeted substrates that have the potential to be used for the biotransformation approach is limonene, since it may lead to the accumulation of intermediate products with high added value (Bier et al., 2011; Sales et al., 2018). Thus, biotransformation of limonene may be regarded as a biotechnological process aligned with pillars of sustainable development and economics (Luna-García et al., 2021). Usually, the reactions in this process are highly selective and can be considered eco-friendly and sustainable due to the low generation of residues (Sharma et al., 2020).

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 the most used monoterpene for this purpose, considering that it can be found in abundance in several essential oils and in some industrial by-products, such as those derived from the citrus industry (De Medeiros et al., 2021).

Aroma compounds are prepared for several types of markets worldwide, mainly to be used as additives in food, beverages, cosmetics, and pharmaceutical products (Sales, Felipe & Bicas, 2020). Nowadays, it is necessary to find new ways to obtain the natural aroma, due to the increasing awareness of the consumer market regarding the link between diet and health. As such, there is a tendency to substitute synthetic additives for natural ones (Luna-García et al., 2021).

The fungal diversity of Brazilian biomes has been explored by different researchers in the search for new biocatalysts, including the discovery of strains with unique properties that can be used in biotechnological applications (Birolli, Lima & Porto, 2019). The biodiversity of the Amazon rainforest is attributed to the high variability of niches within it, making Brazil one of the countries with the highest index of macro and microbiological species. A large number of micro-ecosystems, soil types, and climate conditions are favorable for fungal communities, and provide constant degradation of forest biomass, which makes the Amazon rainforest a substantial source for bioprospection of fungal-originating biocatalysts (Bononi et al., 2021).

Thus, Aroma compounds appeal to many sectors and represent a market value that is 10 to 30 times higher than the initial substrate. In light of the above, there is a significant economic opportunity to be explored in the addition of value to these commodities (de Souza Sevalho et al., 2023). One of the major drawbacks in the biotransformation process is finding an appropriate biocatalyst to be used. Thus, our work sought to bioprospect fungi strains (endophytic, aquatic, and

macrofungi) from the Amazon rainforest with the ability to promote the biotransformation of limonene.

Materials and 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).

Microorganisms

The microorganisms used in this study belong to the working 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). The screening was performed using thirty-one ascomycetes of the genera *Penicillium*, *Trichoderma*, and *Pestalotiopsis*, in addition to sixteen basidiomycetes (Agaricomycetes, Basidiomycota) that had been isolated from different sources found in the Amazon region, Brazil. 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. Information on all the species identified in this study can be found in Table 1.

Table 1. Species studied in this research and their respective origins and molecular identification.

Nº	**ID in the working collection**	**Species**	**Origin of isolates**	**Genbank accession number *ITS***
		Penicillium		
1	LabMicrA-38	*P. glabrum*	*Annona* sp.	JN180489.1
2	LabMicrA-46	*P. adametzii*	*Annona* sp.	KF313079.1
3	LabMicrA-52	*P. paxilli*	*Annona* sp.	JN617709.1
4	LabMicrA-143	*P. oxalicum*	*V. amazonica*	MT588795.1
5	LabMicrA-135	*P. chrysogenum*	*M. flexuosa*	LC325162.1
6	LabMicrA-180	*P. adametzii*	*Strychnos* sp.	JN714932.1
7	LabMicrA-307	*Penicilium* sp.	*P. peltata*	JN851050.1
8	LabMicrA-401	*P. rubens*	*G. elliptica*	MN604092.1
9	LabMicrA-407	*P. oxalicum*	*G. elliptica*	MF186029.1
10	LabMicrA-409	*P. citrinum*	*G. elliptica*	MT597828.1
11	LabMicrA-433	*P. rubens*	*G. elliptica*	KU216703.1
		Pestalotiopsis		
12	LabMicrA-01	*P. clavispora*	*G. augusta*	KM877471.1
13	LabMicrA-474	*P. neglecta*	*E. oleracea*	GU595050.1
14	LabMicrA-448	*P. microspora*	*G. augusta*	KM438014.1
15	LabMicrA-06	*P. mangiferae*	*G. augusta*	JX857168.1
16	LabMicrA-562	*P. clavispora*	*E. oleracea*	EU342211.1
17	LabMicrA-505	*P. mangiferae*	Basidiomycete	KM998724.1
18	LabMicrA-05	*P. microspora*	*G. augusta*	DQ456865.1
19	LabMicrA-506	*P. formicarum*	Basidiomycete	KM199358.1
20	LabMicrA-516	*P. disseminata*	Basidiomycete	KP714294.1
21	LabMicrA-762	*P. clavispora*	*M. guianensis*	KJ590132.1
		Trichoderma		
22	LabMicrA-28	*T. lentiforme*	*Rollinia sp.*	MN262484
23	LabMicrA-269	*T. harzianum*	Unidentified	MN262487

24	LabMicrA-70	*T. afroharzianum*	*V. amazonica*	MN262508
25	LabMicrA-37	*T. koningiopsis*	*M. paniculata*	MN262513
26	LabMicrA-145	*T. asperelloides*	*V. amazonica*	MN262514
27	LabMicrA-140	*T. asperellum*	*H. courbaril*	MN262502
28	LabMicrA-219	*T. atroviride*	Unidentified	MN262492
29	LabMicrA-1136	*T. reesei*	River	MN262488
30	LabMicrA-263	*T. spirale*	*S. micranthum*	MN283156
31	LabMicrA-869	*Clonostachys rosea**	River	MN262501
		Agaricomycetes		
32	LabMicrA-1039 D107	*Sebipora aquosa*	Basidiome	**
33	LabMicrA-1028	*Daedaleopsis flavida*	Basidiome	**
34	LabMicrA-1040	*Fomitopsis nivosa*	Basidiome	**
35	LabMicrA-1010	*Pycnoporus sanguineus*	Basidiome	**
36	LabMicrA-1041	*Flavodon flavus*	Basidiome	**
37	LabMicrA-1032	*Lenzites elegans*	Basidiome	**
38	LabMicrA-1027	*L. elegans*	Basidiome	**
39	LabMicrA-1031	*Ganoderma tornatum*	Basidiome	**
40	LabMicrA-1036	*G. orbiforme*	Basidiome	**
41	LabMicrA-1047	*Coriolopsis polyzona*	Basidiome	**
42	LabMicrA-1046	*C. polyzona*	Basidiome	**
43	LabMicrA-1048	*C. rigida*	Basidiome	**
44	LabMicrA-1049	*P.tephropora*	Basidiome	**
45	LabMicrA-1044	*P. centrali-africana*	Basidiome	**
46	LabMicrA-1038	*Trametes elegans*	Basidiome	**
47	LabMicrA-1050	*T. elegans*	Basidiome	**

* Genus *Clonostachys* (Hypocreales: Bionectriaceae); **Not deposited in GenBank

Inoculum preparation

Each isolate was first inoculated in Petri dishes containing a semi-solid culture medium of potato dextrose agar supplemented with yeast extract (PDA + Y, 200 g/L fresh potato, 20 g/L dextrose, 15 g/L agar, and 2 g/L yeast extract) under sterile conditions. Three fragments of the mycelium of the fungi (tri-point inoculation) were sown at equidistant points and cultivated at 24 ^{o}C (*Pestalotiopsis* species), and 28 ^{o}C (other fungi) 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 24 ^{o}C (*Pestalotiopsis* species), and 28 ^{o}C (other fungi) in a rotary shaker at 120 rpm for 72 h (Souza et al., 2004; De Oliveira et al., 2021). After incubation, the humid biomass was recovered by centrifuging at 4.400 rpm, and 28 ^{o}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. (2015) and Bier et al. (2017) 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 K2HPO_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 the substrate *R*-(+)-limonene to be tested, the flasks were incubated at 24 ^{o}C (*Pestalotiopsis* species), and 28 °C (other fungi) 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.

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 substrate consumption and its derived products was determined on the basis of the chromatographic peak areas using the Xcalibur software (version 2.2) of the own GCMS system. 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

In the screening carried out in this study with forty-seven ascomycetes and basidiomycetes from the Amazon rainforest, only twelve species could biotransform the substrate using *R*-(+)-limonene as the single carbon source, which served as an indicator of biocatalytic activity and its potential for producing an aroma compound. Nine endophytic fungi, two basidiomycetes, and one aquatic fungus were the best adapted microorganisms (Figure 1). The other fungi were incapable of using the substrate and oxygenated derivatives accumulation was not detected (Figure S2).

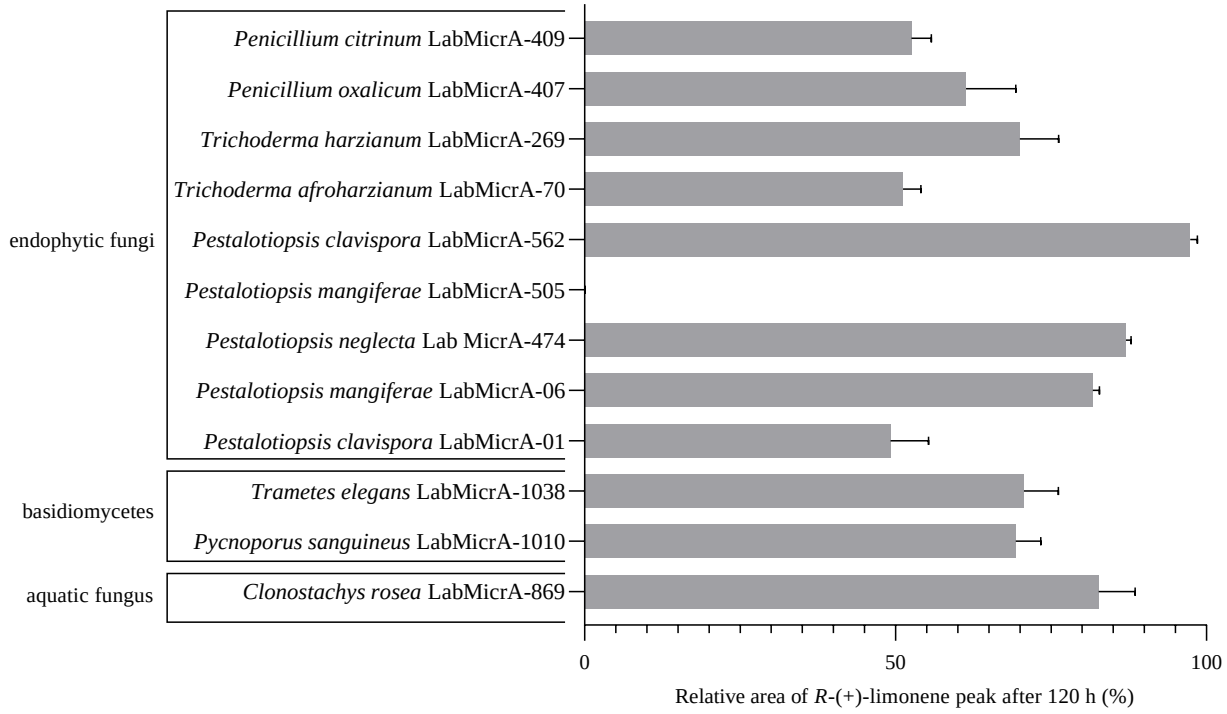


Figure 1. Results obtained after biotransformation process using *R*-(+)-limonene as substrate and fungi strains as biocatalysts after 120 h of reaction.

In the biotransformation, a precursor is added to the process, and the biological systems are induced to follow a specific pathway which allow us to achieve the final product in one chemical reaction. The biological systems are well-known for being efficient and selective biocatalysts (Pessôa et al., 2019). In this regard, reactions catalyzed by fungi transcend areas of chemistry and microbiology due to the fact that biocatalysts are selective, easy to handle and environmentally friendly (green chemistry principles) (De Melo Pereira et al., 2019). Fungal biotransformation has the potential of being more environmentally benign than chemical synthesis and more cost-effective when compared to isolated enzyme catalysis. Among all the existing biological systems, the use of fungi has traditionally been used in bioprocesses (Choudhary et al., 2021).

Many studies have focused on the selection of new fungal species that can be directly applied in limonene biotransformation, since one of the major drawbacks of this process is being able to find an appropriate biocatalyst to be used (De Carvalho, 2016). Generally, in screening assays, the first experiments aim to identify the most adequate and robust microorganisms, since both substrate

(single source of carbon), and new products may cause inhibitory effects on cell growth (Pessôa et al., 2019; Liu et al., 2021).

The biotransformation processes using endophytic fungi promote selective hydroxylation reactions and enantioselective catalytic epoxidation of the carbon chain. These catalytic modifications to the functional groups of limonene produce different metabolic pathways that determine the diversity of acyclic, monocyclic and bicyclic aroma compounds (Choudhary et al., 2021).

In this study, two *Pestalotiopsis*, one *Penicillium* and one *Trichoderma* species were considered potential candidates for limonene biotransformation, since they eventually accumulated interesting compounds after 120 h of reaction (Figure 2). However, only *P. mangiferae* LabMicrA-505 (Figure S1) was the most promising endophytic fungi found in the screening, due to its capacity to use all the *R*-(+)-limonene as the single carbon and energy source in a mineral medium after 96 h of reaction.

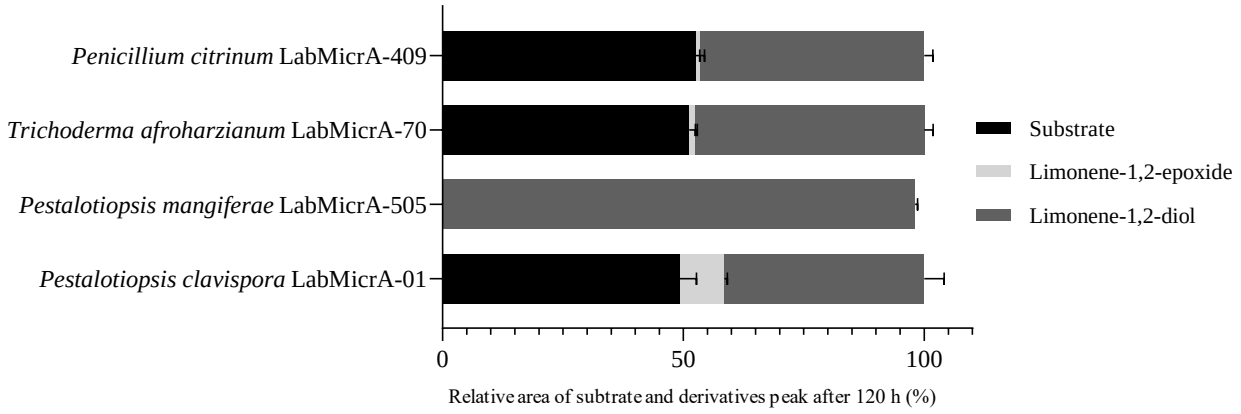


Figure 2. Bioconversion of substrate into aroma compounds via fungal biotransformation after 120 h of reaction.

The volatile compounds accumulated in the ethyl acetate fraction via limonene biotransformation using *P. mangiferae* LabMicrA-505 after 72 h (Table 2), it demonstrates the accumulation of the limonene-1,2-epoxide (1.88±0.08%) and limonene-1,2-diol (74.13±0.81). These derivatives were not detected in the controls conducted using only the microorganism or the addition of the substrate (Figure S3).

Table 2. Composition of ethyl acetate fraction obtained via limonene biotransformation using *P. mangiferae* LabMicrA-505 after 72 h of reaction.

RT	Compounds	Relative amount (%) [a]	RSI (%)
13.38	*R*-(+)-limonene	22.29±0.71	94.8
18.22	Limonene-1,2-epoxide	1.88±0.08	95.0
23.65	Ascaridole	0.81±0.07	84.6
24.27	Nona-3,5-dien-2-ol	0.90±0.12	76.6
25.45	Limonene-1,2-diol	74.13±0.81	92.7

RT - retention time; RSI - Reverse Search Index; [a]Percentage of peak area in the CG-MS analysis.

Interestingly, it was noticed that the oxygenated derivatives were obtained from the ring 1,2-double bond epoxidation of limonene, followed by the limonene-1,2-epoxide formation, resulting in the formation of limonene-1,2-diol. In this case, Sales et al. (2018) found traces of limonene-1,2-epoxide as a by-product of the biotransformation of limonene with *Colletotrichum nymphaea* and *C. acutatum.* The authors support the hypothesis that both strains present a pathway for converting limonene to limonene-1,2-diol via limonene-1,2-epoxide. Figure 3 illustrates the proposed pathway of bioconversion, representing the consumption of substrate and product formation.

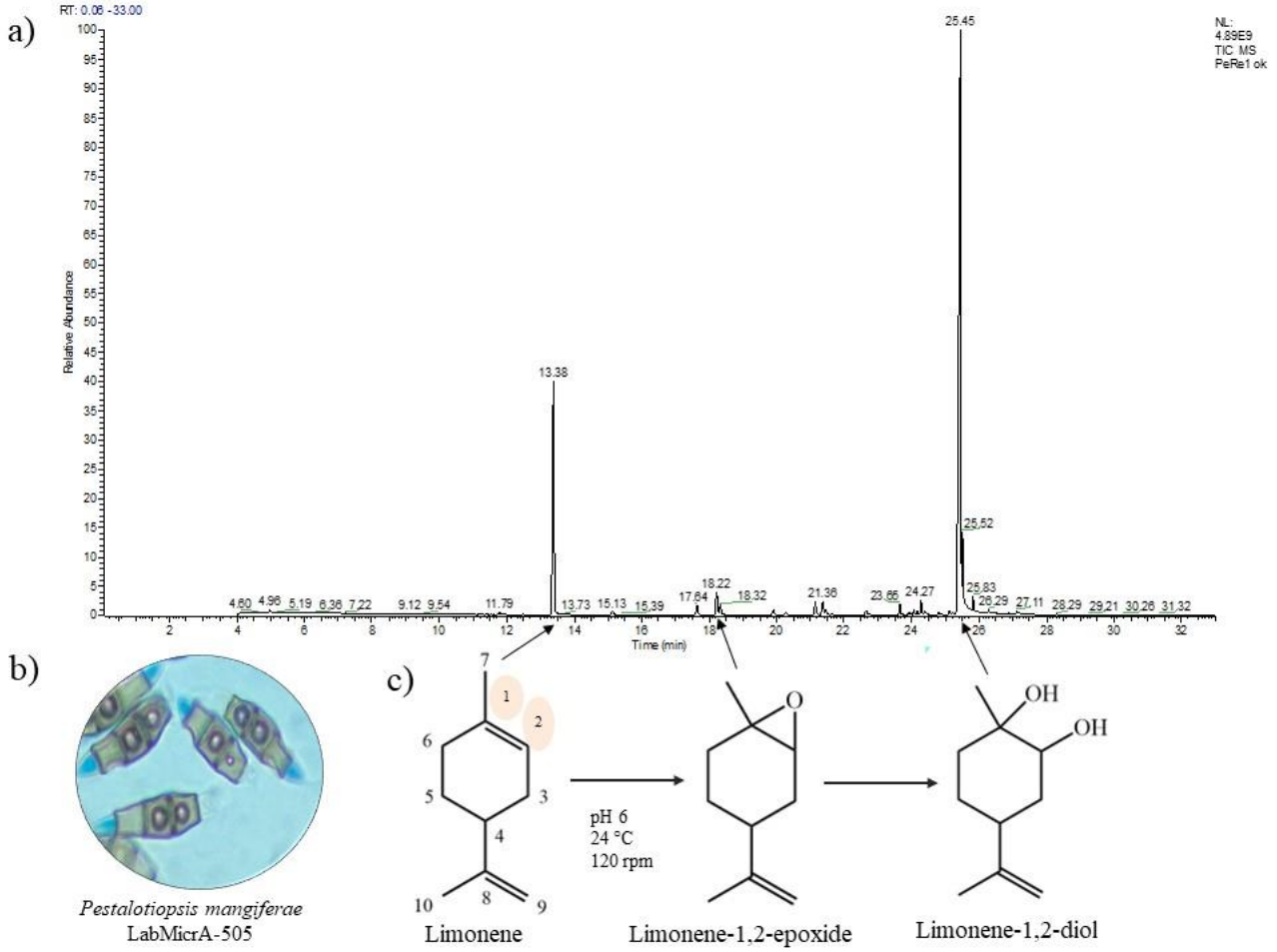


Figure 3. a) GC-MS chromatogram; b) micro-morphological image of *Pestalotiopsis mangiferae* LabMicrA-505; c) proposed pathway of limonene biotransformation into limonene 1,2-diol after 72 h of reaction.

The biotechnological production of limonene-1,2-diol involves the action of two important enzymes, in *Colletotrichum nymphaeae*, such a pathway starts with an attack on the 1,2 double bond of limonene by FAD-binding monooxygenase (Acc No F0X7A8), which oxidizes limonene to form limonene-1,2-epoxide. Subsequently, a very active limonene-1,2-epoxide hydrolase (Acc No F0X7A7) catalyzes the hydrolysis of limonene-1,2-epoxide to limonene-1,2-diol (Sales et al., 2019).

To evaluate the kinetics (total peak area) of limonene-1,2-diol production, we performed a controlled biotransformation process using substrate consumption by *P. mangiferae* LabMicrA-505. The results (Figure 4) show that the increase in the percentage of biocatalysts resulted in

enhanced conversion, obtaining nearly 74.13±0.81% of the limonene-1,2-diol product in 72 h of reaction. Even under non-optimized conditions, this amount is considerably higher and near the maximal concentration recently described for the biotransformation of *R*-(+)-limonene into limonene-1,2-diol by *C. nymphaeae* (Medeiros et al., 2021).

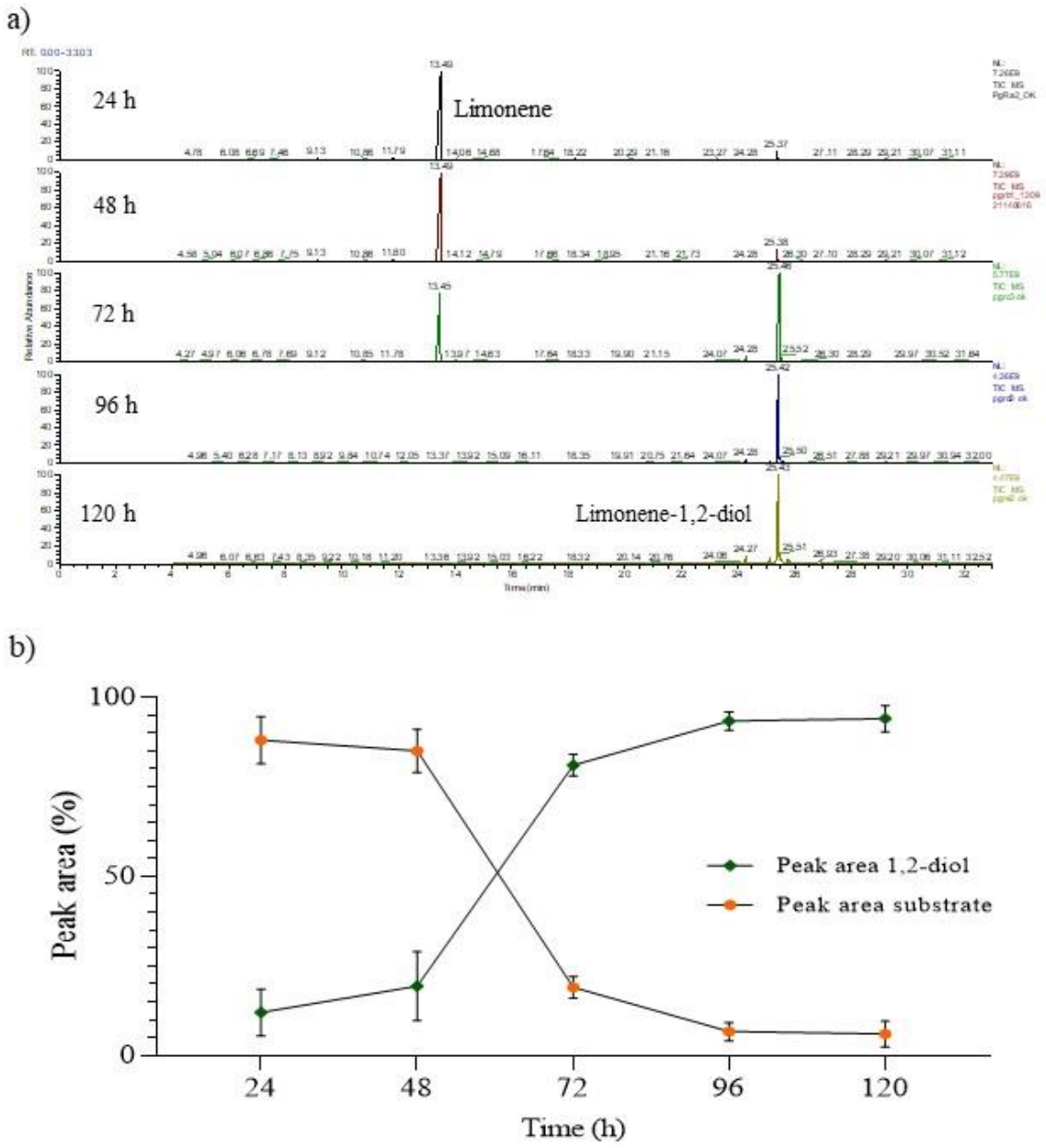


Figure 4. a) GC-MS chromatographic profile b) The biocatalytic reaction of limonene biotransformation to produce limonene-1,2-diol (%) over time using *P. mangiferae* LabMicrA-505.

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

The compounds obtained, mainly the limonene-1,2-epoxide, and limonene 1,2-diol are of great industrial interest and can be used as additives in food and cosmetics due to their potential biological activity. In this perspective, it is interesting to employ 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 the 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 have a market value that is 16 times greater than *R*-(+)-limonene; thus, it is considered that its biotransformation may be a good choice for increasing its added value. This importance can be estimated by its commercial value, using the Molbase database (https://www.molbase.com/) to illustrate this, the reference price of limonene-1,2-diol is around US$ 8,500/kg, while *R*-(+)-limonene has reference price of US$ 34/kg.

In the database of Merck KGaA Brazil (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 R$ 79.10/kg. In this context, it would be a good strategy to invest efforts and resources to better understand *R*-(+)-limonene biotransformation.

Fungal biotransformation is a relevant strategy to obtain high added-value natural compounds under controlled environmentally friendly conditions. Endophytic fungi offer great potential for producing several groups of compounds; however, few studies have evaluated limonene biotransformation (De Souza Sevalho et al., 2022). Furthermore, the use of whole cells of endophytic fungi in biotransformation processes is an emerging field of biotechnology that yields new modified compounds that may be labeled as “natural”, are relatively low cost, and can be considered more economically viable (Liu et al., 2021).

Only *P. mangiferae* LabMicrA-505 was important in this study due to its biotransforming potential, which showed an accumulation of intermediate metabolites in levels that justify further optimization studies. As far as we know, there is no limonene biotransformation study described in the literature using this genus of fungi. Biotransformation processes with all the selected microorganisms were investigated in our laboratory in order to select the aroma-producing strains. Therefore, experiments are underway to evaluate the possible pathways through which these microorganisms degrade the limonene.

Fungi complement the capacity for biotransformation by utilizing various organic compounds such as carbon sources, which enable them to play an important role in the degradation of structural components such as wood, lignin components, leaf litter, and also environmental components such as agro-industrial waste, contamination by heavy metals (Liu et al., 2021). In addition to the importance of the secondary metabolism of fungi, the enzymatic potential of these microorganisms has also attracted the attention of a number of research groups. These fungi have a wide capacity for the production of several enzymes since they are capable of degrading several organic substances in the environment (Santos & Silva, 2019).

Conclusion

This investigation was focused more on the qualitative study of the products obtained than on absolute gains and to select biocatalysts with potential for application in future, more detailed, and larger-scale works. The screening carried out in this study provided twelve endophytic and aquatic fungi, and macrofungi as potential new biocatalysts. However, the highlight of this study was the first biotransformation assay of *R*-(+)-limonene to limonene-1,2-diol using *P. mangiferae* LabMicrA-505, an endophytic fungal isolated from Basidiomycota species collected from Amazon Rainforest fragments in Amazonas state, Brazil. These achievements are important and support the development of the production of natural aromas and demonstrate the potential of using these wild Amazonian fungi in biotechnological processes.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Credit authorship contribution statement

ESS: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft and Writing – review & editing; EFB, FASF, MDPF, and KRPF: Data curation, Writing - review & editing; BNP: Methodology; AQLD: Funding acquisition, Methodology, Supervision and Writing – original draft; ADLS: Funding acquisition, Methodology, Supervision and Writing – review & editing. This study is part of the doctoral thesis of ESS (PPG-BIONORTE-UFAM).

Ethical statement

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

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Supplementary Information

Macro- and micro morphological identification of the fungus *P. mangiferae* LabMicrA-505 505 (most promising endophytic fungi found in the screening), which showed a peculiar growth with a cotton-like mycelium, white in color, sometimes yellowish around the edges. In the center of the plate, a black coloring was displayed due to the many acervuli produced, which is a typical structure for the genus (Figure S1). The genus *Pestalotiopsis* belongs to the Amphisphaeriaceae (Coelomycetes) family, which has been extensively isolated from healthy plant tissues and has been considered the main part of endophytic fungi in the past decade.

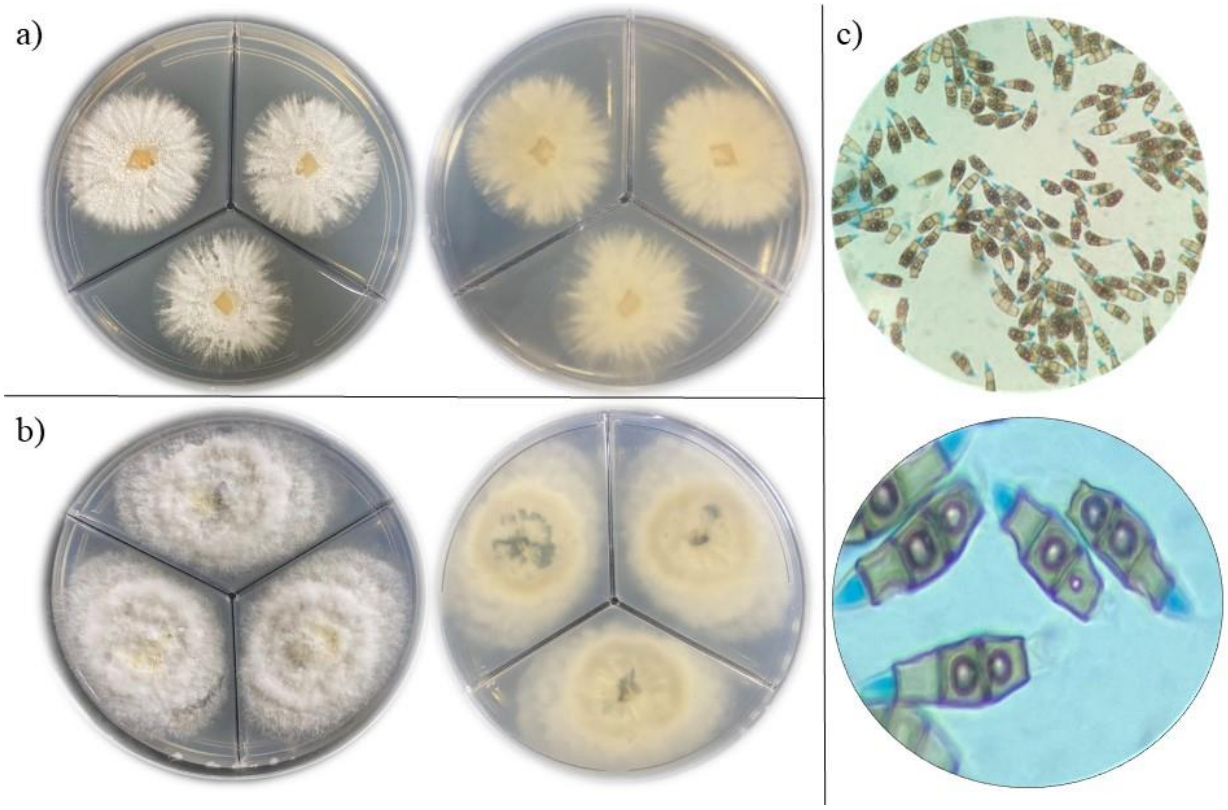

Figure S1. Colony morphology of *P. mangiferae* LabMicrA-505 at 24° C in the PDA + Y semi-solid culture medium: a) macro-morphological image (top and reverse) after 3 days of incubation; b) macro-morphological image (top and reverse) after 20 days of incubation; c) micro-morphological image: conidia set and individual conidia.

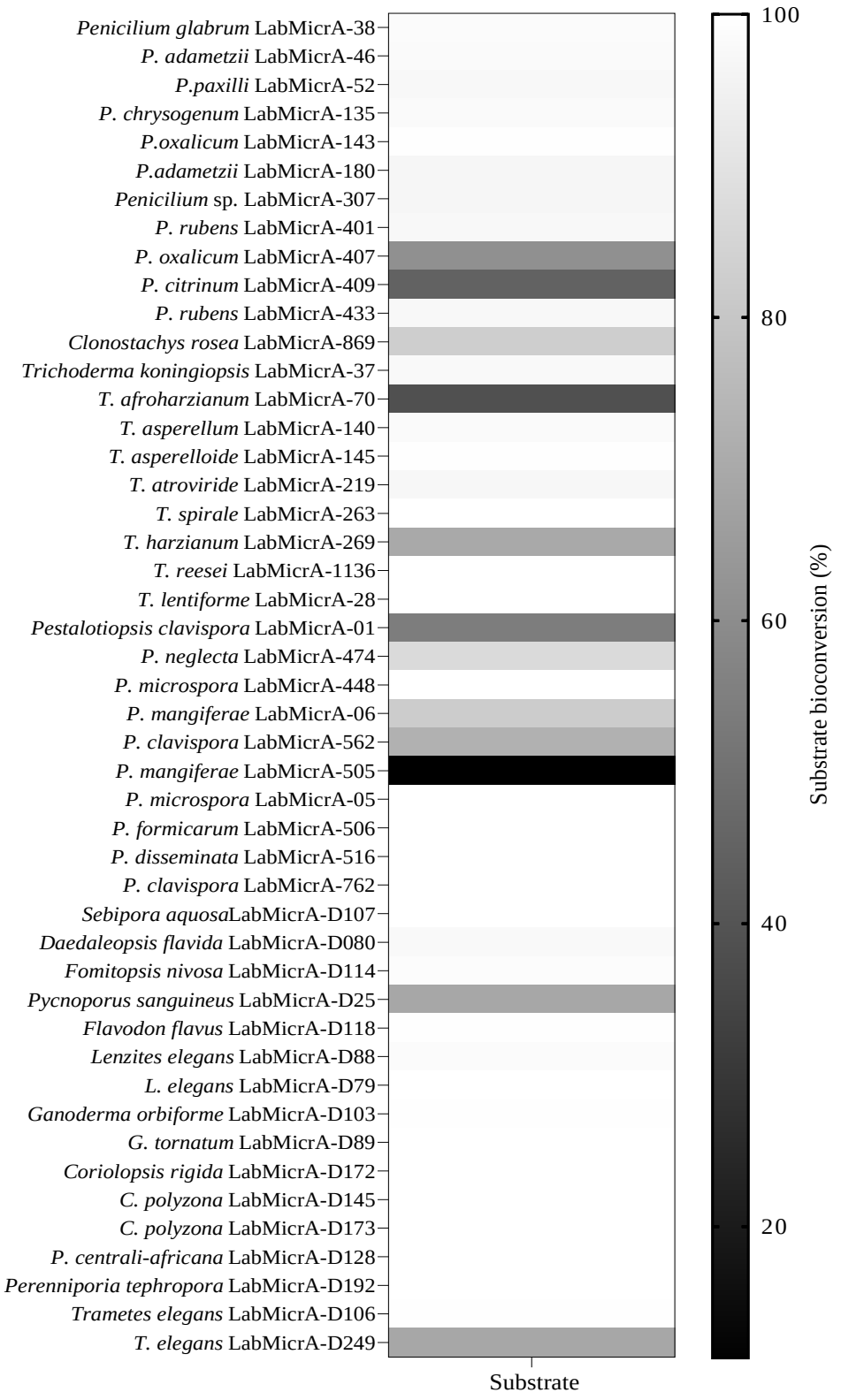

Figure S2. Heatmap analyses of the substrate consumption by the twenty-seven macro-micro fungi.

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. (Figure S3).

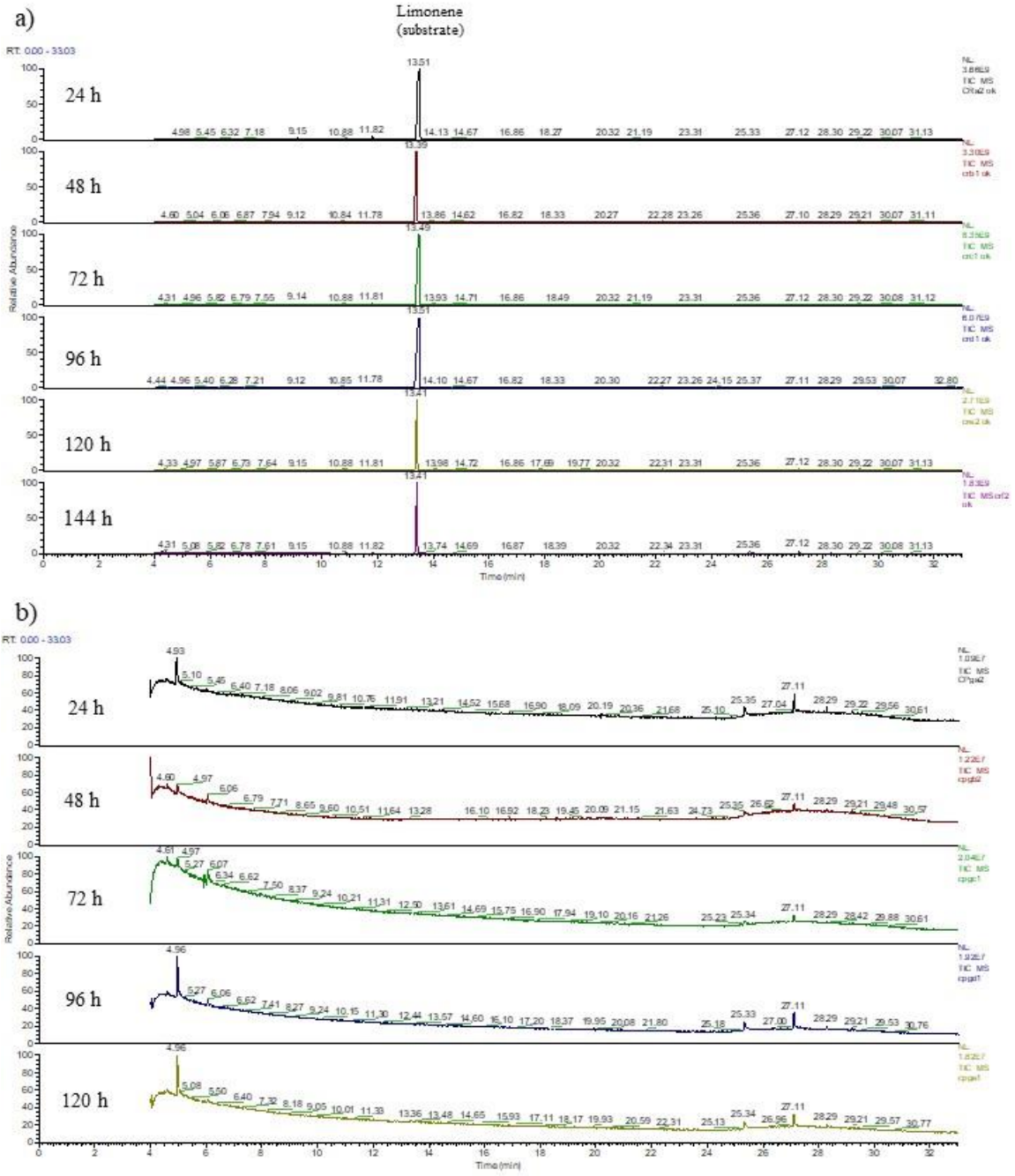

Figure S3. GC-MS chromatogram of controls a) conducted using only the substrate; b) conducted using only *P. mangiferae* LabMicrA-505.

CAPÍTULO III

Monoterpene biotransformation using actinomycetes of the genus *Streptomyces* isolated from the rhizosphere of inga-cipó (*Inga edulis* Martius) [1]

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Abstract

The aim of the study was to investigate the biocatalytic potential of actinomycetes of the genus *Streptomyces* for monoterpene biotransformation. The process of monoterpenes biotransformation was screened using eleven strains of ascomycetes that belong to the working collection of the Laboratory of Bioassays and Microorganisms of the Amazon at the Analytical Center - Center of Multidisciplinary Support at the Federal University of Amazonas. The biotransformation assays were executed in flasks containing the substrates (*R*-(+)- limonene, *S*-(-)-limonene, (+)–α-pinene, and (-)–β–pinene) in a mineral medium for 96 h. Samples were taken from each culture every 24 h, extracted with ethyl acetate, and analyzed using gas chromatography–mass spectrometry and compared with the National Institute of Standards and Technology database. In this study, *Streptomyces* sp. LabMicra B270, *Streptomyces* sp. LabMicra B310, and *Streptomyces* sp. LabMicra B314 were considered potential candidates for biotransformation of (+)–α-pinene, since they eventually accumulated interesting compounds after 96 h of reaction. However, only *Streptomyces* sp. LabMicra B270 was the most promising actinomycete strain found in the screening due to its capacity to use all the α-pinene as the single carbon and energy source in a mineral medium, with the main products *cis*-verbenol, verbenone, and myrtenol being accumulated after 48 h of reaction. These achievements are important and support the development of the production of natural aromas and demonstrate the potential of using these Amazonian ascomycetes from the rhizosphere of inga-cipó (*Inga edulis* Mart.) as new biocatalysts.

Keywords: Bioprospection; α-pinene; *cis*-verbenol; Aroma compounds; Amazon strains.

Introduction

Biotransformation has emerged as an attractive alternative for terpene oxidation since, when compared to the traditional chemical methods, it utilizes mild conditions, has an elevated regio- and enantioselectivity, does not generate toxic wastes, and the products obtained can be labeled as “natural”. Generally made *in vitro*, biotransformation modifies a substrate on single or multistep reactions that are catalyzed in biological systems containing microbial cells (bacteria, yeasts, and filamentous fungi) or with enzymes, purified or not. In addition, the most significant

advantage of biotransformation processes is their ability to produce compounds that are not easily obtained by chemical methods (Molina et al. 2014; de Oliveira Felipe, de Oliveira & Bicas 2017; Sales et al. 2018; Pessôa et al. 2019). We can also add the possibility to repeat indefinitely a biotransformation procedure, without greater damage to the environment.

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 these rises of and interest in biotechnological processes for the production of aromas is the possibility of using agro-industrial by-products that are rich in monoterpene compounds– for example, limonene and pinene since these can be used as substrates for biotransformation (Sharma et al. 2020; Soares-Castro, Soares & Santos 2020). More on the application of monoterpenes as substrates in the production of aroma compounds has been described and reviewed in the last few years, where it becomes clear as an important approach in biotechnology (de Oliveira Felipe, Oliveira and Bicas 2017; de Souza Sevalho et al. 2023).

Limonenes exist in nature as two enantiomers, *R*-(+)-limonene (PubChem CID: 440917) and *S*-(−)-limonene (PubChem CID: 439250). They are the most studied monoterpenes and *R*-(+)-limonene is one of the most abundant in nature, being found as the main constituent of several essential oils of mainly citrus species (Ren et al. 2020). 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 (Vieira et al. 2018). 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 (Soares-Castro, Soares & Santos, 2020).

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). The 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 abundantly produced by-product of the paper and cellulose industry (Salehi et al. 2019; Kutyła et al. 2020). 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 (Vespermann et al. 2017).

Many studies have focused on selecting new biocatalysts to apply in biotransformation processes. Despite the various advantages of biotransformation using microorganisms, including economic and eco-friendly procedures (green chemistry principles), it has, naturally, its challenges (Pessôa et al. 2019). One of the main challenges involving the biotransformation of terpenes concerns their toxicity toward microorganisms. Therefore, one of the first steps in achieving an efficient process is finding a suitable but resistant strain to biotransform a determined terpene (Sales et al. 2018).

At the global level, it is considered that the diversity of microorganisms exceeds in the order of some thousands the diversity of plants and animals. It is estimated that Brazil hosts between 15-20% of the world's biological diversity and a considerable portion of this biodiversity is located in Amazonian ecosystems, an incommensurable source of raw materials, including incalculable microorganisms, for biotechnological application (Borelli et al. 2020). Among the microbial biodiversity, the actinomycetes can be highlighted as occurring in a great diversity in natural and artificial habitats and growing in large variety of substrates (e.g., soil environments) (Farda et al. 2022; Nazari et al. 2022). Besides their importance to Amazon Forest sustainability and as sources of antibiotics, the actinomycetes can also be explored for many biotechnological purposes, including the biotransformation of terpenes, a subject poorly targeted all around the world.

In this context, the objective of this study was to investigate the capacity of actinomycetes of the genus *Streptomyces*, isolated from the rhizosphere soils of inga-cipó (*Inga edulis* Mart.) in the Brazilian Amazon, for the biotransformation of the monoterpenes limonenes and pinenes, two abundant and cheap agro-industrial precursors of many aromas with high added value.

Materials and Methods

Chemicals

All the chemicals for the culture media 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 standards *R*–(+)–limonene (≥93%), *S*–(-)–limonene (≥95%), (+)–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 strains were isolated from the rhizosphere of inga-cipó plants (*Inga edulis* Martius) on the campus area of the Federal University of Amazonas (UFAM) in Manaus city, Amazonas state. 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 AAAAAA. Information on all the *Streptomyces* strains in this study can be found in Table 1.

Table 1. *Streptomyces* strains isolated from *Inga edulis* studied in this research.

Strains isolated from *Inga edulis*	**Similarity (%)**	**Correspondent at GenBank/NCBI**	**16*S r*RNA sequencing**
Streptomyces sp. LabMicra-B267	99.51	*S. pulveraceus* strain NBRC 3855	Partial
Streptomyces sp. LabMicra-B270	96.41	*S. bullii* strain C2	Partial
Streptomyces sp. LabMicra-B271	97.53	*S. graminearus* strain NBRC 15420	Partial
Streptomyces sp. LabMicra-B272	88.60	*S. acidicola* strain K1PN6	Partial
Streptomyces sp. LabMicra-B278	93.78	*S. misionensis* JCM 4497	Partial
Streptomyces sp. LabMicra-B301	96.65	*S. pulveraceus* strain NBRC 3855	Partial
Streptomyces sp. LabMicra-B310	98.96	*S. pulveraceus* strain NBRC 3855	Partial

Streptomyces sp. LabMicra-B314	99.61	*S. olivaceus* strain NBRC 12805	Partial
Streptomyces sp. LabMicra-B318	95.10	*S. similanensis* strain KC-106	Partial
Streptomyces sp. LabMicra-B319	97.93	*S. acrimycini* strain NBRC 12736	Partial
Streptomyces sp. LabMicra-B325	99.19	*S. katrae* strain NRRL B-3093	Partial

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 8 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 fragments of 1 cm^2 of each strain 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 h (Souza et al. 2004). After incubation, the humid biomass was recovered by centrifuging at 4,400 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. (2015) and Bier et al. (2017) 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 K2HPO_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, (+)–α-pinene, and (-)–β–pinene), 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. Periodically, 500 μL samples from each treatment (biotransformation and control experiments) were taken every 24 h in order to monitor the consumption of substrate and product formation. Each sample was extracted with the same volume

of ethyl acetate (1:1, v/v). After phase separation in a vortex chamber, the organic layer was separated and stored at -80 ºC until analyzed.

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.

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 substrate consumption and its derived products was determined on the basis of the chromatographic peak areas using the Xcalibur software (version 2.2) of the own GCMS system. 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

All the *Streptomyces* strains were submitted to the biotransformation process using four monoterpenes as substrates: *R*-(+)-limonene, *S*-(-)-limonene, (+)–α-pinene, and (-)–β–pinene. Among the eleven actinomycete strains, only three were able to biotransform at least and solely

one substrate, the α-pinene, as the single carbon source (Figure S1). No sensitive amount of products were detected for the biotransformation of the hydrocarbons *R*-(+)-limonene, *S*-(-)-limonene, and (-)–β–pinene. The absence of product accumulation suggests that they were completely metabolized in these *Streptomyces* strains.

The three strains able to biotransform (−)-α-pinene, *Streptomyces* sp. LabMicra B270 (Figure S2a), *Streptomyces* sp. LabMicra B310 (Figure S2b), and *Streptomyces* sp. LabMicra B314 (Figure S2c), consumed this substrate completely and accumulated interesting compounds after 96 h of reaction. However, only *Streptomyces* sp. LabMicra B270 was promising in the capacity to use the substrate as the single carbon and energy source in a mineral medium and accumulated products earlier, after 48 h of reaction, consuming all the precursor after 72 h (Figure 1).

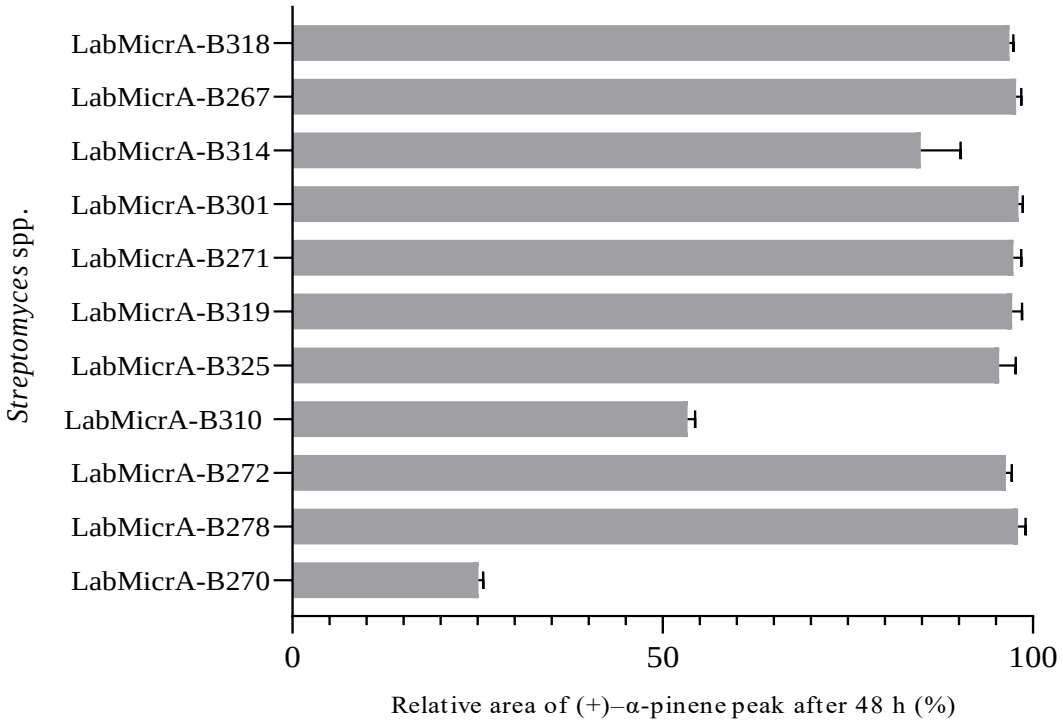
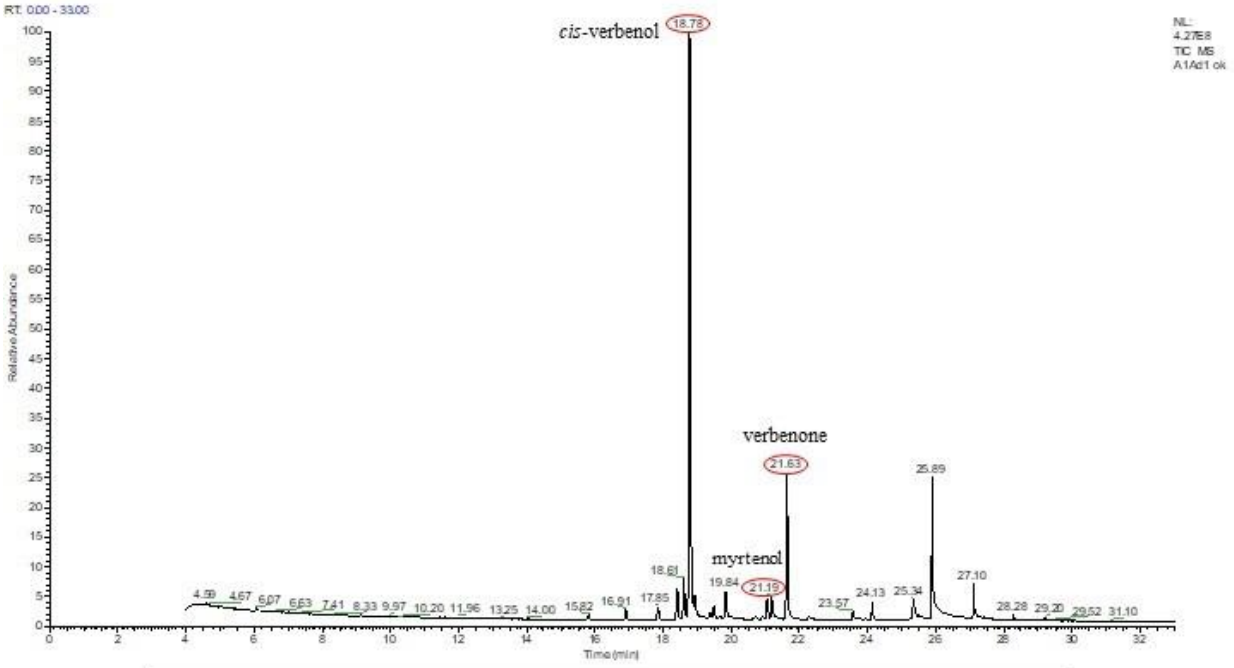


Figure 1. Results obtained after biotransformation process using (+)–α-pinene as substrate and *Streptomyces* strains as biocatalysts after 48 h of reaction.

The oxidation of (+)–α-pinene via *Streptomyces* sp. LabMicra B270 as biocatalysts after 72 h of reaction was examined, being the main products accumulated as *cis*-verbenol, verbenone, and myrtenol. Interestingly, 57,39±0,07% of the total peak area of the ethyl acetate fraction was

accounted for by a single oxygenated metabolite, *cis*-verbenol. The resulting total ion chromatogram for the volatile compounds is shown in Figure 2.

Figure 2. Composition of ethyl acetate fraction obtained via (+)–α-pinene biotransformation using *Streptomyces* sp. LabMicra B270 after 72 h of reaction.



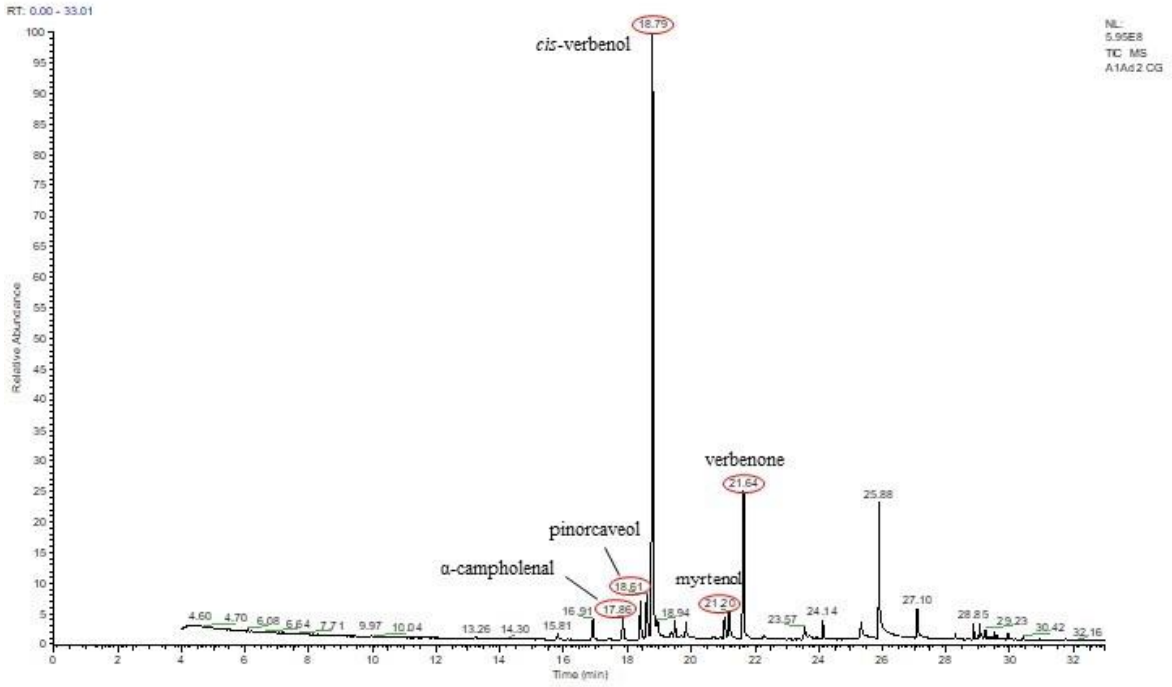
RT (min)	Compounds	Relative amount (%)[a]	RSI (%)	Structure
18.78	*cis*-verbenol	57.39±0.07	92.1	
21.19	myrtenol	4.81±2.02	92.2	
21.63	verbenone	37.80±2.10	95.1	

RT, retention time; RSI, Reverse Search Index; [a]Percentage of peak area in the CG-MS analysis

In the biotransformation processes employing (+)–α-pinene as substrate, the actinomycetes strains identified as *Streptomyces* sp. LabMicra B310 and *Streptomyces* sp. LabMicra B314 also

presented compounds formation. Figures 3 and 4 provide the chromatographic profile obtained after analysis of 96 h reaction samples as well as the chemical structures of the main products of biotransformation.

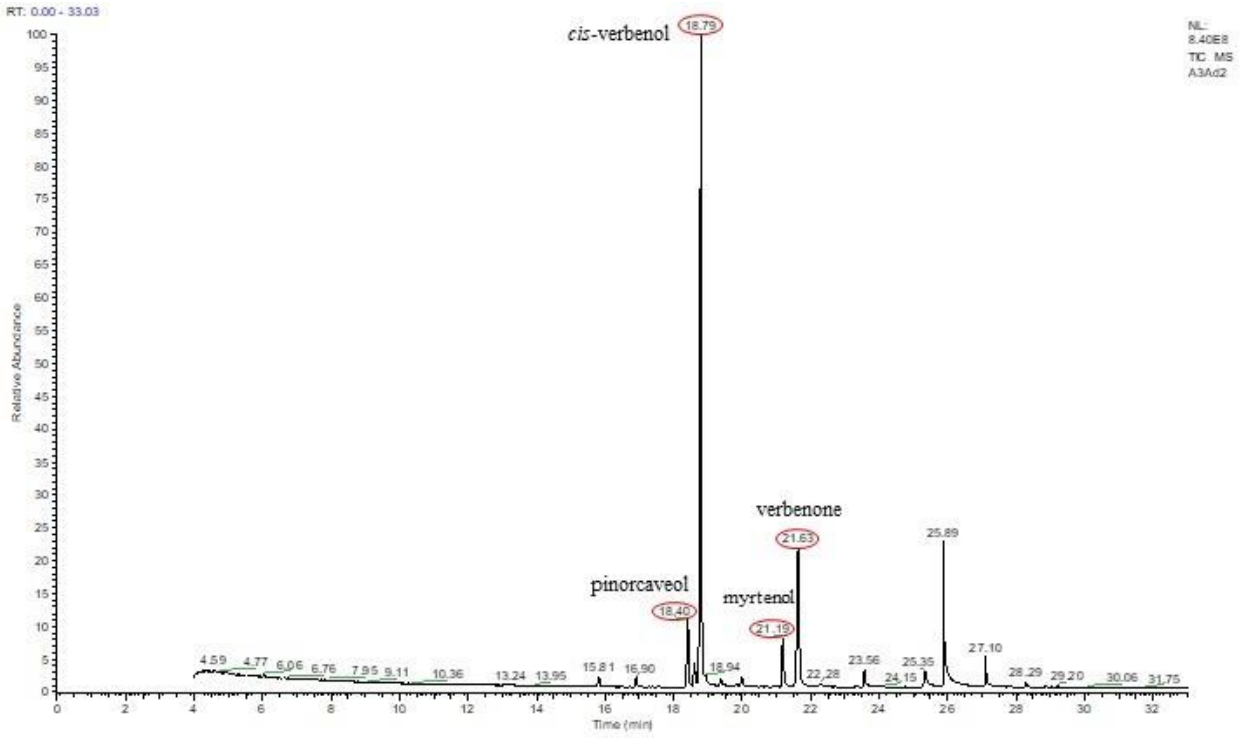
Figure 3. Composition of ethyl acetate fraction obtained via (+)–α-pinene biotransformation using *Streptomyces* sp. LabMicra B310 after 96 h of reaction.



RT (min)	Compounds	Relative amount (%)[a]	RSI (%)	Structure
17.86	α-campholenal	2.45±0.27	92.2	
18.61	pinorcaveol	8.08±1.12	91.8	
18.79	*cis*-verbenol	69.79±0.80	93.0	
21.20	myrtenol	3.09±0.67	91.3	
21,64	verbenone	17.70±0.63	95.6	

RT, retention time; RSI, Reverse Search Index; [a]Percentage of peak area in the CG-MS analysis

Figure 4. Composition of ethyl acetate fraction obtained via (+)–α-pinene biotransformation using *Streptomyces* sp. LabMicra B314 after 96 h of reaction.



RT (min)	Compounds	Relative amount (%)[a]	RSI (%)	Structure
18.40	pinorcaveol	7.68±0.48	91.8	
18.79	*cis*-verbenol	69.22±1.77	93.0	
21.19	myrtenol	5.54±0.63	91.3	
21,63	verbenone	17.49±0.06	95.6	

RT, retention time; RSI, Reverse Search Index; [a]Percentage of peak area in the CG-MS analysis

Derivatives such as *cis*-verbenol, and verbenone myrtenol were not detected in the controls conducted using only the microorganism or the addition of the substrate. The transformed products had a bicyclic structure, similar to the substrate and were identified. The resulting of GC-MS

chromatographic profile for the volatile compounds from oxidation of (+)–α-pinene via *Streptomyces* sp. LabMicra B270, is shown in Figure S3.

Oxidative functionalization of α-pinene, leading to chemical products capitalized on the natural derivatives useful as flavors and fragrances, is considered an alternative with significant potential in industrial applications. Oxidative transformations of α-pinene can take the following two competitive routes: (1) epoxidation of the double bond of the cycle leading to pinene epoxide and getting varied to varied monoterpenoid derivatives and (2) allylic oxidation of α-pinene by the production of verbenol–verbenone and myrtenol–myrtenal mixtures (Gheorghita et al. 2023).

So far, α-pinene oxidation via bacterial biotransformation has only been reported in a few studies. In a recent study, Saidani et al. (2022) showed the biotransformation of α-pinene by cell cultures of *Paenibacillus popilliae* 1C and *Streptomyces rochei* AB1, which resulted in the formation of main compounds such as *trans*-verbenol (59.47% and 40.40%, respectively). Among the value-added aromatic compounds, *cis*-verbenol (PubChem CID: 164888) is a valuable food-flavoring compound with fresh pine odor. It is widely used in soft drinks, soups, meats, sausages and ice cream. It has been reported to have biological activity such as antimicrobial and anti-fungicide properties (Vespermann et al. 2017). 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 (Choi et al. 2010).

Verbenone (PubChem CID: 29025) is a colorless liquid with an odor typical of mint and camphor. It has been associated to have good pesticidal properties such as antiaggregation pheromone activity and pine bark beetle repellent activity; as well as some pharmacological properties like bronchodilating, anti-inflammatory, and haemolytic activities (González-Velasco et al. 2022). 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 (Vespermann et al. 2017).

Economically, the advantages of the biotransformation are clear when comparing the reference prices of substrates and products. This importance can be estimated by its commercial value; using the database of Merck KGaA Brazil (https://www.sigmaaldrich.com/BR/pt), the values of *cis*-verbenol are around US$ 229.05/5 g and US$ 784.91 for 25 g. The reference price of

verbenone is around US$ 72.07 for 1 mL and US$ 245.05 for 5 mL, whereas the reference price of α-pinene is about US$ 34.25 8 for 500 mL. In this context, it would be a good strategy to invest efforts and resources to better understand α-pinene biotransformation.

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 (Vespermann et al. 2017).

Conclusion

In this work, it was possible to explore the potential of the rhizosphere as a source of promissory biocatalysts. Three actinomycete strains proved to be able to use at least one substrate for aroma production. The compounds obtained are of great industrial interest since they can be applied as additives in foods and cosmetics and also due to their potential biological activity. With this perspective, it is interesting to make further efforts in this area in order to improve product concentration and obtain higher yields, thus increasing the potential of natural aroma production through biotechnology.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Credit authorship contribution statement

ESS: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft, and Writing – review & editing; RSR: Methodology and Data curation; BNP: Methodology; AQLD: Funding acquisition, Methodology, Supervision and Writing – original draft; ADLS: Funding acquisition, Methodology, Supervision, and Writing – review & editing. This study is part of the doctoral thesis of ESS (PPG-BIONORTE/UFAM).

Ethical statement

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

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Supplementary Information

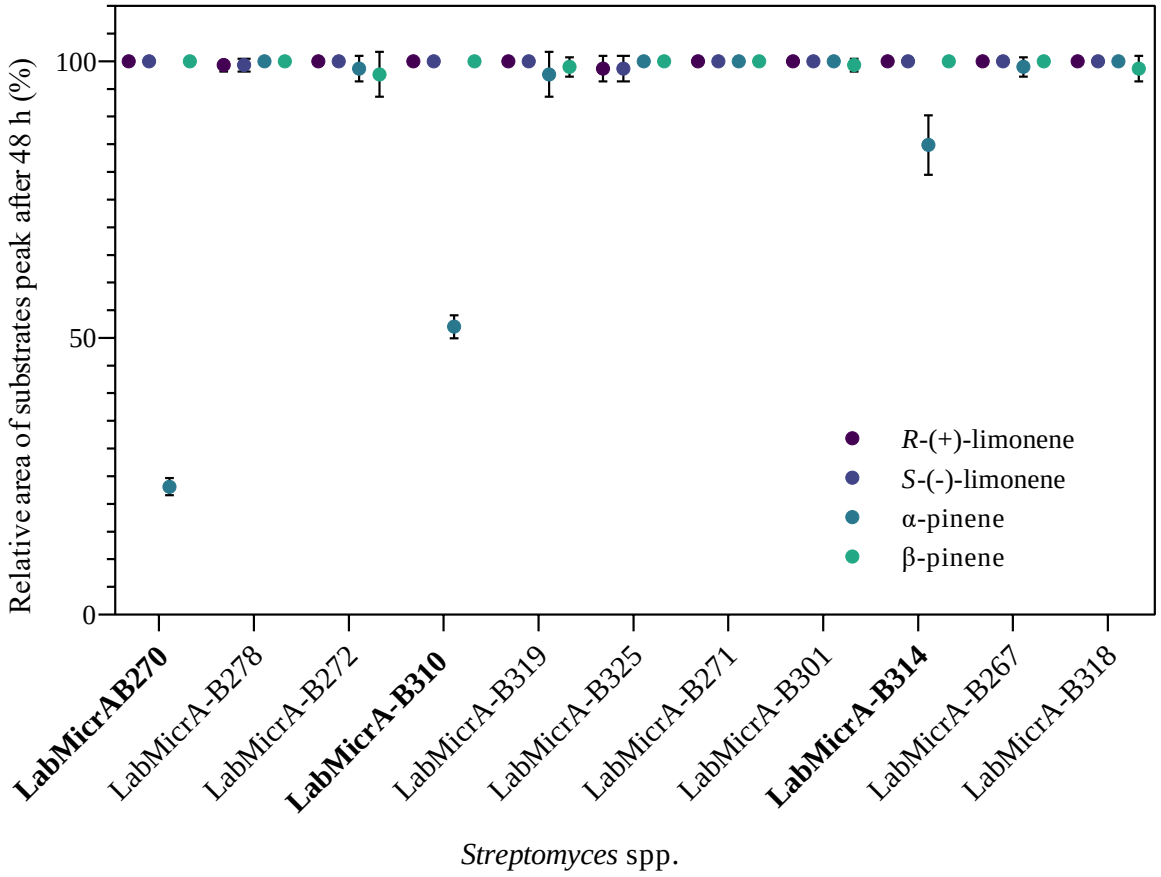

Figure S1. Results obtained after biotransformation process using substrates and *Streptomyces* strains as biocatalysts after 48 h of reaction.

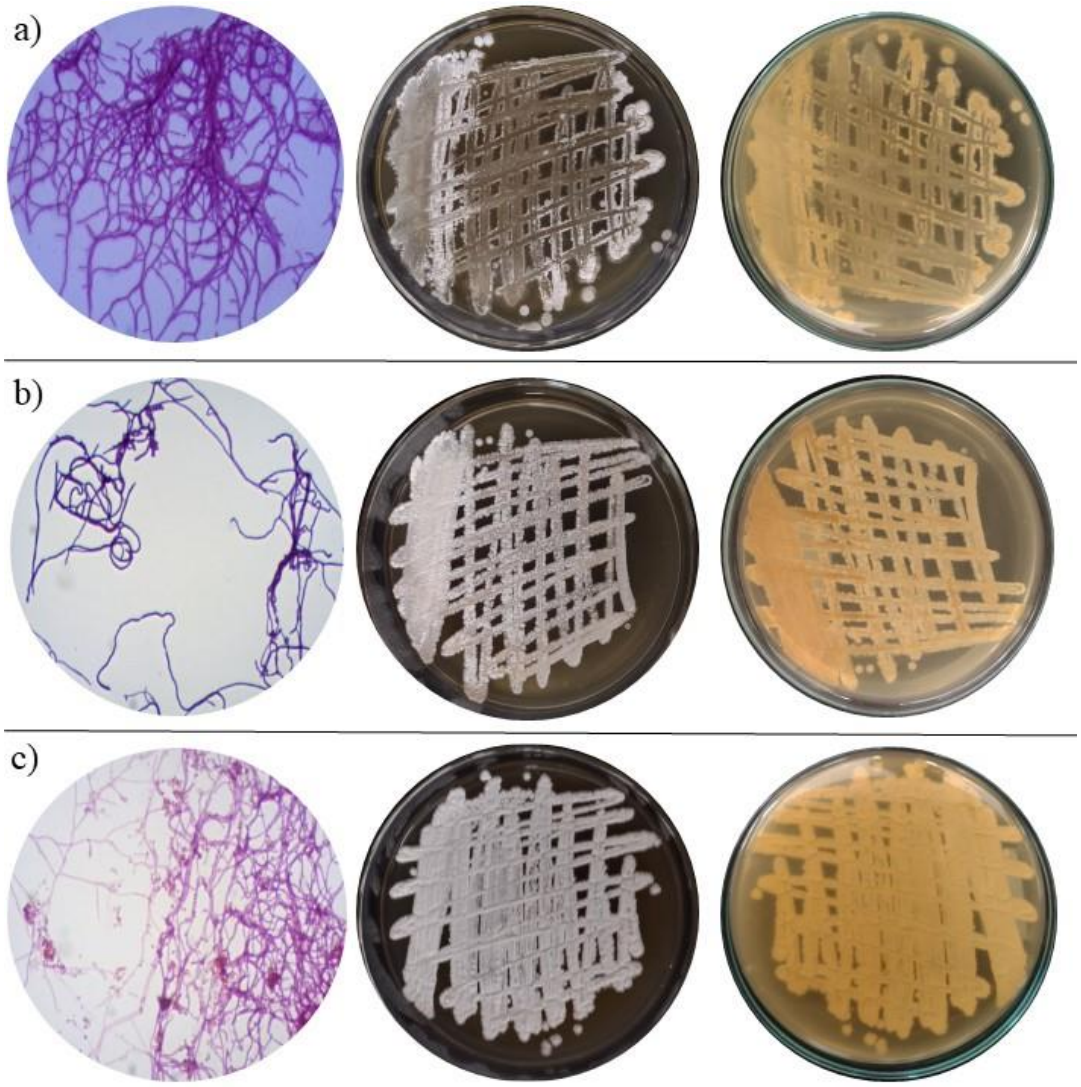

Figure S2. Micro and macro morphological images (top and reverse) of *Streptomyces* strains grown at 28 °C in the ISP2 semi-solid culture medium: a) *Streptomyces* sp. LabMicra B270; b) *Streptomyces* sp. LabMicra B310; c) *Streptomyces* sp. LabMicra B314.

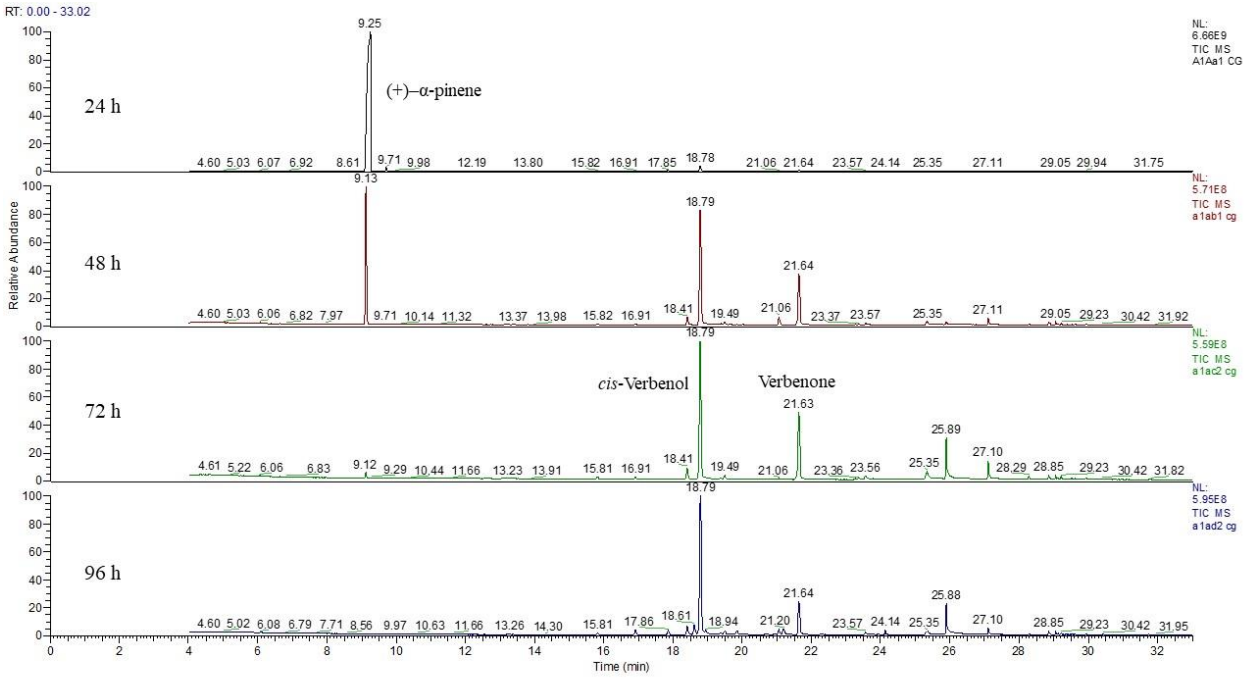

Figure S3. GC-MS chromatographic profile of the biotransformation of (+)–α-pinene into *cis*-verbenol and verbenone over time using *Streptomyces* sp. LabMicra B270.

CAPÍTULO IV

Investigation into the effect of culture conditions and optimization on limonene-1,2-diol production from the limonene biotransformation by *Pestalotiopsis mangiferae* **LabMicrA-505**[1]

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Abstract

Before reaching a commercial scale, a biotechnological process must be well characterized in terms of process conditions. Thus, this study aimed to investigate the culture conditions and optimization of the limonene biotransformation to limonene-1,2-diol by *Pestalotiopsis mangiferae* LabMicrA-505 obtained from the Brazilian Amazon. Therefore, the study started with the investigation of the conditions among the three culture mediums, different inoculum ages, and influence pretreatments on obtain of biomass. Subsequently, a central composite design was employed to optimize the main parameters of the process on limonene-1,2-diol production, modeled in shake flasks. The results demonstrated that the use of yeast extract-malt extract broth as a culture medium supports the growth of biomass. In terms of inoculum age, 72 hours-old biomasses performed better when compared to 48 or 96 hours-old inoculums. Among influence of the fresh cells and effect of freezing–thawing upon fungal biomass evaluated, did not significant difference change on limonene-1,2-diol production, indicating that the biomass could be used following the pretreatments. Finally, the contour plots and surface responses of a central composite design revealed that the optimal biotransformation conditions were 4 g of inoculum, 2,0 % of the substrate at temperature 24 °C, 120 rpm, and pH 6. Under the optimized conditions, it accumulated up to 98.34±1.53% of limonene-1,2-diol after 96 h of reaction. Considering the results of this extensive optimization process, the endophytic fungus *Pestalotiopsis mangiferae* LabMicrA-505 proved to be a potent biocatalyst with great potential for the development of new aroma compounds from the limonene biotransformation.

Keywords: Aroma compounds; Central Composite Design; Influence of culture; Amazon endophytic fungus.

Introduction

Natural additives as aroma compounds are a potential alternative for the replacement of traditional chemicals, and are extensively employed in the food technology, cosmetic, and pharmaceutical industry. In the last few years, many chemical companies have increased their portfolios with the inclusion of naturally-obtained compounds, through the use of biotechnology-

based approaches in order to substitute chemical synthesis (de Oliveira Felipe, de Oliveira and Bicas, 2017). In this context, a joint effort between science and industry for the production aroma compounds being one of the main targets, is fundamental for the development of appropriate solutions in order to meet the demand for natural compounds (Paulino et al. 2021).

The limonene biotransformation is a biotechnological approach for the production of aroma compounds aligned with sustainable development, due to the use of agro-industrial by-products, 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. In this context, *R*-(+)-limonene, (4*R*)-1-methyl-4-prop-1-en-2-ylcyclohexene (PubChem CID: 440917*)*, also known as d-limonene 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 (Sharma et al. 2020; de Souza Sevalho et al. 2023a).

Economically, the advantages of the 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 it added value. This importance can be estimated by its commercial value, using Molbase database (https://www.molbase.com/) to illustrate this, the reference price of limonene 1,2-diol is around US$ 8,500/kg, while *R*-(+)-limonene has reference price of US$ 34/kg.

Limonene-1,2-diol, 1-methyl-4-prop-1-en-2-ylcyclohexane-1,2-diol (PubChem CID: 94217) is a colorless to slightly yellowish oil with a fresh mint aroma. The conversion of limonene to limonene-1,2-diol may also be economically attractive due to its potential application in the food industry as an aroma additive. It also has insect-attractant properties, besides being used as a flavoring for beverages, chewing gum, gelatins, and puddings. Limonene-1,2-diol has been associated with a significant inhibitory effect on the pro-inflammatory activities of $CD4^+$ and $CD8^+$ T lymphocytes; a potential anticancer activity (Sales et al. 2019; de Souza Sevalho et al. 2023a).

Fungal biotransformation in the limonene 1,2-diol production is also important and some authors have reported the epoxidation reaction of the 1,2 double bond from limonene by fungi strains as biocatalysts, such as *Fusarium oxysporum* 152B (Molina et al. 2015); *Phomopsis* sp. (Bier, Medeiros and Soccol, 2017); *Alternaria alternata* Eb03 and *Neofusicoccum* sp. (Cecati et al.

2018); *Colletotrichum nymphaeae* CBMAI 0864 (Sales et al. 2018; Sales et al. 2019; Medeiros et al. 2021); *Pestalotiopsis versicolor* LabMicrA-478 (de Souza Sevalho et al. 2023b).

To better understand and to optimize a biotransformation process, it is first essential to characterize its parameters, including the culture condition, resistance to substrate toxicity, inoculum age, and enzyme induction, among others (Shields et al. 2021). In summary, understanding some properties of the biocatalyst and its relation with the surrounding environment may help to achieve greater production just by adjusting some process conditions, besides providing fundamentals for studies on scale-up and product recover (Sales et al. 2019).

Generally, classical optimization methods consist of varying the parameters one at a time while maintaining the other variables constant. The Central Composite Design (CCD) is a screening approach that helps to statistically select the significant variables of numerous factor experiments, aiming to reduce the number of trials in the final design. This statistical methodology analyzes how the studied variables and their interaction impact a process. This technique culminates in the proposal of a mathematical model that describes the behavior of the analyzed factors and establishes their optimal values (Zhang et al. 2020; Basturk et al. 2021).

Considering that one of the main challenges involving the limonene biotransformation is related to their toxicity towards biological systems, the use of phytopathogens in these processes may be a good choice, since such microorganisms are presumably more resistant to terpene (Sales, Pastore & Bicas, 2019). In this approach, the aim of this work is to evaluate the effects of culture conditions and analyze five variables (agitation, temperature, pH, substrate concentration, and biocatalyst biomass) in the biotransformation of limonene to limonene-1,2-diol production by an endophytic fungus *Pestalotiopsis mangiferae* LabMicrA-505 obtained from the Brazilian Amazon.

Materials and Methods

Substrate and microorganism

The standard *R*-(+)-limonene (≥93%) used as substrate was acquired from Sigma-Aldrich Brazil (São Paulo, Brazil). All other reagents used in the study were of analytical grade. The endophytic fungus *Pestalotiopsis mangiferae* LabMicrA-505 (Genbank access number KM998724.1) previously isolated from the fruiting bodies (basidiome) of basidiomycete, was used

in the current investigation. The strain belongs to the working collection of the Laboratory of Bioassays and Microorganisms of the Amazon (LabMicrA) from the Analytical Center - Center of Multidisciplinary Support at the Federal University of Amazonas (CA/CAM/UFAM) (Brazilian SISGEN registration: AC1746C).

Influence of culture conditions

To evaluate the production of fungal biomass used on limonene biotransformation, three different liquid culture mediums were tested on *Pestalotiopsis mangiferae* LabMicrA-505 growth. The fungal strain was cultured in the basic medium of potato-dextrose (PD), following potato-dextrose 0.2% yeast extract broth (PDY) (Souza et al., 2004), and yeast extract-malt extract broth (YM) (Molina et al., 2015). For testing the effect of different inoculum ages, independent biotransformations were carried out with resting cells at three different times: 48 h (inoculum A), 72 h (inoculum B), and 96 h (inoculum A) (Sales et al. 2019).

Conidia suspension (30 µl) (McFarland No. 6) was inoculated in 125 ml Erlemeyer flasks containing 50 ml of the liquid culture mediums (PD, PDY, and YM) and incubated at 24 °C with shaking at 120 rpm. Samples were collected every 48 h, 72 h, and 96 h, the culture broth was separated into supernatant and mycelium by centrifugation (Eppendorf Centrifuge 5702, Merck KGaA, Darmstadt, Alemanha) at 4.400 r.p.m., and 28° C for 10 min. The supernatant was discarded and the resulting fresh mycelial pellets by centrifugation were washed twice with sterile ultrapure water. A comparison of the influence pretreatments used on biomass recovered: a freezing-thawing step, and lyophilization procedure on biotransformation performance was evaluated (Sales et al. 2019). Part of the biomass obtained was frozen at − 18 °C for 72 h, and another part was lyophilized (Martin Christ Lyophilizer, Alpha 1–2 LD Plus, Osterode am Harz, Germany) under pressure 1.3 x 10^{-1} mbar with drying chamber temperature at – 30 °C. The samples obtained were held frozen at −18 °C for further analysis.

Experimental design procedure

To determine the influence of the optimization conditions on limonene-1,2-diol production was performed according by Molina et al. (2015). Central composite design (CCD) was applied

as an optimization tool of response surface methodology (RSM), to explore the impact of the independent variables on limonene-1,2-diol production process. To determine the optimum conditions, three process independent variables were considered: inoculum biomass (X_1), substrate (X_2), agitation (X_3), temperature (X_4), and pH (X_5) were investigated.

The CCD involves factorial points (2^n), axial points ($2n$), and central points (n_c). The center points determine the experimental error and how well the data can be reproduced. The axial points are taken in a way to ensures ratability, and the model prediction variance is constant at every point equidistant from the center of the design. The total experimental runs conducted was computed by Equation 1 (Bayuo, Abukari, & Pelig-Ba, 2020):

$$N = 2^n + 2n + n_c$$
$$N = 2^{5-1} + 2(5) + 1 = 27 \quad \text{(Eq. 1)}$$

where n is number of independent variables (factors), n*c* is number of center points and *N* is the overall total of experimental runs. This indicated that twenty-seven experimental runs comprising sixteen factorial runs, ten axial runs and one replicates at central points were prerequisites for the modeling and optimization process. The experimental design and data obtained were studied by means of Statistica® 12.5 software (StatSoft Inc, Oklahoma, USA) (www.statsoft.com). The level of the considered independent variables and their experimental ranges for limonene-1,2-diol production is presented in Table 1.

Table 1. The coded levels of the independent variables in the central composite design.

Code	Independent variable	Units	Levels		
			-1	0	+1
X_1	Inoculum	(g)	3	4	5
X_2	Substrate	(%)	1,5	2	3
X_3	Agitation	(rpm)	80	100	120
X_4	Temperature	(°C)	20	24	28
X_5	pH	-	4.0	6.0	8.0

General biotransformation procedure

Following the procedure adapted previously described by Molina et al. (2015), the biomass obtained was inoculated in three Erlenmeyer flasks (125 mL) containing 50 mL of mineral medium (0,5 g l^{-1} $MgSO_4$, 3 g l^{-1} $NaNO_3$, 1 g l^{-1} $K2HPO_4$, 0,5 g l^{-1} KCl, and 0.01 g l^{-1} Fe2SO4, dissolved in Ultrapure water and mixed well). Under different culture conditions, 0.5% (v/v) substrate was added to these flasks, and both were incubated on a rotary shaker operating at 24° C and 120 rpm for 120 h. Furthermore, experimental design conditions were performed according to the CCD, and their responses are presented in Table 1. Two negative controls were incubated in parallel under the same conditions. 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).

Gas chromatography-mass spectrometry analysis

The analysis of the extract was performed using 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. 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. The sample was injected and detected through an auto-sampler injector done in split mode (split ratio of 1:30) at temperature of 250 °C, ionization energy 70 eV, and the scan range *m/z* 35-400 amu, without delay.

Statistical Analysis

The compounds were identified using the national institute of standards and technology (NIST) library (similarities <90% were discontinued). The ratio between the substrate consumption and its transformed products was determined on the basis of the peak area of the mass spectroscopy

graph. Three independent assays were run for each experiment. Data of the peak area of the chromatograms obtained from the tests of culture conditions were collected, tabulated (mean ± standard deviation), and subjected to statistical analysis using GraphPad Prism version 9.5.1 for Windows, GraphPad Software, San Diego, California USA (www.graphpad.com). One-way ANOVA was used to determine the presence/absence of differences between the various studied groups. Also, Tukey'spair-wise post-hoc test was employed where needed with *p*-value <0.05 considered statistically significant.

The optimization tool of RSM, to explore the impact of the independent variables on the limonene-1,2-diol production was performed using Statistica® 12.5. The correlation values were generated by the computational analysis of the software. The significance of the model and the effects of the interaction between the three factors were tested based on the *t* test and *p*-values. The effects were considered to be significantly different at *p*-value <0.05 and a confidence level of 95%, which is acceptable for biotechnological processes (Sales et al 2019).

Results and Discussion

Establishment of the culture conditions

Considering the chromatographic profile (total peak area), it demonstrates the low accumulation of the intermediate limonene-1,2-epoxide in the limonene biotransformation via *P. mangiferae* LabMicrA-505. In this case, Sales et al. (2018) found traces of limonene-1,2-epoxide as a by-product of the biotransformation of limonene with *Colletotrichum nymphaea* and *C. acutatum.* The authors support the hypothesis that both strains present a pathway for converting limonene to limonene-1,2-diol via limonene-1,2-epoxide. However, as observed on the experiments, the limonene-1,2-diol was confirmed as 92.7% similarity by the mass spectrometry.

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 are 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 (de Souza Sevalho et al. 2023b).

To determine the optimal culture conditions, three different parameters as a comparison of the liquid culture mediums (BD, BDL and YM) were tested initially to investigate biomass recovered by growing *P. mangiferae* LabMicrA-505 under laboratory conditions. Figure 1a graph shows that there were statistical differences among effect of YM with two others medium in the percentage of limonene1,2-diol. The influence of biomass with three different times (48, 72 and 96 h) is presented in Figure 1b. The 72 h-old biomasses presented a significant statistically when comparing as the biomass de 48 and 96 h, inducing the reduces of the percentage of production, and productivity of limonene1,2-diol.

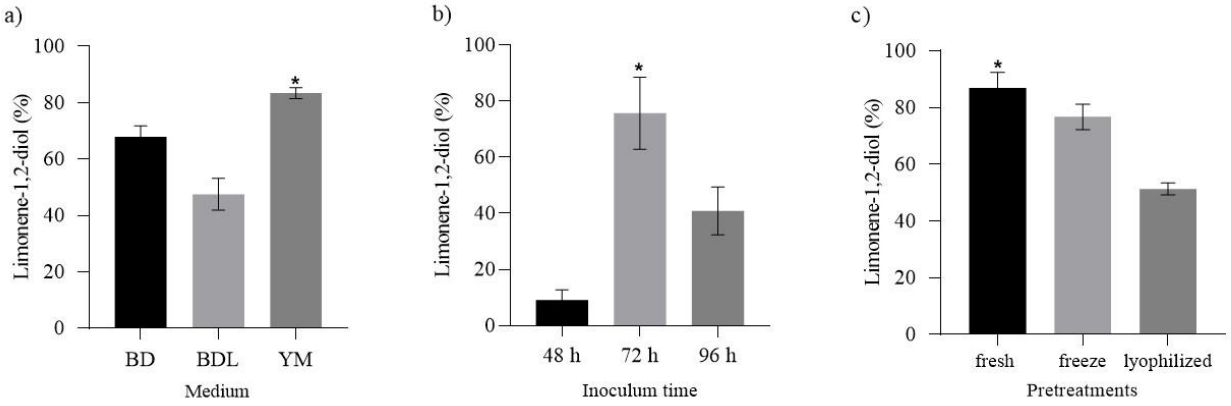

Figure 1. Production of limonene-1,2-diol from biotransformation of *R*-(+)-limonene by *P. mangiferae* LabMicrA-505 biomass previously treated with three parameters (a) liquid culture medium, (b) influence of inoculum time, and (c) pretreatments.

The biotransformation with fresh, freezing-thawing and lyophilized cells were evaluated by possibility of maintaining the biocatalytic activity of the obtain biomass after 72h. Figure 1c, shows the accumulation of limonene-1,2-diol using biomasses submitted to pretreatments. As may be noticed, none of the treatments (fresh and freeze) revealed significant differences in limonene-1,2-diol production. In any case, the biotransformation profile of freeze cells is similar to fresh cells, confirming that this technique may be suitable for storage. The lyophilized biomass showed biotransformation activity as well. This would be of great interest from a technical point of view, since it standardizes and simplifies the inoculation besides enabling biocatalyst storage. However, the maximal limonene-1,2-diol peak area was nearly 60% lower.

Something observed by Sales et al. (2019) for the fungus *C. nymphaeae* CBMAI 0864, a freeze-thawing process accelerated the biotransformation of limonene to limonene-1,2-diol (i.e., increased limonene-1,2-diol productivity). This behavior was explained by the semi permeabilization effect of this treatment, which could release part of the intracellular enzymes and improve the solute transfer rate through the cell membrane (Bicas et al 2010). Moreover, as observed for *P. mangiferae* LabMicrA-505 biomass could be stocked in frozen form before being used for limonene biotransformation, and this is very useful from a practical point of view.

Central composite design

Generally, in the design of a statistically based experiment, the first step is the choice of the performance characteristic or the response variable, which will be closely monitored. The second step is the identification of variables or factors that contribute to this response variable, which will be studied. The next step is the choice of different treatment stages or levels, at which these factors will be tested for individual experiments. The final step is identifying uncontrollable or noise factors that may influence the process in any way. Statistical procedures follow the general principles of randomization, replication, and duplication to predict the actual behavior of a process (Ranganathan et al. 2016; Schmidt et al. 2022).

In order to understand how process parameters affect on limonene biotransformation and optimize limonene-1,2-diol production, the variables biocatalyst biomass, substrate concentration, agitation, temperature, and medium pH values were evaluated in a central composite design with twenty-seven experiments. Considering the results obtained in this study, the limonene-1,2-diol production of run 18 and 27 (central point) showed good reproducibility, with an average high from 96 h. The results obtained from 72 h to 120 h-biotransformation for each experiment in the central composite design are provided in Table 2.

Table 2. Experimental matrix design, values of the factors and responses and peak area of limonene-1,2-diol in GC/MS after 72 h and 120 h of biotransformation for each assay.

Run	Coded variables					Actual variables					Peak area (%) *		
	X_1	X_2	X_3	X_4	X_5	X_1 (g)	X_2 (%)	X_3 (rpm)	X_4 (°C)	X_5	72 h	96 h	120 h
1	-1	-1	-1	-1	1	3	1,5	80	20	8	2,45±0,56	15.49±2.82	2,45±0,56
2	-1	-1	-1	1	-1	3	1,5	80	28	4	3,19±0,18	19.19±0,16	4,31±0,54
3	-1	-1	1	-1	-1	3	1,5	120	20	4	9,25± 0,60	12,36±2,29	10,19±2,00
4	-1	-1	1	1	1	3	1,5	120	28	8	19,29±1,58	22,19±2,02	19,23±2,04
5	-1	1	-1	-1	-1	3	3	80	20	4	10,88±1,26	15,28±2,11	11,83±0,84
6	-1	1	-1	1	1	3	3	80	28	8	8,69±0,50	12,11±1,17	8,96±1,05
7	-1	1	1	-1	1	3	3	120	20	8	15,01±1,12	19,23±1,67	9,63±1,05
8	-1	1	1	1	-1	3	3	120	28	4	9,59±0,83	12,13±1,33	8,13±0,56
9	1	-1	-1	-1	-1	5	1,5	80	20	4	17,19±1,03	20,13±1,51	15,13±0,82
10	1	-1	-1	1	1	5	1,5	80	28	8	19,73±0,90	24,28±1,04	11,83±0,84
11	1	-1	0	-1	1	5	1,5	100	20	8	23,01±0,51	45,33±2.07	10,36±0,71
12	1	-1	0	1	-1	5	1,5	100	28	4	15,91±1,10	17,23±0,96	10,69±1,02
13	1	1	-1	-1	1	5	3	80	20	8	10,72±0,91	12,36±2,29	7,19±0,54
14	1	1	-1	1	-1	5	3	80	28	4	8,63±0,47	12,11±1,17	6,13±0,55
15	1	1	1	-1	-1	5	3	120	20	4	15,01±0,51	21,23±1,17	12,29±1,22
16	1	1	0	1	1	5	3	100	28	8	20,23±1,05	27,39±1,84	16,28±0,72
17	-2	0	1	0	0	2	2	120	24	6	37,19±1,41	11.21±1.69	28,21±4,56
18	2	0	1	0	0	6	2	120	24	6	43,19±1,63	98.34±1.53	58,29±2,95

19	0	-2	0	0	0	4	0,5	100	24	6	46,28±3,45	19.23±1,67	73,61±1,56
20	0	2	0	0	0	4	3,5	100	24	6	53,01±2,07	26,36±0.51	57,29±1,42
21	0	0	-2	0	0	4	2	60	24	6	37,23±1,09	49,27±2,10	29,21±13,37
22	0	0	2	0	0	4	2	140	24	6	3,59±0,47	12.74±1.21	4,29±0,70
23	0	0	0	-2	0	4	2	100	16	6	2,28±0,98	13,41±0,58	2,31±0,56
24	0	0	0	2	0	4	2	100	32	6	3,82±0,46	11.83±1.17	2,82±0,85
25	0	0	0	0	-2	4	2	100	24	2	47,53±0,59	53,29±1,15	37,96±0,65
26	0	0	0	0	2	4	2	100	24	10	37,25±1,43	45,47±2,06	28,61±5,17
27 C	0	0	1	0	0	4	2	120	24	6	70,23±0,59	96,36±0,66	63,52±1,46

*Mean ± SD of limonene-1,2-diol area peak

Moreover, simple changes in the bioprocess conditions (temperature, pressure, pH, substrate concentration, inoculums etc.) can significantly improve the productivity of a biotransformation. Therefore, response surface methods are considered important tools to overcome the low yields obtained for the biotransformation of terpenes, since this approach help in cutting down the number of experiments required in multi-factorial systems (Molina et al. 2019).

As a result, 72 h and 120 h-reaction periods presented no practical advantage over 96 h, which was considered to be the optimum time for the statistical evaluation. The independent variable chosen to be used as a response (96 h-reaction) was treated by the Statistica® 12.5 software, which generated the regression coefficients and respective statistical analysis of the parameters considered. The significance of each coefficient was determined by the *t*-test and *p*-value. The greater the magnitude of the *t*-value and the lower the *p*-value, the more significant the corresponding coefficient. This implies that the linear and quadratic models were statistically significant, considering a $p < 0,05$. This was not valid for the model of interaction between study variables. These results are presented in Table 3.

Table 3. The least-squares and significances of the regression coefficients of the model parameters (96 h-biotransformation).

Parameters	RC	SE	*t* (60)	*p*-value
Mean	**-2141.81**	**176.50**	**-12.13**	**0.00**
Linear				
X_1	**74.21**	**21.94**	**3.38**	**0.00**
X_2	**129.49**	**25.83**	**5,01**	**0.00**
X_3	**11.28**	**1.23**	**9,15**	**0.00**
X_4	**106.84**	**7.28**	**14,67**	**0.00**
X_5	**47.55**	**10.09**	**4,71**	**0.00**
Quadratic				
X_1^2	**-9.84**	**1.86**	**-5.29**	**0.00**
X_2^2	**-32.94**	**3.15**	**-10.45**	**0.00**
X_3^2	**-0.06**	**0.00**	**-12.37**	**0.00**
X_4^2	**-2.22**	**0.14**	**-16.25**	**0.00**
X_5^2	**-4.84**	**0.46**	**-10.41**	**0.00**
Interaction				
X_1 X_2		Not significant		> 0.64
X_1 X_3		Not significant		> 0.20
X_1 X_4		Not significant		> 0.42
X_1 X_5		Not significant		> 0.30
X_2 X_3		Not significant		> 0.30
X_2 X_4		Not significant		> 0.28

X_2 X_5	Not significant	> 0.53
X_3 X_4	Not significant	> 0.46
X_3 X_5	Not significant	> 0.06
X_4 X_5	Not significant	> 0.92

RC = regression coefficients; SE = standard error; *t* (60) = value for 52 degrees of freedom under Students *t*-test; X_1= Inoculum (g); X_2=Substrate (%); X_3= Agitation (rpm); X_4=Temperature (ºC); X_5= pH. Parameters in bold are statistically significant for the model (p <0,05).

To screen the tested variables, limonene was added to the mineral médium at concentrations ranging from 1,5 % (level −1) to 3 % (level +1). Table 3 shows that the substrate concentration positively affected (p <0,05) the response (limonene-1,2-diol productivity). In an optimization study, Sales et al. (2019) observed that limonene exerted (above 3%) a negative effect on limonene-1,2-diol concentration, probably because *R*-(+)-limonene (above 3%) was toxic to *C. nymphaeae* CBMAI 0864.

The design also examined the effect of biomass (biocatalyst) concentration. In general, the highest the biocatalyst content, the highest is the conversion rate. Fontanille and Larroche (2003), for example, found that biocatalyst content influenced the maximum product recovery positively in the case of α-pinene oxide biotransformation into isonovalal. In the present study, biomass content in a range of 3 g (level −1) to 5 g (level +1) presented effect (p <0,05) on limonene-1,2-diol production (Table 3). A similar behavior was also observed for the biotransformation of limonene to limonene-1,2-diol by *Sphingobium* sp, whose biomass content was statistically significant to improve product production (Molina et al., 2019).

Finally, the screening design indicated how culture conditions (temperature and agitation) affected limonene-1,2-diol production. It is widely known that temperature directly influences biological reactions and that medium agitation promotes mass transfer, homogenization and cell-substrate interaction. However, in addition to increasing the process energy costs, high temperatures and agitation rates might enhance substrate and product loss as well as side reactions. Thus, it is essential to seek an ideal balance, to obtain the best results (Bicas et al., 2008).

In the case of agitation speed, this variable presented a positive effect (p <0,05, Table 3) on limonene-1,2-diol production, which means that an increase in agitation would promote limonene conversion, probably due to increased mass transfer and cell-substrate contact. Thus, optimal agitation may be situated at values above the maximum tested in the screening design (120 rpm),

suggesting the use of a wider range for this variable in the central composite design. Considering statistically the model (p <0,05), temperature did impact the biotransformation process significantly (Table 3), which means that this process is quite robust in terms of temperature control, since yields would be roughly constant in the range of 24–28 °C. For the central composite design, temperature was kept constant at 24 and 28 °C. This was close to the optimum temperature for the biotranformation of limonene to limonene-1,2-diol by *C. nymphaeae* CBMAI 0864 (Sales et al., 2019). Authors have reported that for *F. oxysporum* 152B (Bicas et al., 2008b, 2010b) the bioconversion decreased dramatically at temperatures above 32, 30, and 28 °C, respectively.

After the measurement of the regression coefficients, an analysis of variance (ANOVA) was performed to verify the validity of the linear and quadratic models. The calculated F value was higher than the respective listed value, while the p-value of the model was lower than 0,05. Although it is not ideal, R^2 value = 0.85 is a perfectly acceptable value for biological systems. Moreover, Sales et al. (2019) found similar R^2 values that ranged from 0.84 to 0.86 in the study of optimization of limonene biotransformation to limonene-1,2-diol by *C. nymphaeae* CBMAI 0864. With these data, it was possible to obtain a mathematical model that predicts limonene-1,2-diol peak area. The regression coefficients which compose the model are adjusted with only significant parameters ($p < 0.05$), given by Equation 2.

$$Y = -2141.81 + 74.21\,X_1 - 9.84\,X_1^2 + 129.49\,X_2 - 32.94\,X_2^2 + 11.28\,X_3 - 0.06\,X_3^2 + 106.84\,X_4 - 2.22\,X_4^2 + 47.55\,X_5 - 4.84\,X_5^2 \quad \text{(Eq. 2)}$$

Where Y is the dependent variable limonene-1,2-diol area peak in percentage, and X are the coded independent variables (X^1 = fungal biomass, X^2 = substrate concentration, X^3 = agitation, X^4= temperature, and X^5 = medium pH).

As the model obtained was signed it was built the surface responses and contour plots from illustrate the behavior of each variable in the model. Figures 2, 3, and, 4 graphically present the Equation 2. In both figures, it is possible to observe that a maximum plateau is observed for inoculum, subtrate, agitation, temperature, and pH values in the range of 2,0 %, 4 g, 100 rpm, 24 °C, and 6.0, respectively, regardless the response considered.

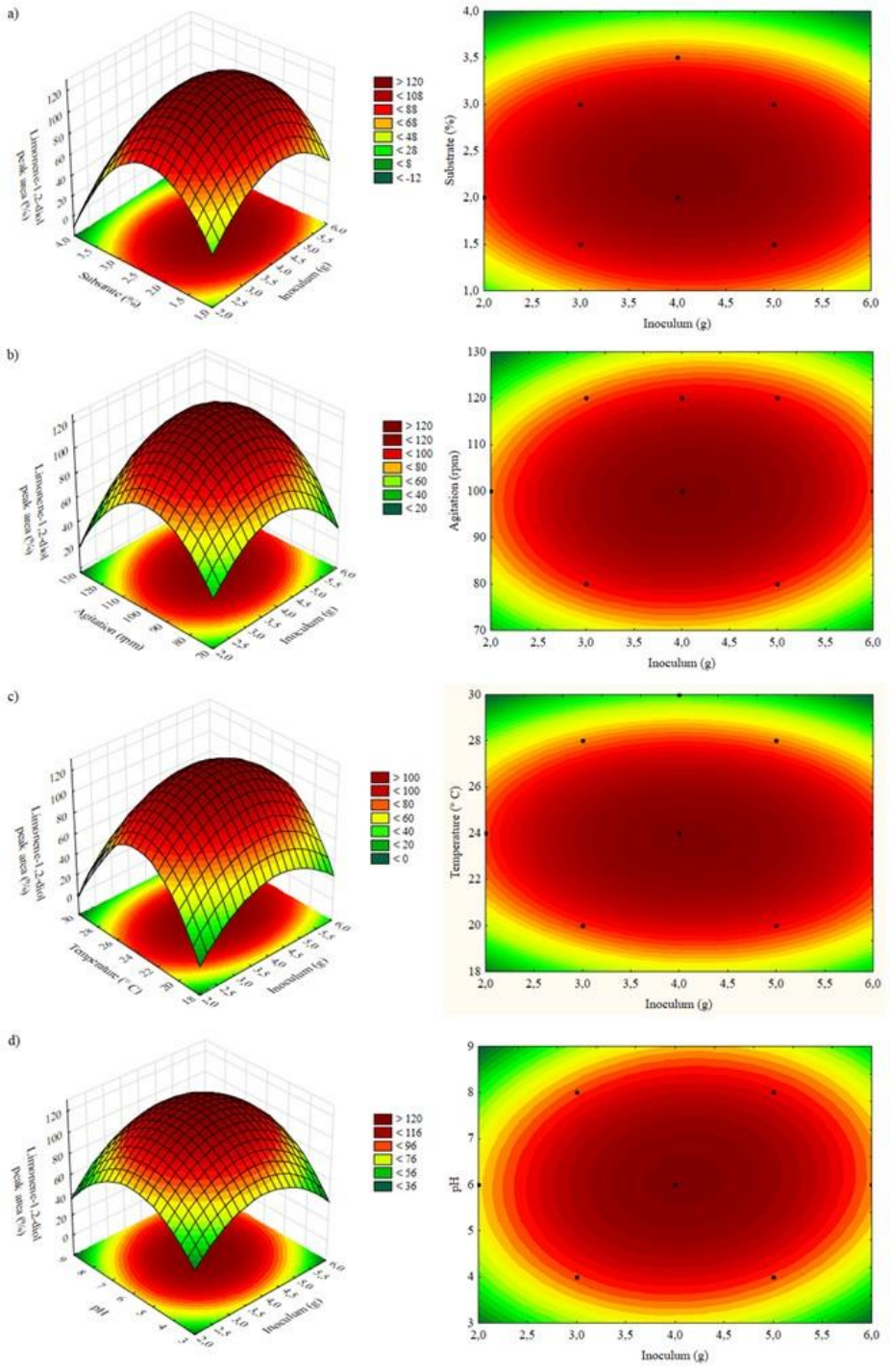

Figure 2. Response surfaces an contour plots for limonene-1,2-diol peak area after 96 h-biotransformation as a function of: incoculum and bubstrate (a); incoculum and agitation (b); incoculum and temperature (c); incoculum and pH (d). For each figure, the non-cited variables were fixed at their center points

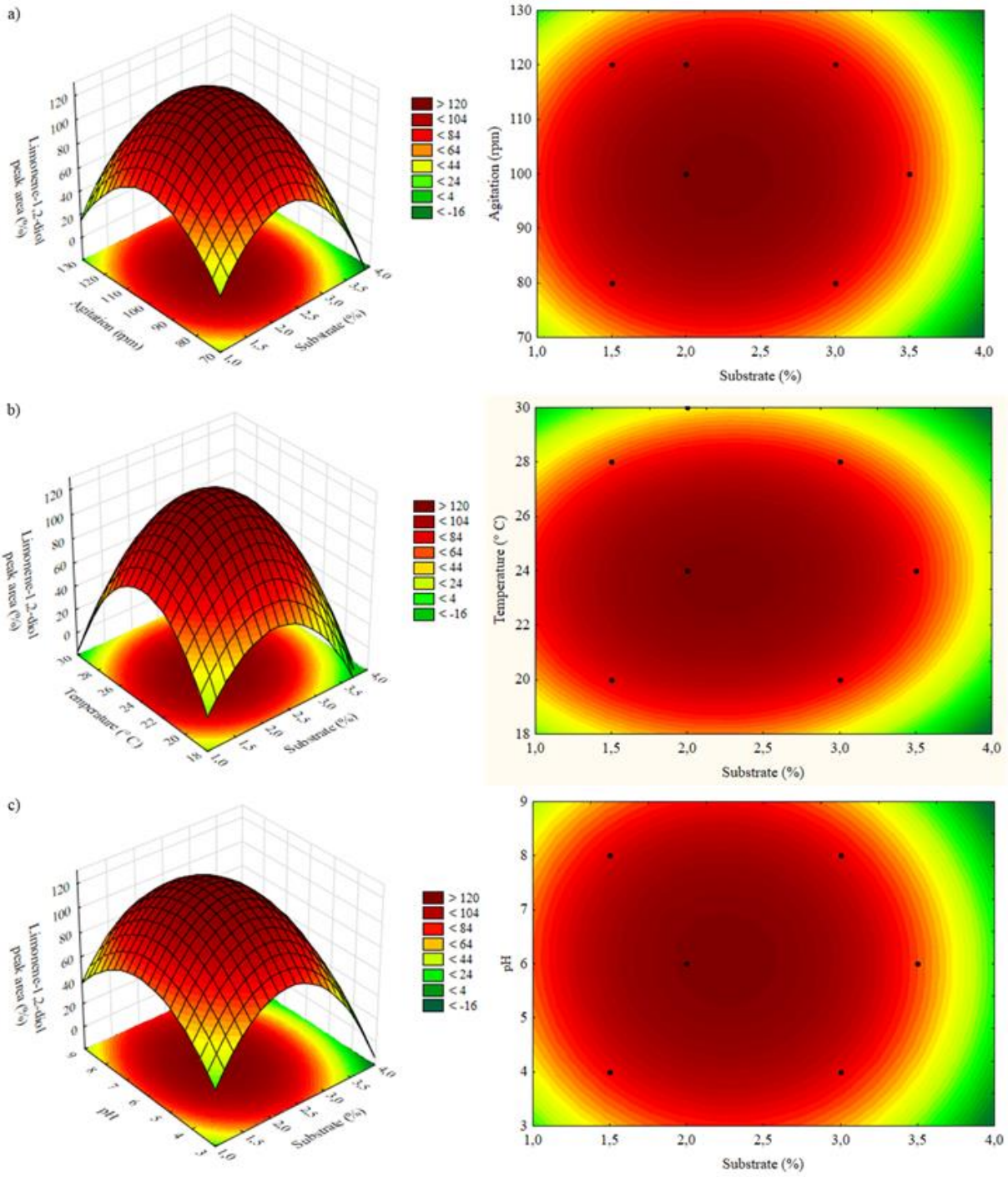

Figure 3. Response surfaces an contour plots for limonene-1,2-diol peak area after 96 h-biotransformation as a function of: substrate and agitation (a); substrate and temperature (b); incoculum and pH (c). For each figure, the non-cited variables were fixed at their center points

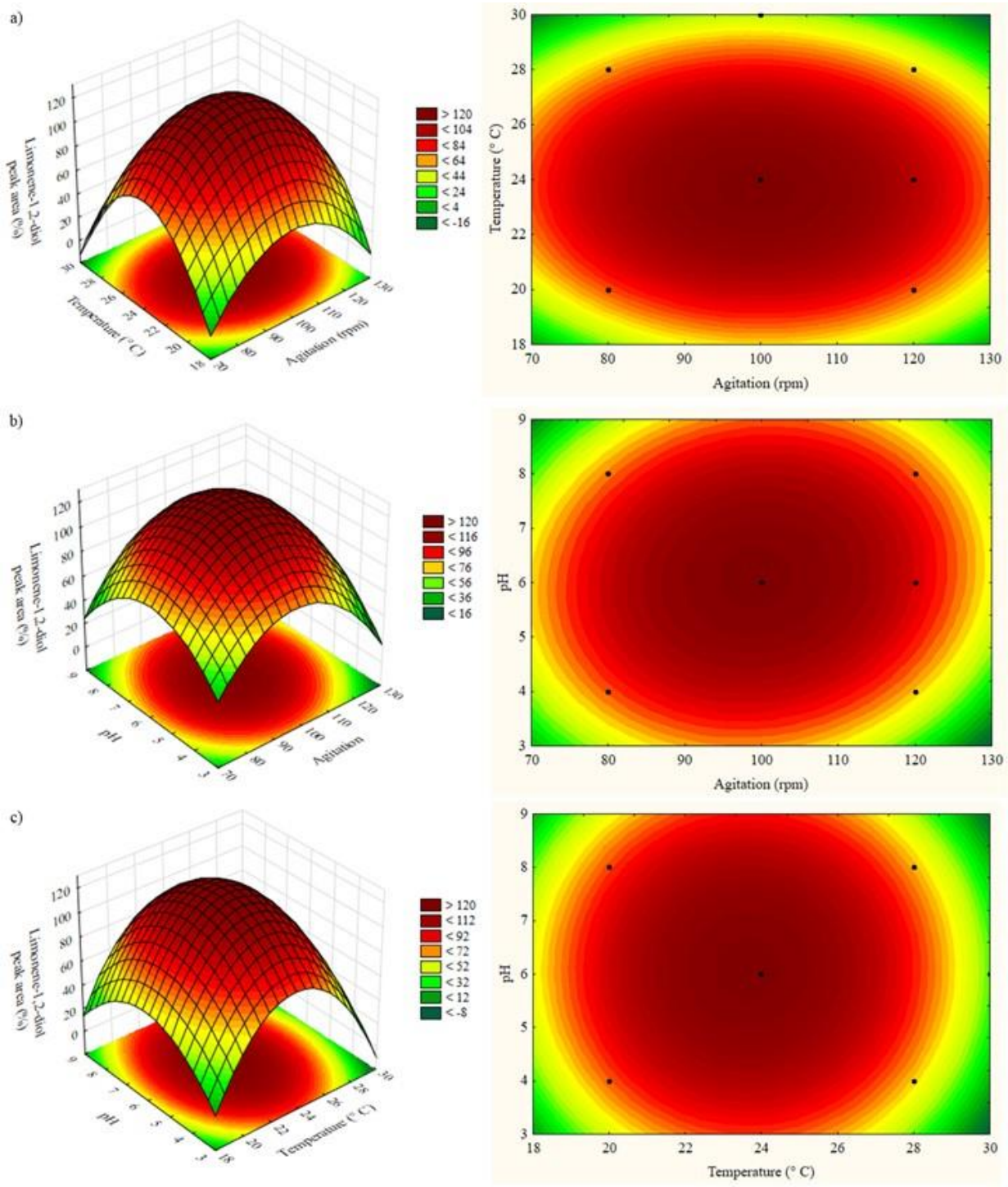

Figure 4. Response surfaces an contour plots for limonene-1,2-diol peak area after 96 h-biotransformation as a function of: agitation and temperature (a); agitation and pH (b); temperature and pH (c). For each figure, the non-cited variables were fixed at their center points

Authors of studies on optimization indicated that the substantial increase in the concentration of products is feasible due to the relevant parameters (temperature, pH, and agitation) in the limonene biotransformation. Some reports of such an approach are available in the literature for optimizing the production of natural aroma compounds, including the limonene biotransformation into limonene-1,2-diol. (Sales, Pastore & Bicas, 2019).

Other authors have used multi-response analysis to establish the optimal process conditions for other systems of limonene biotransformation. For example, Bicas et al. (2008) obtained the best results using 0.5% (v/m) *R*-(+)-limonene, inoculum/medium ratio of 0.25 (m/m), and 72 h cultivation at 26 °C and 240 rpm when they employed a *F. oxysporum* strain. In another study, Rottava et al. (2011) reported production of approximately 1.7 g/L α-terpineol in optimized conditions, comprising of substrate concentration of 1.75%, mass of inoculum of 2 g, and substrate-toethanol volume ratio of 1:1. The biotransformation of limonene to limonene-1,2-diol by *Colletotrichum nymphaeae* CBMAI 0864 in shake flasks presented the best behavior when 13.2 g/L biomass was incubated in the presence of 20 g/L of substrate at 27 °C, 250 rpm and pH of 6.0 (Sales et al., 2019b).

Conclusion

Reduction of manufacturing costs is a constant challenge to all kinds of industries. One of the simplest strategies is shortening the processing time, the rational use of all raw materials and the full exploration of the process. The characterization of a given process is essential to understand the requirements and assess its applicability in larger scale. This study revealed, for instance, that the biotransformation of limonene to its corresponding diol by *P. mangiferae* LabMicrA-505 is a process in that the bioconversion parameters (product productivity) were better when using a 72 h-old biomass and grown in medium YM. Moreover, we showed that the biomass could be stored in a freezer (-18 °C) without significant loss in biotransformation activity even after freeze and lyophilized. Knowing the optimal conditions of a bioprocess is essential for reaching viable large-scale processes. This study suggests that the use of 2,0 % substrate, 4 g biomass as inoculum, 120 rpm, 24 °C, and 6.0 pH, could maximize the production of limonene-1,2-diol via biotransformation of limonene by *P. mangiferae* LabMicrA-505, therefore, this microorganism may be a good proved to be a potent biocatalyst for the biotechnological production of such compound. The experiment

was an effective process for this type of study and these results are very important to guide the scale-up of limonene-1,2-diol in this biotransformation process.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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ESS: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft and Writing – review & editing; BNP: Methodology; AQLD: Funding acquisition, Methodology, Supervision and Writing – original draft; ADLS: Funding acquisition, Methodology, Supervision and Writing – review & editing. This study is part of the doctoral thesis of ESS (PPG-BIONORTE).

Ethical statement

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

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6 **CONCLUSÕES GERAIS**

Visando explorar o potencial dos microrganismos isolados a partir de diferentes substratos da Região Amazônica brasileira para a prospecção de biocatalisadores a serem utilizados na produção de aromas naturais pela biotransformação de limoneno e pineno. O primeiro capítulo da Tese compreendeu uma ampla revisão bibliográfica sobre o potencial dos fungos na biotransformação desses monoterpenos. O trabalho teve como intuito abordar os exemplos mais importantes e promissores para a produção de diferentes compostos de aroma, abordando suas características químico-estruturais, atividades biológicas e potenciais aplicações em biotecnologia. Devido a importância desta revisão para a área e seu amplo caráter como material de referência, o trabalho foi publicado na revista *Brazilian Journal of Chemical Engineering* (Qualis A3 – JCR 1.7).

O segundo capítulo trata-se do *screening* de 47 linhagens de fungos dos gêneros *Penicillium*, *Trichoderma* e *Pestalotiopsis*, além de basidiomicetos (Agaricomycetes, Basidiomycota) capazes de biotransformar limoneno, um substrato monoterpênico presente como subproduto de processamento de frutas cítricas. Das linhagens testadas apenas 12 linhagens, sendo nove fungos endofíticos, dois macrofungos e um fungo aquático demonstraram esse potencial. Contudo, a linhagem mais promissora foi o fungo endofitico *Pestalotiopsis. mangiferae* LabMicrA-505, conseguindo bioconverter todo limoneno em limoneno-1,2-diol. Para avaliação e identificação qualitativa dos compostos de aroma formados após a biotransformação, foi usado a cromatografia a gás (GC-MS).

O terceiro capítulo descreve a prospecção de biocatalisadores a serem utilizados na produção de aromas naturais pela biotransformação de monoterpenos, onze cepas do gênero *Streptomyces* isoladas de solos rizosféricos de ingá (*Inga edulis* Mart.) coletados em áreas do *Campus* da Universidade Federal do Amazonas (UFAM) no estado do Amazonas. Estas foram testadas para biotransformação dos monoterpenos *R*–(+)–limoneno *S*–(-)–limoneno, (+)–α-pineno e (-)–β–pineno, sendo que três linhagens foram capazes de metabolizar pelo menos um substrato para a produção de seus compostos oxigenados. A linhagem de actinomiceto identificada como *Streptomyces* sp. LabMicra 270 mostrou-se capaz de biotransformar α-pineno produzindo *cis*-verbenol (54.94%), myrtenol (2.36%) e verbenona (17.11%).

O quarto capítulo consiste no estudo de condições de cultivo e otimização do processo de biotransformação de limoneno a limoneno-1,2-diol utilizando uma linhagem fúngica de *Pestalotiopsis. mangiferae* LabMicrA-505, através do Delineamento Composto Central Rotacional (DCCR) com 5 variáveis. Os parâmetros de bioconversão foram melhores ao se utilizar uma biomassa de 72 h cultivada em meio YM. A biomassa pode ser armazenada

congelada sem perda significativa na atividade de biotransformação mesmo após o processo de liofilização e congelamento-descongelamento. A variável dependente foi a concentração de limoneno-1,2-diol em 96 horas de processo. De acordo com a análise das superfícies de respostas e curvas de contorno, as melhores condições para a maior produção de limoneno-1,2-diol foram 24°C, 4g de inóculo, 2,0% de substrato e agitações superiores a 120 rpm.

Portanto, o destaque do estudo até o presente momento foi relatar o primeiro ensaio de biotransformação de *R*-(+)-limoneno em limoneno-1,2-diol por espécies do gênero *Pestalotiopsis*. Estas conquistas são importantes para apoiar o desenvolvimento da produção de aromas naturais e para demonstrar o potencial do uso desses microrganismos da Amazônia para o campo da biotecnologia. Este estudo também demonstrou a aplicabilidade de ferramentas estatísticas em processos biotecnológicos de produção de aromas, sendo que a otimização de processos é um importante e vantajoso abordagem que permite o estabelecimento de condições em que há um aumento do rendimento em estudos de biotransformação em escala laboratorial permitindo assim possíveis aplicações em estudos de *scale-up*.

6.1 SUGESTÕES PARA TRABALHOS FUTUROS

Este trabalho atingiu os objetivos proposto de demostrar o potencial de diferentes microrganismos naturalmente isolados em diferentes fontes para serem utilizados na biotransformação destes compostos, e que, portanto, é válido investir recursos e esforços nesta área de prospecção de novos biocatalisadores para a produção de bioaromas. Como perspectivas futuras, estudos que deem continuidade e complementaridade a este trabalho podem ser realizados. Considerando-se os resultados aqui apresentados, sugere-se:

- Realizar a otimização da produção de *cis*-verbenol, myrtenol e verbenone pela linhagem *Streptomyces* sp. LabMicra 270, usando α-pineno como substrato e considerando as condições de cultivo e variáveis estudadas;

- Quantificar os derivados obtidos a partir da biotransformação de limoneno e α-pineno usando cromatografia quiral;

- Usar a resina XAD-2 para a recuperação dos aromas obtidos no processo de biotransformação dos monoterpenos.;

- Quantificar a atividade enzimática dos microrganismos promissores;

- Utilizar novos substratos para os ensaios de biotransformação;

- Utilização de resíduos (agro)industriais como fonte de terpenos para os ensaios de biotransformação;

- Aprofundar os estudos na atividade antimicrobiana e antitumoral dos compostos de aromas derivados do processo de biotransformação, assim como, avaliar sua capacidade antioxidante.

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25. Revisor do artigo Revisor do artigo "Caracterização *in silico* de isoformas dos genes de lacase de Basidiomicetos comestíveis e medicinais" para o periódico Research, Society and Development, 2020.

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- **Outras Informações**

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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

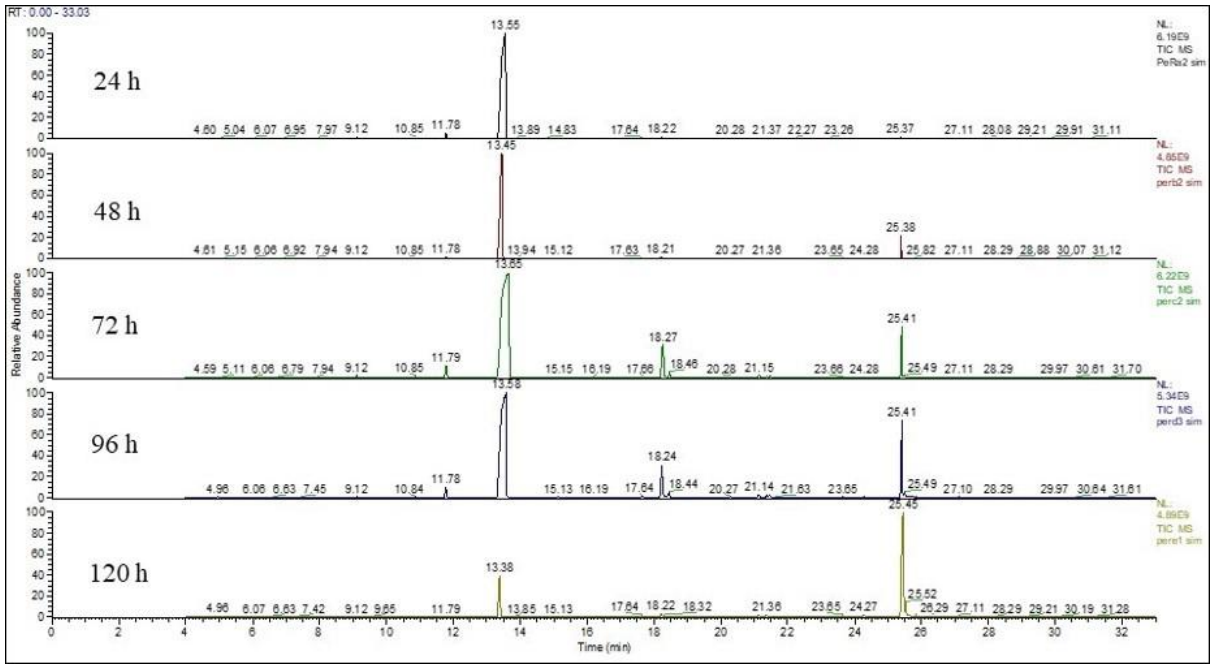
The qualitative analysis was performed by Gas chromatographic Trace Ultra coupled to mass spectrometer ISQ Single Quadrupole – GC/MS (Thermo Scientific™) equipped with a Trace™ TR-5 capillary column with 30 m length x 0.25 mm i.d. x 0.25 µm of film thickness. Helium was used as carrier gas with a flow 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.

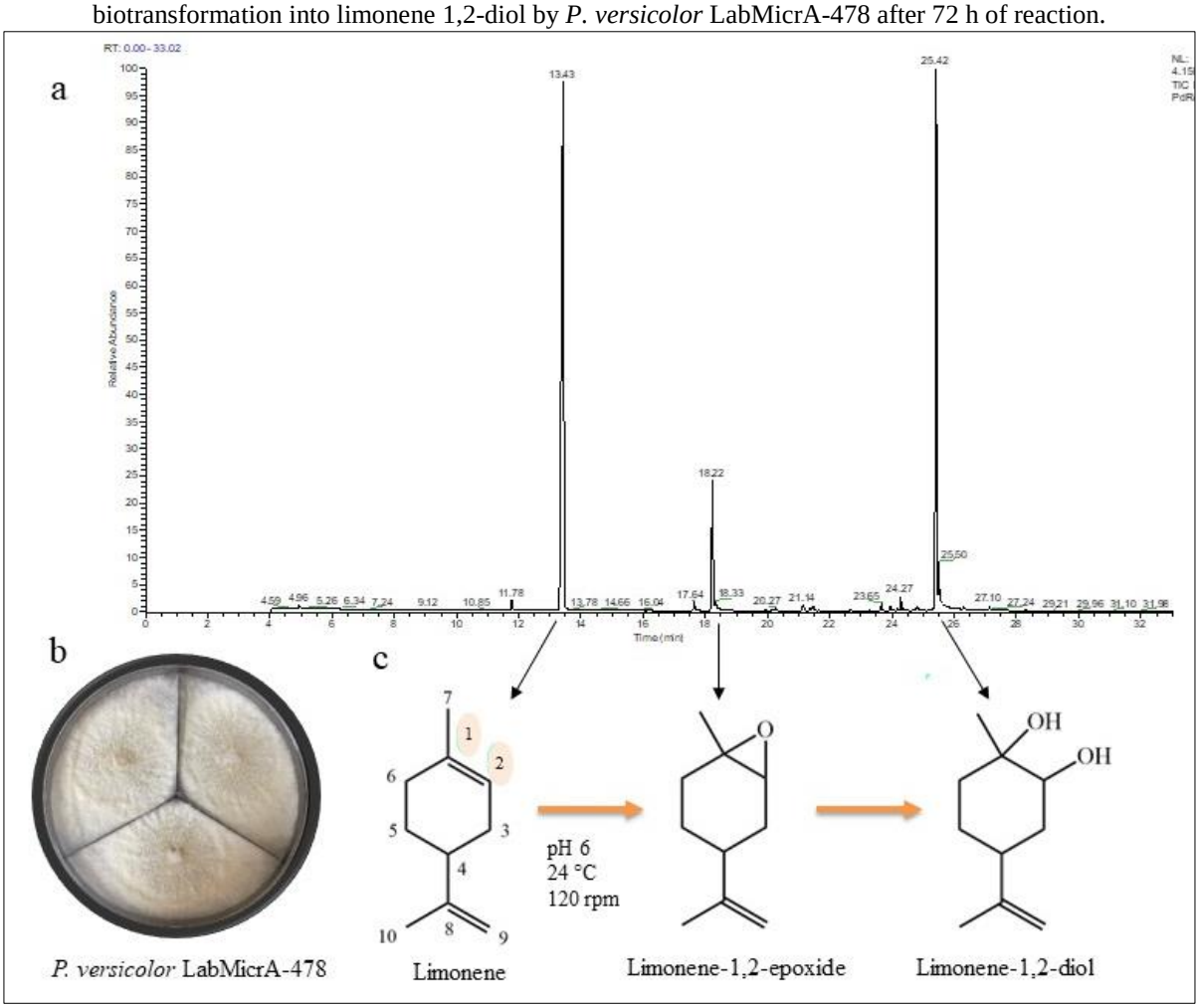


Source: Sevalho (2022)

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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DISCLOSURE STATEMENT

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.

REVISTA DELOS DESARROLLO LOCAL SOSTENIBLE

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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ANEXOS

SPRINGER NATURE LICENSE
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CNPJ 46.323.444/0001-69

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I hereby certify that the manuscript "**Monoterpene biotransformation using actinomycetes of the genus** *Streptomyces* **isolated from the rhizosphere of inga-cipó (***Inga edulis* **Mart.)**" was revised by me and that by being a native English speaker and by holding a degree in Portuguese Language and a post-graduate degree in English, and as a professional translator and reviewer, I am qualified to carry out such work.

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I hereby certify that the manuscript "**Investigation into the effect of culture conditions and optimization on limonene-1,2-diol production from the limonene biotransformation by** *Pestalotiopsis mangiferae* **LabMicrA-505**" was revised by me and that by being a native English speaker and by holding a degree in Portuguese Language and a post-graduate degree in English, and as a professional translator and reviewer, I am qualified to carry out such work.

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Ministério do Meio Ambiente
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Número do cadastro:	**AC1746C**
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CPF/CNPJ:	**04.378.626/0001-97**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Pestalotiopsis mangiferae
Pestalotiopsis versicolor
Pestalotiopsis mangiferae
Pestalotiopsis clavispora
Pestalotiopsis clavispora
Pestalotiopsis neglecta
Trichoderma harzianum
Trichoderma afroharzianum
Clonostachys rosea
Penicillium citrinum
Penicillium oxalicum
Trametes elegans
Pycnoporus sanguineus
Streptomyces sp.
Streptomyces sp.
Streptomyces sp.
Streptomyces sp.
Streptomyces sp.
Streptomyces sp.
Kitasatospora sp.
Streptomyces sp.
Streptomyces sp.
Streptomyces sp.
Kitasatospora sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Streptomyces sp.

Impossibilidade de identificação

Pestalotiopsis microspora

Pestalotiopsis formicarum

Pestalotiopsis disseminata

Trichoderma lentiforme

Trichoderma koningiopsis

Trichoderma asperelloides

Trichoderma sp.

Trichoderma reesei

Penicillium glabrum

Penicillium adametzii

Penicillium sp.

Penicillium rubens

Sebipora aquosa

Daedaleopsis flavida

Fomitopsis nivosa

Lenzites elegans

Ganoderma tornatum

Flavodon flavus

Ganoderma orbiforme

Coriolopsis polyzona

Perenniporia tephropora

Perenniporia centrali-africana

Coriolopsis rigida

Penicillium citreosulfuratum

Título da Atividade: **Potencial biotecnológico de fungos filamentosos e actinobactérias da Amazônia brasileira**

Equipe

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Antonia Queiroz Lima de Souza	**Universidade Federal do Amazonas**
Elison de Souza Sevalho	**Universidade Federal do Amazonas**
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