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PROSPECTING ENDOPHYTIC BIOCATALYSTS FOR BIOFLAVORS PRODUCTION

PROSPECÇÃO DE BIOCATALISADORES ENDOFÍTICOS PARA PRODUÇÃO DE BIOAROMAS

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Tese apresentada a Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Ciência de Alimentos

Orientadora: Profª Drª Gláucia Maria Pastore

ESTE EXEMPLAR CORRESPONDE A VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA MARINA GABRIEL PESSÔA E ORIENTADA PELA PROFª DRª GLÁUCIA MARIA PASTORE

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RESUMO

Esta tese de doutorado teve como objetivos principais a prospecção de novos biocatalisadores fúngicos para serem utilizados no processo de biotransformação de monoterpenos (*R*-limoneno, *S*-limoneno, α-pineno, β-pineno e citronelol) para obtenção de aromas naturais, assim como otimizar as condições de processo para a maximização da concentração dos produtos obtidos. A primeira etapa do trabalho prático deste projeto envolveu o isolamento de micro-organismos endofíticos e seleção das melhores linhagens para a produção de bioaromas. Estes micro-organismos foram isolados de frutos verdes de jenipapo (*Genipa americana* L.), um fruto da flora brasileira rico em monoterpenos. Quinze linhagens diferentes de fungos filamentosos endofíticos foram isoladas de frutos verdes de jenipapo, dos quais, três mostraram a capacidade de metabolizar os monoterpenos testados. A linhagem identificada como JV-7 foi capaz de bioconverter α-pineno e produzir verbenol e verbenona, compostos muito visados na indústria de aromas e fragrâncias. Na biotransformação de β-pineno, foi possível a obtenção de pinocarveol, utilizando-se a linhagem JV-15. Este composto tem maior valor agregado comparado a seu precursor, e este é um dos poucos trabalhos envolvendo sua produção. Os isômeros de limoneno foram biotransformados pela linhagem JV-10 a dois produtos majoritários diferentes: neodihidrocarveol e limoneno-1,2-diol a partir de *R*- e *S*- limoneno respectivamente. Visando aumentar a concentração dos compostos de aroma previamente obtida utilizando as linhagens de fungos endofíticos, as variáveis agitação, temperatura, concentração inicial de substrato e quantidade de biomassa fúngica adicionada foram estudadas em dois delineamentos experimentais. No primeiro delineamento composto central rotacional (DCCR), as condições de processo para a biotransformação de α-pineno em verbenol foram otimizadas: 200 rpm, 22 ºC, concentração de substrato igual a 1,25 % (v/v) e 5 g de biomassa de biocatalisador em 50 mL de meio. Nessas condições, foi possível aumentar aproximadamente 1,4 vezes a concentração final de verbenol, de 222,4 ± 2 mg/L para 318,4 ± 2,4 mg/L. O segundo DCCR envolveu a biotransformação de β-pineno para produção de pinocarveol. Neste caso, as condições otimizadas foram: concentração de substrato de 1 % (v/v), biomassa fúngica 5 g/50 mL de meio, agitação de 150 rpm e 30 ºC, o que resultou em um aumento na concentração de produto de 64,8 ± 0,6 mg/L para 141,32 ± 2,6 mg/L, ou seja, o dobro da concentração inicial pode ser obtida. O estudo da otimização das condições de processos para a produção de aromas naturais é uma das estratégias mais promissoras que vem sendo utilizadas para o aumento da concentração de produto e consequentemente, para redução de custos e

viabilização da sua produção. Além disso, este trabalho demonstra o potencial de micro-organismos endofíticos, encontrados naturalmente em fontes ricas em terpenos para serem utilizados como novos biocatalisadores na biotransformação destes compostos e obtenção de extratos com características sensoriais inovadoras e interessantes.

ABSTRACT

This work aimed to prospect novel fungal biocatalysts to be employed in biotransformation of terpenes (*R*-limonene, *S*-limonene, α-pinene, β-pinene e citronellol) for natural aroma production, as well as the optimization of process conditions in order to increase final product concentration. Initially, the practical work comprised the isolation of endophytic microorganisms and the screening of the best strains for bioflavors production. These microorganisms were isolated from unripe genipap (*Genipa americana* L.) fruits, a Brazilian unexploited source of monoterpenes and iridoids. Fifteen endophytic filamentous fungi strains were isolated from unripe genipap, from which, three strains showed the ability to metabolize the tested monoterpenes. A strain identified as JV-7 was able to bioconvert α-pinene into verbenol and verbenone, interesting compounds for flavors and fragrance industries. In the biotransformation of β-pinene, the major product obtained was pinocarveol, using the strain JV-15. Pinocarveol has higher reference price when compared to its precursor, and, there are few works regarding its production. The isomers of limonene were metabolized by JV-10 strains and two different products could be obtained: neodihydrocarveol and limonene-1,2-diol, from *R*- and *S*-limonene respectively. Aiming to increase final product concentration obtained with endophytic strains, the variables agitation, temperature, substrate concentration and amount of biocatalyst biomass were evaluated in two experimental designs. In the first central composite design (CCD), the biotransformation of α-pinene into verbenol could be optimized using the process conditions: 200 rpm, 22 ºC, substrate concentration of 1.25 % (v/v) and fungal biomass of 5 g in 50 mL media, increasing verbenol concentration from 222.4 ± 2 mg/L to 318.4 ± 2.4 mg/L. The second CCD, aimed to optimize β-pinene biotransformation for pinocarveol production. In this case, the optimum conditions were substrate concentration of 1% (v/v), 150 rpm, 30 ºC and 5g of fungal biomass per 50 mL media. It was achieved a 2-fold increase in pinocarveol concentration, from 64.8 ± 0.6 mg/L to 141.32 ± 2.6 mg/L. The study of process parameters for natural aroma production is one of the most promising tools that are being used to increase product concentration and consequently to reduce costs in its production. In addition, this work shows the potential of endophytic microorganisms that can be naturally found in terpene rich sources to be employed as new biocatalysts in biotransformation assays, obtaining extracts with novel and interesting sensorial characteristics.

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INTRODUÇÃO GERAL

1. Aditivos naturais na indústria de alimentos

A aceitação de produtos do mercado de alimentos está diretamente relacionada com a forma na qual este é apresentado e as impressões causadas aos consumidores. Geralmente a primeira característica observada é a aparência do alimento, evidenciada principalmente pela sua coloração, seguida pelas características aromáticas, que influenciam tanto no odor, quando no sabor do alimento (Fanaro et al., 2016).

A coloração dos alimentos é utilizada para inferir grau de maturação, frescor, composição e qualidade. Experiência prévia, heranças culturais e instinto estão relacionados à associação de tonalidades de cor às classes específicas de alimentos (de Carvalho et al., 2016). Corantes são adicionados em alimentos nos casos onde a cor é perdida durante seu processamento ou armazenamento, para intensificar a cor já presente, para minimizar diferenças entre produtos do mesmo lote ou até para proteger outros componentes do alimento (de Carvalho et al., 2016; Molina et al., 2016).

De maneira semelhante, os aromas reforçam a qualidade sensorial do produto sem apresentar função nutritiva, influenciando tanto o sabor quanto o odor dos alimentos (Longo; Sanromán, 2006). São relatados pelo menos 8000 compostos voláteis, portanto, há inúmeras possibilidades de combinação entre estes, resultando em uma única característica aromática para cada tipo de alimento. Devido a essa particularidade, o aroma pode ser considerado o principal atributo dos alimentos para definir sua aceitação (Fanaro et al., 2016).

Devido a sua evidente importância, há uma crescente preocupação em suprir a demanda de aditivos alimentares, principalmente daqueles classificados como naturais. Atualmente, o mercado consumidor tem preferência pela substituição de compostos sintéticos por naturais, devido à sua conscientização da relação entre saúde e nutrição (Mapari et al., 2010). A filial da empresa Nestlé (Suíça) localizada nos Estados Unidos, por exemplo, anunciou em fevereiro de 2015 a eliminação de corantes e aromatizantes artificiais de seus chocolates até o final do mesmo ano em resposta a essa preferência de seus consumidores, conforme já havia ocorrido nas filiais de outros países (Leffingwell; Leffingwell, 2015). A empresa buscou por frutos e vegetais como fontes alternativas de corantes, como por exemplo, a substituição dos corantes *Red 40* e *Yellow 5* do chocolate Butterfinger™ por

corantes obtidos em sementes de urucum (*Bixa orellana*) e a substituição do aroma artificial de baunilha pelo mesmo composto, de origem natural no chocolate Crunch™ (Nestlé USA – Press Release). Neste contexto, a biotecnologia é uma área em expansão que surge como uma alternativa para a obtenção de aditivos alimentares produzidos naturalmente.

Biotecnologia pode ser definida como "uma aplicação tecnológica que emprega sistemas biológicos, organismos vivos ou derivados (por exemplo, enzimas), para a produção ou modificação de produtos ou processos para uso específico" (FAO, 2000; Molina; Fanaro, 2016). O uso de micro-organismos em processos biotecnológicos é vantajoso, uma vez que estes são biocatalisadores versáteis, capazes de produzir uma vasta gama de enzimas, essenciais nas reações químicas para obtenção dos produtos e metabólitos secundários que podem ser purificados e aplicados diretamente como aditivos (Molina; Fanaro, 2016; Pessôa et al., 2017). No caso da utilização de micro-organismos como biocatalisadores, membranas e paredes celulares são barreiras físicas que protegem as enzimas das forças de cisalhamento e outros fatores, além da possibilidade de regeneração de cofatores, como NADH e NADPH (de Carvalho; da Fonseca, 2006).

O emprego da biotecnologia é considerado sustentável uma vez que os processos ocorrem em condições brandas, geram menor quantidade de efluentes tóxicos, emissões e subprodutos quando comparados com processos químicos tradicionais. Embora promissora, esta área ainda precisa de esforços em pesquisa e desenvolvimento, principalmente no ramo de aditivos (Neri-Numa et al., 2016; Felipe et al., 2017).

2. Aromas

Aromas são compostos de baixo peso molecular, geralmente menores que 400 Da, que podem ser percebidos por via nasal (odor) ou retronasal (aroma) (Berger, 1995). 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, ou seja, não apresentam uma função química específica (Bicas et al., 2009). Além disso, apresentam grande diversidade quanto à polaridade, solubilidade, volatilidade e temperatura e pH de estabilidade (Berger, 1995; Berger, 2007).

Dentre os aromas, os ésteres pertencem ao grupo mais importante dos compostos com característica frutada, sendo aplicados em produtos com esse aroma característico e laticínios. Alguns exemplos são: acetato de etila (frutal), acetato de butila (odor frutal forte,

com reminiscente de abacaxi), acetato de isoamila (banana), butirato de etila (reminiscente de abacaxi) e hexanoato de etila (morango, kiwi, maçã e abacaxi). As lactonas também são reconhecidas pelo seu aroma frutal, como por exemplo γ-decalactona (pêssego) e 6-pentil-α-pirona (coco) (Dionísio et al., 2012; Fanaro et al., 2016).

No grupo dos álcoois, 1-octen-3-ol tem odor herbáceo, com notas de lavanda e o composto 2-feniletanol possui odor característico de rosa, podendo ser aplicado em perfumes, cosméticos e alimentos (Burdock, 2010). Entre os aldeídos, benzaldeído e vanilina são os mais importantes e amplamente utilizados na indústria. O benzaldeído possui aroma de amêndoa e a vanilina é bastante empregada na indústria de alimentos devido ao seu aroma de baunilha (Dionísio et al., 2012). Uma classe de destaque são os terpenos, sendo os mais abundantes na natureza uma vez que são responsáveis pela constituição da maior parte dos óleos essenciais de plantas (Molina et al., 2014). Estes compostos serão descritos com maior destaque no tópico abaixo.

Apesar de estarem presentes em concentrações muito baixas, os aromas são muito potentes, com limites de detecção na ordem de partes por bilhão (ppb), e podem representar cerca de 50% do custo total do produto (Fanaro et al., 2016). Em 2016, a indústria de aromas e fragrâncias movimentou cerca de US$ 24,5 bilhões, sendo as empresas Givaudan, Firmenich e IFF responsáveis por 18,7 %, 13,5 % e 12,5 % do mercado, respectivamente (Leffingwell, 2017).

2.1. Formas de obtenção dos compostos de aroma

A obtenção de compostos de aroma é possível através de três métodos: síntese química, extração de recursos naturais ou síntese biotecnológica. 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 não apresentam régio- ou enantioseletividade ao substrato, podendo formar uma mistura de isômeros indesejada. Além disso, esse tipo de processo gera imensos impactos ambientais devido a liberação de resíduos não-biodegradáveis. Em relação à extração de aromas a partir de vegetais, os compostos são obtidos em concentrações muito baixas e há instabilidade quanto a sua extração, pois depende de fatores sazonais e climáticos e pode causar problemas ecológicos devido ao extrativismo. Desta forma, a biotecnologia é a forma mais promissora de se obter compostos de aroma (Bicas et al., 2010; Felipe et al., 2017).

Os processos biotecnológicos podem ser divididos em síntese *de novo* e biotransformação/bioconversão. No primeiro caso, micro-organismos são cultivados em meios de cultura simples e todo o arsenal metabólico é ativado para a produção de uma mistura de compostos (Maróstica Jr.; Pastore, 2007). Por outro lado, a biotransformação ocorre devido à biocatálise de precursores adicionados ao meio de cultura e que são metabolizados por micro-organismos formando um produto principal através de uma única reação enzimática e que possui estrutura química muito semelhante a do seu precursor (Berger, 1997; Berger, 2007).

Um dos principais desafios da implementação da biotransformação é a obtenção de linhagens de micro-organismos adequadas para este processo. É necessário que o micro-organismo seja robusto e resistente tanto ao substrato, como aos produtos formados, que podem causar efeitos inibitórios no crescimento das células. Além disso, as linhagens devem conseguir metabolizar o substrato como única fonte de carbono e acumular metabólitos de interesse (Bicas; Pastore, 2007).

Industrialmente, estão sendo desenvolvidos diversos métodos para a produção biotecnológica de aromas, a maioria deles empregando leveduras no processo. A empresa Amyris produz β-farneseno a partir de cana de açúcar, utilizando uma linhagem geneticamente modificada de *Saccharomyces cerevisiae*. Este sesquiterpeno também tem potencial aplicação como precursor para a formação de outros compostos de aroma (Felipe et al., 2017; Schempp et al., 2017). Esta mesma empresa em parceria com a Firmenich desenvolveu um substituto para o óleo de patchouli (*Pogostemon* sp.) (Clearwood), com características sensoriais mais agradáveis que o óleo essencial natural (Leffingwell; Leffingwell, 2015). Também tem sido reportada a produção de valenceno e seu derivado, nootkatona pelas empresas Evolva e Isobionics. No caso da empresa Isobionics utiliza-se uma linhagem de *Rhodobacter sphaeroides* que contém os genes para a biossíntese de monoterpenos (Huembelin et al., 2014).

No caso da produção biotecnológica de vanilina, geralmente são utilizados micro-organismos geneticamente modificados e as maiores produtoras são as empresas Evolva-IFF, Mane, Solvay-Rhodia e BASF (Felipe et al., 2017). Em destaque, a empresa chinesa Shanghai Apple Flavor & Fragrance Group Co. Ltd. que utiliza uma linhagem isolada de amostras de solos de pomar para a conversão de ácido ferúlico em vanilina, demonstrando que linhagens

selvagens também tem potencial aplicação da produção industrial de bioaromas (Xu et al., 2009).

Neste contexto, muitos pesquisadores têm dedicado esforços para o isolamento e seleção de linhagens para serem aplicadas em processos biotecnológicos para a produção de aromas naturais (Rottava et al., 2010; Molina et al., 2013; Palmerín-Carreño et al., 2015; Bier et al., 2011). Endofíticos são micro-organismos encontrados naturalmente no interior de frutos e outros tecidos vegetais, porém não causam doenças à planta hospedeira. O uso de micro-organismos endofíticos é vantajoso uma vez que estes podem ser selecionados a partir de fontes ricas em monoterpenos, demonstrando o potencial de resistência dessas linhagens ao composto a ser utilizado como substrato. (Pimentel et al., 2011; Abrahão et al., 2013; Molina et al., 2013).

Além disso, os baixos rendimentos e concentrações encontrados em processos biotecnológicos podem ser superados com o emprego de modelos matemáticos e métodos estatísticos, como, por exemplo, a utilização de delineamentos experimentais para a otimização de processos. Métodos clássicos de otimização geralmente são realizados pelo estudo dos fatores separadamente, ou seja, apenas um dos fatores é variável enquanto os demais são mantidos em valores constantes. No caso de delineamentos compostos centrais rotacionais, todas as variáveis são avaliadas ao mesmo tempo, assim como a interação entre elas. Com essa metodologia, é possível propor um modelo matemático que irá descrever o efeito das variáveis estudadas sobre a resposta desejada (Rodrigues; Iemma, 2005).

3. Terpenos

Terpenos são metabólitos secundários de plantas, produzidos com a função de defesa contra micro-organismos e insetos (terpenos voláteis) e também como pigmentos acessórios para captação de luz e agentes antioxidantes protetores aos danos causados pela luz (tetraterpenos não voláteis). Além disso, estão relacionados às características odoríferas e à atração de polinizadores pelas plantas (Schempp et al., 2017). Estruturalmente, são constituídos de resíduos de isopreno (C_5H_8), podendo ser classificados de acordo com o número de carbonos presente na molécula, como monoterpenos (dez carbonos), sesquiterpenos (quinze carbonos), diterpenos (vinte carbonos), triterpenos (trinta carbonos) e tetraterpenos ou carotenos (quarenta carbonos) (de Carvalho; da Fonseca, 2006).

Esses compostos não possuem grupos funcionais em sua estrutura, já os seus derivados oxidados, denominados terpenóides, possuem o mesmo esqueleto carbônico do terpeno original, mas ainda a presença de grupos funcionais como álcoois, aldeídos, ácidos, cetonas ou epóxidos terpênicos em sua composição, o que colabora para suas características sensoriais interessantes (Duetz et al., 2003; Pimentel, 2012).

Os terpenos são encontrados em grandes quantidades em óleos essenciais, cujo aroma é atribuído principalmente aos mono e sesquiterpenos (terpenos mais voláteis) presentes em sua constituição. Na maioria dos casos, o odor característico de frutas, ervas e especiarias é devido aos terpenos voláteis (Krings et al., 2006). Devido à suas características, estes compostos são muito visados pela indústria de fragrâncias e aromas (de Carvalho; da Fonseca, 2006).

Entre os monoterpenos, há a classificação de acordo com a ciclização de sua cadeia, podendo ser monoterpenos acíclicos, monocíclicos ou bicíclicos (Figura 1) (Bicas et al., 2009; Fontanille et al., 2002). Em alguns casos, mono e sesquiterpenos podem ser encontrados em rejeitos industriais, portanto possuem um baixo custo. Isso torna viável a utilização destes terpenos como precursores para sua biotransformação em compostos oxidados de maior valor agregado. Dois exemplos são o limoneno e o α-pineno, monoterpenos abundantes na natureza e amplamente utilizados como substrato para biotransformação (Burdock, 2010).

3.1. Genipina

Outro importante monoterpenóide, da classe dos iridoides é a genipina. Este composto é um metabólito secundário incolor e sem aroma característico, encontrado em frutos de *Gardenia jasminoides* J. Ellis (gardênia) e frutos verdes de *Genipa americana* L. (jenipapo), ambos da família Rubiaceae (Buchweitz, 2016). Estruturalmente, a genipina é caracterizada pela presença de uma unidade ciclopentanoide com um anel dihidropirano, sendo que o grupo hidroxila na posição C1 pode ser substituída por 1 ou 2 unidades de glicose, formando seus derivados glicosilados: geniposídeo (genipin-1-O-β-glucosídio) e genipin-1-O-β-D-gentibiosídio, respectivamente (Figura 1) (Brauch, 2016).

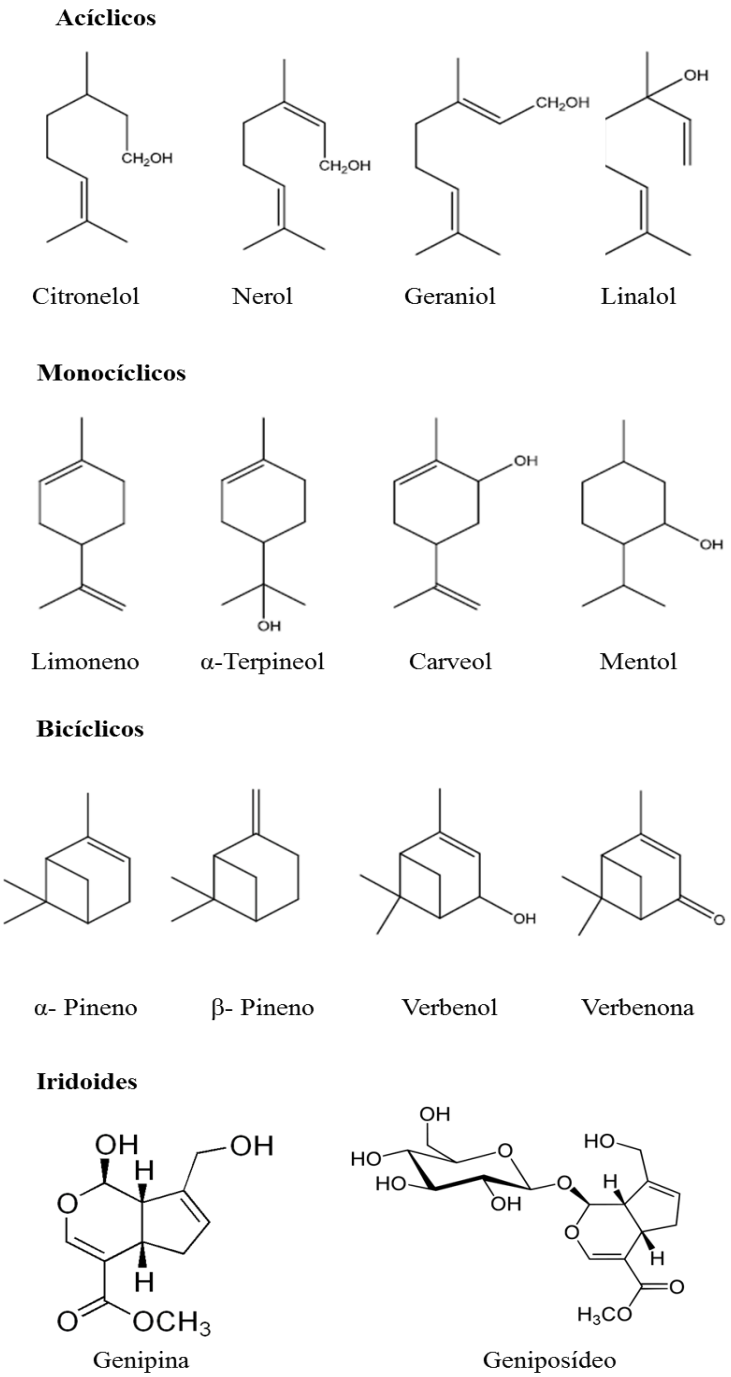

Figura 1. Estruturas de alguns dos monoterpenos mais estudados

Enquanto genipina livre (aglicona) pode ser encontrada em frutos verdes de jenipapo, apenas a forma glicosilada (geniposídeo) pode ser obtida em frutos de gardênia, portanto, para a obtenção de genipina livre, é necessário o tratamento do geniposídeo com β-glicosidases (Ramos-de-la-Peña et al., 2016).

Na presença de oxigênio, a genipina reage com aminoácidos primários ou proteínas formando pigmentos roxo-azulados solúveis em água. O mecanismo pelo qual a

formação de pigmentos ocorre ainda não está completamente elucidado, porém é extremamente dependente do pH (Wu, 2013; Tokareva et al., 2017). Devido a essa característica, a genipina tem grande potencial para ser explorada como corante azul a ser aplicada como aditivo em determinados alimentos, embora ainda sejam necessários estudos considerando sua estabilidade e a sua reatividade.

Devido ao fato de frutos verdes de jenipapo apresentarem uma grande quantidade de monoterpenos da classe dos iridóides, este fruto pode ser considerado uma potencial fonte de biocatalisadores resistentes a esse tipo de composto e que, portanto, poderiam ser aplicados em processos de biotransformação de monoterpenos para a produção de aromas naturais. Uma vez identificado o biocatalisador adequado, estudos envolvendo delineamentos experimentais devem ser aplicados para melhorar o processo fermentativo e possibilitar uma possível implementação da produção de aromas naturais.

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OBJETIVOS

1. Objetivo geral

O objetivo principal desta tese de doutorado foi avaliar o desempenho de micro-organismos endofíticos isolados de frutos verdes de jenipapo na biotransformação de monoterpenos, visando obter novos biocatalisadores para serem utilizados na produção biotecnológica de aromas naturais. Além disso, a sequencia deste trabalho visou estudar o efeito dos parâmetros temperatura, agitação, concentração inicial de substrato e concentração de biomassa de catalisador, através de um delineamento experimental a fim de se otimizar as condições do processo de biotransformação de terpenos e aumentar as concentrações de produtos obtidas.

2. Objetivos específicos

Capítulo 1: Elaborar uma revisão bibliográfica sobre a utilização de monoterpenos como substrato em processos biotecnológicos para a produção de aromas naturais.

Capítulo 2: Isolar fungos endofíticos de frutos verdes de jenipapo e selecioná-los de acordo com a sua capacidade de utilizar substratos monoterpênicos e produzir compostos de maior valor agregado para a indústria de aromas e fragrâncias.

Capítulo 3: Avaliar a influência dos parâmetros de processo na biotransformação de α-pineno a verbenol e otimizar condições de cultivo para a obtenção de maiores concentrações de verbenol.

Capítulo 4: Otimizar a produção de pinocarveol a partir de β-pineno visando conhecer os efeitos de cada variável e encontrar as melhores condições de processo.

CAPÍTULO 1 – REVISÃO BIBLIOGRÁFICA

THE USE OF MONOTERPENES AS SUBSTRATE IN BIOTRANSFORMATION PROCESSES FOR PRODUCTION OF NATURAL AROMA COMPOUNDS

ABSTRACT

The biocatalytic bioconversion or biotransformation of monoterpenes allows significantly enhanced accumulation of a desired flavor product, since they are a structurally related precursor molecule. As a prerequisite for this strategy, the precursor must be present in nature and its isolation in sufficient amounts from the natural source must be easily feasible in an economically viable fashion (e.g., the monoterpenes limonene and α-pinene). This chapter is intended to review some monoterpenes with potential to be used as substrates to obtain new natural flavor compounds with economic and commercial interest. This chapter also describes the properties and uses of the most promising monoterpenes used as substrates, which could be important for an industrial standpoint increasing their importance as starting materials to obtain new molecules for biotechnological and biological applications.

Keywords: Terpenes, Biotransformation, Bioconversion, Aroma compounds, Bioflavors

1. Introduction

The methods for obtaining flavor compounds include the direct extraction from nature, chemical transformations and biotechnological transformations (which include microbial and enzymatic biotransformations, de novo synthesis and the use of genetic engineering tools) (Franco, 2004.) The scientific literature contains many examples of reviews dealing with the chemical reactions of terpenes to produce flavors and the biotransformation of volatile terpenes for aroma production (Swift, 2004; Bicas et al., 2009; de Carvalho; da Fonseca, 2006; Van der Werf et al., 1997).

Despite the great industrial application of aroma compounds produced via chemical synthesis (still responsible for a large portion of the market due to the satisfactory yields), the bioprocesses possess a number of inherent advantages when compared with the classical chemical processing, since it occurs at mild conditions, presents high regio- and enantio-selectivity, does not generate toxic wastes and the products obtained may be labeled as "natural" (Bicas et al., 2009; Berger, 2007). Also, biotechnological processes usually involve less damaging process conditions for the environment and yield desirable enantiomeric flavor compounds. Thus, bioflavors appeal to many sectors and represent a high market value (Gatfield, 1997; Krings; Berger, 1998).

Therefore, the biocatalytic conversion of a structurally related precursor molecule (bioconversion or biotransformation processes) is often a more adequate strategy which allows significantly enhanced accumulation of a desired flavor product. As a prerequisite for this strategy, the precursor must be present in nature and its isolation in sufficient amounts from the natural source must be easily feasible in an economically viable fashion (e.g., the monoterpenes limonene and α-pinene). Among the most targeted substrates for biotransformation/bioconversion approaches are the monoterpenes (Bicas et al., 2009; Krings; Berger, 1998).

Thus, this chapter is intended to review some monoterpenes with potential to be used as substrates to obtain new natural flavor compounds with economic and commercial interest. This chapter also describes the properties and uses of the most promising monoterpenes used as substrates, which could be important for an industrial standpoint increasing their importance as starting materials to obtain new molecules for biotechnological and biological applications.

2. Monoterpene biosynthesis

Terpenes are secondary metabolites of plants produced in part for defense against microorganisms and insects. Previously, it was believed that these compounds were derived solely from the mevalonate pathway. However, some inconsistencies were observed, leading to the discovery of new biosynthetic pathways independent of the mevalonate pathway (Eisenreich et al., 2001; Rohmer, 1999). Anyway, all terpenes are from common intermediates - the isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) - whose synthesis is based on the path of mevalonate or towards deoxixilulose phosphate (Baser; Demici, 2007).

In other words, by having the same basic biochemistry these terpenes presents structural features and are grouped into a single class. The common feature of all terpenoids refers to the fact that they have a structure that can be decomposed into isoprene units (C_5H_{10}) and, therefore, have the general formula $(C_5H_{10})n$. The volatile terpenes, i.e., mono (n = 2), and sesquiterpenes (n = 3) are major constituents of a variety of essential oils (Table 1), due to their organoleptic properties, are widely used in the flavor industry, as fragrances or as ingredients in foods and cosmetics.

Table 1. Major components of some essential oils (Rowe, 2005)

Essential Oil	**Main components***
Caraway	*S*-(+)-Carvone, limonene, myrcene, α-phelandrene, α-pinene, β-pinene
Dill	*S*-(+)-Carvone, α-phelandrene, limonene
Eucalyptus	1,8-Cineol, α-pinene, p-cimene, limonene
Ginger	Zingiberene, α-curcumene, β-sesquiphelandrene, bisabolene, camphene, β-phelandrene, 1,8-cineol
Orange peel	Limonene, myrcene, linalool, citronelal, neral, geranial,valencene, α- and β-sinensal
Rose	Citronellol, geraniol, nerol, eugenol, geranilacetate, rose oxide
Mint	*R*-(–)-Carvone, limonene, myrcene, 1,8-cineol, dihydrocarveol

*The compounds listed are terpenes or their derivatives.

3. Monoterpenes as substrates for biotransformation processes

3.1. Limonene

Limonene is the most abundant naturally occurring monoterpene and represents up to 90% of orange peel oil, an interesting and inexpensive citrus byproduct (Bauer et al., 1990). The expansion of the orange juice industry in Florida between 1945 and 1960 increased the percentage of processed oranges from 1% to 80%, and consequently the availability of large amounts of low-cost *D*-limonene from the peel oil (Murdock and Allen, 1960). Therefore, limonene has become one of the most studied precursors in bioconversion processes for production of high-value derivatives, which may be a good strategy for enrich the commercial value of agro industrial residues, since the bulk price of this compound is around US$ 1-2/Kg, while its oxygenated counterparts, such as menthol and carvone, cost around US$ 30-60/Kg (Maróstica jr. and Pastore, 2007; Mazzaro, 2000). Figure 1 shows the structure of limonene and the other monoterpenes selected in this chapter, with potential to be used as substrate for the bio-production of new compounds.

Research and development on the degradation of limonene continued to draw much attention on a wide variety of conversion products such as perillic compounds, carveol, carvone at significant amounts, which could be more valuable in the fields of cosmetics, food ingredients, drug, and chemical synthesis. A number of reports in the literature concerned with the biotransformation of limonene leading to many oxygenated derivatives were reviewed by Maróstica and Pastore (2007) and Duetz et al. (2003).

More recently Bicas et al. (2008a) reported the capability of *P. fluorescens* to metabolize limonene in α-terpineol at high concentrations of about 11 g/L, besides other products like limonene-1,2-oxide. *Pseudomonas putida* GS1 was able to convert limonene to perillic acid (up to 11 g/L) when the bacteria was cultivated in fed-batch culture with non-limiting amounts of glycerol, ammonium, and limonene (Mars et al., 2001).

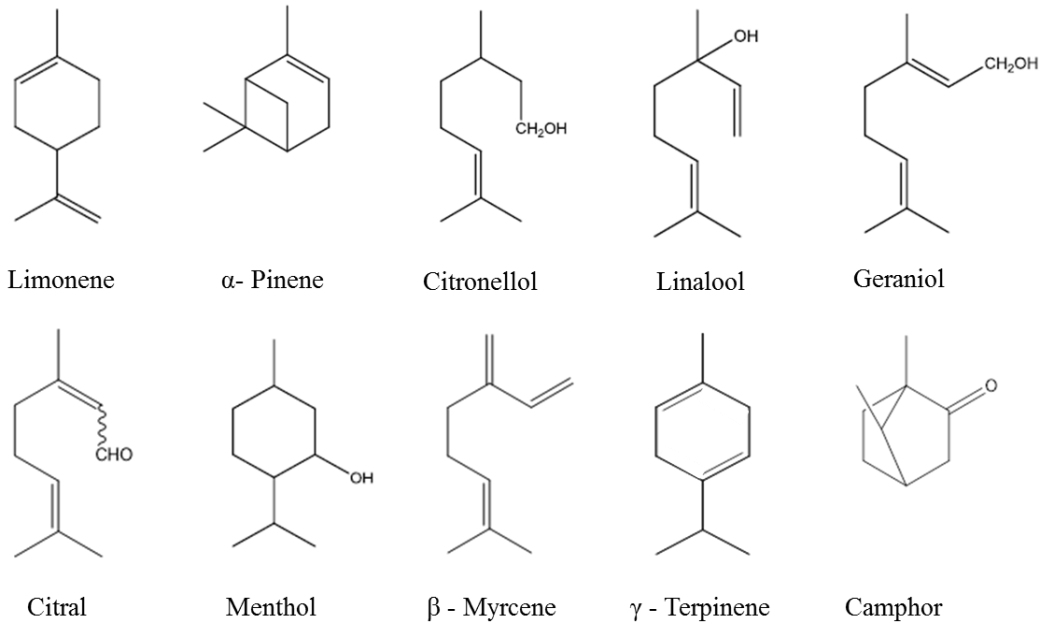

Figure 1. Structure of monoterpenes with potential to be used in bioprocess as starting material.

Later on, an efficient integrated bioprocess was developed using a method for *in situ* product recovery (ISPR) to overcome the inhibition of bioconversion activity, leading to cumulative perilic acid concentration of 31 g/L after 7 days by *Pseudomonas putida* DSM 12264 (Mirata et al., 2009). More recently, other studies reported fungi as potential biocatalysts for limonene degradation leading to the formation of α-terpineol at concentrations of about 3.45 g/L by *Penicillium* sp. (Rottava et al., 2010) and 2.4 g/L by *Fusarium oxysporum* 152b (Bicas et al., 2008b) which was further optimized to almost 4 g/L after 48 h of fermentation (Bicas et al., 2010).

Carvone and carveol, high value-added compounds, were also reported as a conversion products from limonene by several microorganisms such as *Pseudomonas aeruginosa*, yielding about 0.63 g carvone/L (Acosta et al., 1996), basidiomycete *Pleurotus sapidus* achieving a sum of carvone and carveol yield of up to 0.1 g/L (Onken; Berger, 1999a), and *Rhodococcus opacus* obtained *trans*-carveol with 94 to 97% conversion rate (Duetz et al., 2001). It is expected that these advances will soon result in viable processes from an industrial point of view.

3.2. Citronellol

Citronellol is a linear monoterpene alcohol naturally occurring in various citrus plants. The *R*-(+)-isomer is commonly found in essential oils of plants of *Rutaceae*, whereas *S*-(-)-isomer is found in geranium and citronella oil and is much less common (Sousa et al., 2006). Citronellol is an interesting flavor compound for food and flavor industries due to the presence of floral notes (Schrader; Berger, 2001), but is also used as flavor agent in cosmetics, detergents, and "biosafe" insect repellents. It may also be applicable as a precursor to produce other aroma compounds such as rose oxide, one of the most interesting citronellol derivatives (Boersma et al., 2005; Kaminska et al., 1989).

First studies on microbial transformation of terpenes were carried out by Mayer and Neuberg as early as 1915, who reported the synthesis of (+)-citronellol from (+)-citronellal mediated by yeast cells. Further investigations performed by Seubert (1960) were based on the use of linear terpenes as sole carbon source by micro-organisms, thus a soil pseudomonad named *Pseudomonas citronellolis* was reported with the ability to use citronellol and related compounds as the sole source of carbon and energy. Subsequent studies elucidated probable enzymatic steps of the degradation pathway of citronellol in *P. citronellolis* (Seubert et al., 1963; Seubert; Remberger, 1963; Seubert; Fass, 1964a; Seubert; Fass, 1964b).

The presence of β-methyl groups makes the oxidation of linear terpenes harder, thus the first steps of the catabolic pathway of citronellol are the oxidation of primary alcohols to respective aldehydes and acids, and subsequent conversion to the corresponding CoA esters citronellyl-CoA (Hösxhle et al., 2005). Then, β-oxidation is activated by a key enzyme (genaryl-CoA carboxylase) that converts the β-methyl group to acetate, via carboxylation. The carboxymethyl group is removed by another enzyme, 3-hydroxy-3-isohexenylglutaryl-CoA lyase, and the product is able to be degraded by the leucine pathway (Cantwell et al., 1978; Aguilar et al., 2006). Other strains, *P. mendocina* and *P. aeruginosa*, were also reported to degrade citronellol (Cantwell et al., 1978; Tozoni et al., 2010). Investigations on the gene level through molecular biological tools also contributed for the understanding of the acyclic terpene catabolism by *Pseudomonas* species (Diaz-Peres et al., 2004; Förster-Fromme and Jendrossek, 2010).

Botrytis cinerea is an important example of fungal biocatalyst used in the bioconversion of citronellol, citral and its analogous alcohols nerol and geraniol. The

infection of this fungus is especially interesting in fully ripe grapes where it delivers a desirable sweet flavor for winemaking (Demyttenaere, 2001). Brunerie et al. (1987 and 1988) published one of the first studies on the bioconversion of citronellol by the fungus *Botrytis cinerea* using grape must as medium culture. The main conversion products were the hydroxylation product (*E*)-2,6-dimethyl-2-octen-1,8-diol and its reduction product 2,6-dimethyl-1,8-octanediol. When using a small amount of grape must in a synthetic medium, other products were additionally found such as 2-methyl-2-hepten-6-one, 2-methyl-2-hepten-6-ol and citronellic acid. Later, another research group reported the formation of 8-hydroxy citronellol as a conversion product of citronellol by a strain of *Aspergillus niger* in the presence of NADPH and O_2. Rapp and Mandery (1988) also detected hydroxylation products as the main metabolites in the biotransformation of citronellol by *B. cinerea*.

The biotransformation of citronellol into 3,7-dimethyl-1,6,7-octanetriol in an aerated-membrane bioreactor by the basidiomycete *Cystoderma carcharias* was described by Onken and Berger (1999b). The increase of aeration promoted greater rates of microbial growth and biotransformation. They also reported the formation of an important side-product, 3,7-dimethyl- 6,7-epoxy-1-octanol, and minor products such as 2,6-dimethyl-2-octene-1,8-diol, 3,7-dimethyl-5-octene-1,7-diol and 3,7-dimethyl-7-octene-1,6-diol. In minor amounts, microbial formation of rose oxide was observed for the first time in biotransformation of citronellol. The diol products are generated by the photo oxygenation reaction of citronellol, from which the 3,7- dimethyl-5-octene-1,7-diol leads directly to *cis*/*trans* rose oxide upon dehydration and cyclisation (Onken; Berger, 1999a). Another group found the (S)-3,7-dimethyl-5-octene-1,7-diol, a monoterpene from petals of *Rose damascene* Mill., to be the major genuine precursor for isomeric rose oxides through acid-catalyzed conversion (Knapp et al., 1998).

Rose oxide (4-methyl-2-(2-methyl-1-propenyl)-tetrahydropyran) is a high value flavor compound, which occurs in trace amounts in essential plant oils such as Bulgarian rose oil and geranium oil (Onken; Berger, 1999a; Wüst et al., 1998). Thus, the biotechnological production of rose-oxide is especially interesting to flavor industry, since it is one of the most important components in creating rosy notes in perfumery.

One of the last studies in the production of rose oxides by bioconversion of citronellol was published by Maróstica and Pastore (2006). They performed the biotransformation procedure by using agro-industrial residues in the process in order to

provide alternative substrate, cost reduction, and minimize pollution problems. Liquid cassava waste is originated from pressing cassava roots and contains “harmful” pollutants substances. Nevertheless, it was used as an interesting alternative for nutritive medium in bioconversion of citronellol, since the production of rose oxide reached yields of more than 70 and 30 mg/L of *cis* and *trans*-forms, respectively.

Fungal strains of *Aspergillus niger* were found to bioconvert citronellol into *cis*- and *trans*-rose oxides and nerol oxide, by using solid-phase microextraction (SPME) as a fast monitoring technique for microbial screening. The bioconversion products *cis*- and *trans*-rose oxide and nerol oxide presented relative contents up to 54, 21 and 12 % in the headspace SPME extracts, respectively (Demyttenaere et al., 2004).

3.3. α- and β-Pinenes

Pinenes are naturally occurring bicyclic monoterpenes, found in major amounts in turpentine oils from most plants of the *Pinaceae* family (Savitiry et al., 1998). Turpentine is a fluid obtained by the distillation of pine resin arising from the pulp and paper industries, where is collected as a by-product (Yoo; Day, 2002). It is composed by terpenes, mainly the monoterpenes α- and β-pinene and other monocyclic terpenes such as limonene and 3-carene, however the ratio between the different terpenes in turpentine varies according to origin and this greatly influences its value and end use (FAO, 1995).

Likewise, the pulp and paper industry generates hundreds of thousands of tons of turpentine byproduct whose composition has high amounts of α-pinene a bicyclic monoterpene very important as a starting substrate in industrial synthesis, with an annual output of 160.000t (Hanake, 2002; Surburg; Panten, 2006; Schewe et al., 2011). In Brazil, for example, the annual production of turpentine should reach the scale of a few tens of thousands of tons (estimates based on data from the Brazilian participation in the production of turpentine presented in Karthikeyan and Mahalakshmi (2007)).

Considering that turpentine is an abundant and inexpensive source of α- and β-pinenes, it would be interesting to develop methods for converting these monoterpenes into more valuable compounds in order to increase the commercial value of the turpentine oil. Therefore, efforts have been done to select biocatalysts with the ability of selective oxidation of pinenes into value-added compounds, such as verbenone and verbenol. This is feasible, for

example, by hydroxylating α-pinene to produce verbenol, which can be further converted to verbenone through dehydrogenation (Bhattacharyya et al., 1960; Tkacz; Lange, 2004).

Verbenol is a highly valued food-flavoring compound that is widely used in soft drinks, soups, meats, sausages and ice cream (Agrawal et al., 1999). Verbenone is the major constituent of the strawberry, raspberry, and spearmint flavor complexes and is in great demand in the food industry due to its flavor notes of camphor and menthol, and can also be used as a precursor for the synthesis of Taxol®, a pharmaceutical drug used in chemotherapy (Tkacz; Lange, 2004; Agrawal; Joseph, 2000a; Agrawal; Joseph, 2000b; McMorn et al., 2000). Moreover, verbenone and verbenol have also application in agriculture as antiaggregation and aggregation pheromones, respectively, for the control of southern pine beetle infestations (Huber; Borden, 2001; Lindgreen; Miller, 2002; Milller; Lafontaine, 1991).

In fact, α-pinene has been widely studied in the bio-oxidation of terpenes using microbial cell cultures. Since 1960, selective oxidation of α-pinene to verbenol, verbenone, and *trans*-sobrerol by a strain of *Aspergillus niger* was described, and the process was later optimized by the same research group (Bhattacharyya et al., 1960; Prema; Bhattacharyya, 1962). In this approach, the potential to biotransform α-pinene was recognized using the yeast *Hormonema* sp., where the two biotransformation products formed were *trans*-verbenol and verbenone, reaching concentrations of 0.4 and 0.3 g/L after 96 h, respectively (Van Dyk et al., 1998). Lindmark-Henriksson (2003) studied the same pathway to obtain these compounds using as biocatalyst a cell suspension of *Picea abies*. Another interesting report achieved a 15-fold increase in the biotransformation efficiency of α-pinene into verbenol by an *Aspergillus niger* and *Penicillium* sp., when these strains were subjected to mutation in UV light (Agrawal et al., 1999).

The ability of *Pseudomonas fluorescens* NCIMB 11671 to utilize α-pinene as sole carbon and energy sources was described by Best et al. (1987). The microorganism attacked the molecule through epoxidation reaction followed by two rings cleavage by α-pinene oxide lyase to form the isonovalal and novalal. Further reports described another *Pseudomonas* sp. strain, *P. rhodesiae* CIP 107491 capable of catalyzing the cleavage of both rings of α-pinene oxide to form isonovalal (Fontanille et al., 2002). Later, the same research group optimized the process to convert α-pinene oxide into isonovalal, with recovery of 400 g/L after only 2.5 h reaction (Fontanille; Larroche, 2003). Afterwards, the microorganism *P. rhodesiae* CIP 107491 was investigated for the bioconversion of α- and β-pinene by using different pinene

sources. The results demonstrated the substitution of α-pinene by turpentine have given almost the same products profile, with isonovalal as main product (80 g/L after 4 h) (Bicas et al., 2008a).

Other oxidative compounds derived from α-pinene, such as trans-sobrerol (Prema; Bhattacharyya, 1962; Draczyńska-Lusiak; Siewiński, 1989), myrtenol (Savithiry et al., 1998; Limberger et al., 2007), α-pinene oxide (Van Keulen et al., 1998), pinocarveol and pinocarvone, pinocamphone and others have also been reported (Bicas et al., 2009).

Likewise, β-pinene also yields commercially attractive compounds after its oxyfunctionalization. Toniazzo et al. (2005) reported the conversion of β-pinene to α-terpineol by using *Aspergillus niger* ATCC 9462. The same product could also be obtained through the oxidation of α-pinene by *Candida tropicalis* or *Serratia marcescens* (Chatterje et al., 1999; Wright et al., 1986). Another study investigated the biotransformation of the substrates (+)- and (–)-limonene, α- and β-pinene and camphor by *Aspergillus niger* IOC-3913, and identified verbenone and α-terpineol as main derivative products from α- and β-pinene, while no bioconversion products from limonene and camphor were observed (Rozenbaum et al., 2006). The same monoterpenes were applied as substrates for bioconversion procedures by yeast isolated from pine forest litter, identified as *Hormonema* sp. UOFS Y-0067, resulting in *trans*-isopiperitenol from limonene, a mixture of verbenone and *trans*-verbenol from α-pinene, and pinocamphone from β-pinene, which was latter hydroxylated to 3-hydroxy-pinocamphone (Van Dyk et al., 1998).

Table 2 summarizes some products obtained from these substrates and the others discussed in this chapter, including the biocatalysts used in each process. Over the past few decades, a large number of biotransformations of α-pinene have been reported by several authors, using fungi, bacteria, and plant cell culture (Rao et al., 2003; Savithiry et al., 1998; Lindmark-Henriksson et al., 2003; Vanek et al., 2005).

Table 2. Monoterpenes used as substrates in bioprocesses for the production of natural flavor compounds

Terpene substrate	Biotransformation product	Biocatalyst	Reference
Acyclic monoterpenes			
Citronellol	Rose Oxide	*Aspergillus niger*	Demyttenaere et al., 2004
Geraniol	α-Terpineol	*Aspergillus niger*	Demyttenaere et al., 2000
Linalool	Linalool oxides	*Corynespora cassiicola*	Mirata et al., 2008
β-Myrcene	Dihydrolinalool and others	*Pseudomonas putida*	Esmaeili et al., 2011
Citral	Thymol	*Penicillium digitatum*	Esmaeili and Tavassoli 2010
γ-Terpinene	*p*-Cymene-9-ol	*Stemphyluim botryosum*	Krings et al., 2005
Monocyclic monoterpenes			
Limonene	α-Terpineol	*Pseudomonas fluorescens*	Bicas et al., 2008a
	Carveol, carvone	*Rhodococcus opacus*	Duetz et al., 2001
	Perillic acid	*Pseudomonas putida* DSM 12264	Mirata et al., 2009
Menthol	α-Pinene, sabinene and others	*Penicillium* sp	Esmaeili et al., 2009a,b
Bicyclic monoterpenes			
α-Pinene	Verbenol	*Aspergillus niger*	Agrawal et al., 1999
β-Pinene	*α*-Terpineol	*Aspergillus niger* ATCC 9462	Toniazzo et al., 2005

3.4. Linalool

Linalool is a very important molecule to flavor and fragrance industry due to its flower-like odor. This compound has two isoforms ((*S*)-(+)-linalool and (*R*)-(-)-linalool), both of them are used in decorative cosmetics, shampoos, soaps, household cleaners and detergents (Lapczynski et al., 2008).

This acyclic monoterpene can be found in many plants like *Cananga dorata*, *Citrus aurantium*, *Citrus bergamia*, *Coriandrum sativum* seed oil, *Melissa officinalis*, *Pelargonium roseum* and *Salvia sclarea* (Cheng et al., 2012) and in a variety of essential oils, as recently described in "Nutmeg Geranium" (*Pelargonium x fragrans* Willd.), honeybush (*Cyclopia subternata*), Longjing tea (*Camellia sinensis*) and in *Cinnamomum osmophloeum*

ct. linalool essential oil, in which linalool found as pure *S*-(+)- isomer (Verma et al., 2013; Le-Roux et al., 2012; Lin et al., 2012; Cheng et al., 2012). This monoterpene is also a potential therapeutic candidate for treatment of various diseases, because of its anti-inflammatory and antinociceptive properties, its bactericidal effect on some microorganisms and its ability of inducing apoptosis of human leukemia cells, besides other bioactive properties (Huo et al., 2013; Liu et al., 2012; Gu et al., 2009).

When used as substrate in biotransformation procedures, linalool can result in a variety of high–value compounds, depending of the microorganism used. In general, the metabolites obtained have a floral, creamy odor and are used in perfume industry (Demyttenaere et al., 2001). The degradation of linalool by *Pseudomonas* sp. was accomplished by reactions of oxidation and hydroxylation, leading to compounds such as furanoid linalool oxide, 2-vinyl-2-methyl-tetrahydrofuran-5-one, 8-hydroxylinalool (2,6-dimethyl-2,7-octadiene-1,6-diol), 8-carboxylinalool, oleuropeic acid, α-terpineol and perillic acid (Demyttenaere; Willemen, 1997).

When the fungus *Botrytis cinerea* was grown in grape must, linalool was converted mainly in (E)-2,6-dimethyl-2,7-octadiene-1,6-diol by a direct enzymatic hydroxylation of this substrate. In lower concentrations, 2-vinil-2-methyl-tetrahydrofuran-5-one, four furanoid and pyranoid forms of (*E*)- and (*Z*)- linalool oxides and (*E*)- and (*Z*)-acetates of pyranoid linalool oxides were found (Bock; Benda, 1986). Despite the diversity of the compounds obtained, there is no information on their flavor potential.

In other experiment, Mirata et al. (2008) screened 19 types of fungi, of which, four strains were capable of bioconverting linalool. These researchers were the first to observe lilac aldehydes and lilac alcohols as by-product of fungal biotransformation of linalool. *A. niger* and *B. cinerea* were able to produce isomers of lilac aldehyde and lilac alcohol via 8-hidroxylinalool, but linalool oxides and 8-hidroxylinalool were the major products of this biotransfomation. The major productivity was obtained by *Corynespora cassiicola,* reaching 120mg/L.day of linalool oxides. This fungus was identified as a highly stereoselective linalool biotransforming biocatalyst by the authors (Mirata et al., 2008). This last strain was further evaluated in a biotransformation process, where bioreactors and a system for substrate feeding and product removal were used in order to avoid toxic effects (Bormann et al., 2012).

In another fungal screening, Molina et al. (2013) showed that among the 36 strains isolated from Brazilian fruits, few strains were able to use this substrate and

accumulate derivative compounds. Authors found the formation of products such as linalool oxide, geraniol, and α-terpineol.

To confirm the enantioselective biotransformation of linalool by *A. niger*, a racemic mixture of linalool and pure (*R*)-(-)-linalool were used as substrate in liquid cultures. The results showed that the mixture of linalool isoforms was converted into a mixture of *cis*- and *trans*-furanoid and pyranoid linalool oxide. When pure (*R*)-(-)-linalool was used, only *trans*-furanoid and *trans*-pyranoid oxide were found. It was also shown that the growing conditions were not the reasons of these differences (Demyttenaere; Willemen, 1997). Changes in culture conditions were performed by Demyttenaere et al. (2001) in order to improve linalool biotransformation rates. They found that (*S*)-(+)-linalool was much better metabolized than the (*R*)-(-)-isomer and that the co-solvent applied affected the bioconversion rate.

3.5. Geraniol

Geraniol is an acyclic terpene alcohol, obtained from natural oils of several plants, such as rose (*Rosa damascena*), geranium (*Pelargonium graveolens*), citronella (*Cymbopogun winterianus*) and palmarosa (*Cymbopogun martini*). This rose-like flavor compound is mainly used as substrate in biotransformation, allowing the production of a variety of other terpenes, like citronellol, linalool, nerol, hidroxygeraniol, α-terpineol, cineol and 6-methyl-5-hepten-2-one (MHO) (Katiki et al., 2011; Limberger et al., 2003). Geraniol has also been described with anthelmintic and nematicidal activities besides inhibiting *Salmonella* sp. growth when combined with other food antimicrobials (Katiki et al., 2011; Ntalli et al., 2011; Kobilinsky et al., 2007). Moreover, some studies relate the efficacy of geraniol as pontential chemopreventive agent against renal carcinogenesis, since it suppresses renal oxidative stress and tumor incidence (Ahmad et al., 2011). Geraniol also inhibits prostate cancer growth and proliferation of hepatocarcinoma cells, because it modulates the expression of cell cycle regulators and induces apoptosis (Kim et al., 2011; Polo et al., 2011).

The biotransformation of geraniol to form value-added compounds such as citronellol, linalool, hidroxygeraniol, α-terpineol and 6-methyl-5-hepten-2-one has been the focus of many researchers. Changes in growth conditions and search for enzymes and microorganisms to perform the biotransformation have been made aiming improve yields.

In order to find new microorganisms that are able to modify geraniol as substrate, the filamentous fungi *Bipolaris sorokiniana* was identified with a potential application in monoterpenes biotransformation due to the presence of a sesquiterpene phytotoxin that suggests an active terpenoid metabolism. After 5 days incubation in potassium phosphate buffer, the culture showed an oxidative profile with 74.6% yield of conversion of geraniol to 6-methyl-5-hepten-2-one, showing that *B. sorokiniana* is also capable of bioconverting geraniol into higher-value compounds, as already described in *Penicillium digitatum*, *P. italicum* and *Pseudomonas incognita* (Limberger et al., 2003). Studies on biotransformation of geraniol by cultures of *Aspergillus niger* and *Penicillium* sp. were made by Demyttenaere et al. (2000). The authors described that in liquid cultures, geraniol was converted mainly into linalool and α-terpineol, whereas in sporulated surface cultures, linalool was predominantly produced from geraniol, resulting in higher yields.

One of the biggest problems of biotransformation is that the product frequently is very toxic for living cells and has inhibitory effects on microorganisms. Fisher et al. (2011) described that variations on pH affects the conversion of geraniol into linalool and nerol. They monitored this chemical reaction over a 65-day period and reported that at pH 3.4, 20.6% geraniol was converted to linalool, while at pH 7 no linalool formation was observed. For the biotransformation of geraniol into nerol, the major conversion rate was observed at pH 7.0 (14%). At lower pH (3.4), only 1.7% geraniol was converted.

Arifin et al. (2011) described a new way of growing *Saccharomyces cerevisiae* for biotransformation of geraniol into citronellol in a gas-phase system, avoiding direct contact between the reaction medium, geraniol and citronellol. Previous experiments used *S. cerevisiae* in a resting cells system that also separates the cell growth and biotransformation process, however, using a continuous-closed-gas-loop bioreactor (CCGLB) system, they obtained a maximum concentration of 1.18 g/L of citronellol. In the same approach, Bluemke et al. (2001) developed and integrated bioprocess where was possible to remove the products from the cells, consisting in a bioreactor and a downstream unit with a pervaporation membrane module. In this sense, a fast removal of the products was possible, minimizing the interaction cell-product and increasing microbial growth rates and reducing the product loss during biotransformation.

3.6. Citral

Citral is a mixture of stereoisomers where the *E*-form is known as geranial and the *Z*-form is known as neral. This mixture of aldehydes is a readily available terpenoid that can be found in large amounts at reasonable costs from several herbs such as lemon grass, ginger, and some varieties of sweet basil (Förster-Fromme; Jendrossek, 2010; Iijima et al., 2006). Citral is considered a valuable flavor component for the perfumery and food industries due to its "lemony" scent odor characteristics (Iijima et al., 2006).

Several previous investigations have reported the bioconversion of citral into more valuable compounds with interesting properties to the flavor and food industries. For instance, thymol is an important natural preservative that has been used in food products such as cheese to prevent fungal growth (Esmaeili; Tavassoli, 2010). The production of thymol (21.5%) as the major derivative from the biotransformation of citral by *Penicillium digitatum* was reported by Esmaeili and Tavassoli (2010). The authors also emphasized the effectiveness of the sporulated surface cultures method (SSCM) for biotransformation processes.

Afterwards, the same research group reported the bioconversion of citral into citronellol by spores of cells of *Saccharomyces cerevisiae* (Esmaeili et al., 2012). Fungal transformation of citral using SSCM approach also resulted in the production of 6-methylhept-5-en-2-one by spores of *Penicillium digitatum*; similar results were obtained in the bioconversion of nerol (Demyttenaere; De Pooter, 1998).

The enantiospecific reduction of citral to produce citronellal by filamentous fungi, yeast and bacteria strains was reported by several authors. According to Hall et al. (2006), the reductase activity may compete with an alcohol dehydrogenases leading to the formation of nerol/geraniol and citronellol, depending on the microorganism. Strains of *Pseudomonas* sp. were also reported as potent biocatalysts in bioconversion of citral resulting, for instance, in the production of geranic acid by *P. convexa* (Hayashi et al., 1967). Likewise, citral was converted to geranic acid (62%) as the main conversion product followed by other compounds such as 1-hydroxy-3,7-dimethyl-6-octen-2-one (0.75%), 6-methyl-5-heptenoic acid (0.5%), and 3-methyl-2-butenoic acid (1%) by *Pseudomonas aeruginosa* (Joglekar; Dhavlikar, 1969).

3.7. Menthol

Menthol is one of the most important monoterpene alcohol worldwide. It is mainly applied in cigarettes, cosmetics, toothpastes, chewing gum, candies, and medicines (Surburg; Pante, 2006; Burdock, 2010; Caputi; Aprea, 2011). Chemically, menthol is a cyclic monoterpene alcohol with three asymmetric carbon atoms and consequently four pairs of optical isomers are possible: (+)-and (–)-menthol, (+)- and (–)-neomenthol, (+)- and (–)-isomenthol and (+)- and (–)-neoisomenthol, being (–)-menthol the isomer that occurs most widely in nature (peppermint and other mint oils). This compound imparts a mint-like odor and exerts a cooling sensation when in contact to skin and mucosal surfaces, which is one of its most attracting attribute for industry (Koroch et al., 2007). The oil of plants from the genus *Mentha* in the family *Lamiaceae* is one of the main sources of menthol, that is generally found in the free state (Caputi; Aprea, 2011).

Besides its importance in the flavor industry, menthol has other applications. It acts as a topical analgesic and helps the percutaneous penetration of others anesthetic agents in the skin (Liu et al., 2005); it is capable of enhancing ocular drug delivery (Xu et al., 2011) and inhibits bone absorption, suggesting a protective effect against osteoporosis (Mühlbauer et al., 2003); it also attenuates respiratory irritation responses to multiple cigarette smoke irritants (Willis et al., 2011) and decreases the viability of cancer cell lines (Lu et al., 2007; Slominski, 2008).

Menthol can be also used as substrate in biotransformation generating higher-value compounds. Hydroxylation products were obtained for biotransformation of (+)- and (-)-menthol by *A. niger*. In this case, (-)-menthol was converted into 1-,2-,6-,7-,8- and 9-hydroxymenthols, whereas (+)-menthol was mainly biotransformed into 7-hydroxymenthol, with 1-, 6-, 8- and 9-hydroxymenthol as minor products (Asakawa et al, 1991). When the fungus *A. niger* is grown in a sporulated surface culture, it was possible to observe the formation of *cis*-*p*-menthan-7-ol, whereas in sporulates surface cultures of *Penicillium* sp., the products obtained were limonene, *p*-cymene and γ-terpinene (Esmaeili et al, 2009a). The products α-pinene, sabinene, *trans*-*p*-menthan-1-ol, *p*-menth-1-ene, 1,8-cineole and limenone were also identified in a subsequent study with *Penicillium* sp. (Esmaeili et al., 2009b).

The fungal biotransformation of menthol was also performed by *Cephalosporium aphidicola* and *Macrophomina phaseolina*. In a 12-day incubation of *C. aphidicola* with (1*R*, 2*S*, 5*R*)-(-) menthol, yielded six products: 10-acetoxymenthol, 4α-hydroxymenthol, 3α-

hydroxymenthol, 7-, 9-, and 10-hydroxymenthol (Atta-ur-Rahman et al., 1998). The biotransformation of (+)-menthol with *M. phaseolina* generated 8,9-, 6*R*-, 1*R*-hydroxymenthol and others, with the C-8 position of menthol preferentially oxidized (Musharraf et al., 2011). This terpene is an interesting example of the importance of these substrates and the products arising from bioprocesses, considering that the current demand of menthol exceeds the supply from natural sources. In this sense, many efforts are needed to obtain menthol by natural means and from other more readily available raw materials, in addition to synthetic and semi-synthetic routes (Caputi; Aprea, 2011).

3.8. Myrcenes

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is an acyclic monoterpene found in essential oils of several plants. It was identified as major constituent in *Artemisia scoparia* oil and also found in lemongrass (*Cymbopogon citratus*), hop, bay and verbena (Singh et al., 2009; De-Oliveira et al., 1997a). This monoterpene can be used in food flavor additives, cosmetics, soaps, and detergents, but it has also been described as analgesic, anti-mutagenic, and as a tyrosinase inhibitor (Santos and Sá-Correia, 2009). Some studies show that β-myrcene can induce liver isoenzymes (De-Oliveira et al., 1997b), interfere in the xenobiotics' metabolism by inhibition of CYP2B1 monooxygenase (De Oliveria et al., 1997a) and it is a potential bioherbicide since this compound has phytotoxic character and inhibits the growth of *Avena fatua*, *Cyperus rotundus* and *Phalaris minor* through generation of ROS-induced oxidative damage (Singh et al., 2009).

The products of biotransformation of myrcenes have great importance for flavor and fragrance industries due to their lilaceous fragrance. The biotransformation of myrcenes by *Pseudomonas putida* generate dihydrolinalool, cis-β-dihydroterpineol, linalool and cis-ocimene-8-oxo as major products, varying according to the incubation time (Esmaeili et al., 2011).

In the same approach, a similar study was made using *Pseudomonas aeruginosa* for the biotransformation of this monoterpene where the time of incubation was also observed as an important parameter. Different products were obtained in high yields and selectivity, such as dihydrolinalool (79.5 %) and 2,6-dimethyloctane (9.3 %) after 1.5 days of incubation, and α-terpineol (7.7 %) and 2,6-dimethyloctane (90.0 %) after 3 days of process (Esmaeili; Hashemi, 2011).

Thompson et al. (2010) used a strain of *Rhodococcus erythropolis* for the biotransformation of β-myrcene into geraniol. They observed that at least four proteins were overproduced when *R. erythropolis* was growth on β-myrcene. Three of them were identified: an aldehyde dehydrogenase, an acyl-CoA dehydrogenase and a chaperone-like protein, involved in β-myrcene degradation pathway. Other studies were performed in order to find new strains capable of biotransformation of β-myrcene and the enzymes and pathways involved in this process. Iurescia et al. (1999) isolated a β-myrcene-utilizing strain, identified as *Pseudomonas* sp. M1. This strain was able to grow on β-myrcene as the sole carbon source. They also obtained a β-myrcene-negative mutant, which only accumulates myrcen-8-ol as product of biotransformation of β-myrcene. The analyses of the genome of these microorganisms, led to the identification of the β-myrcene catabolism genes through sequencing. They found four open reading frames named *myrA*, *myrB*, *myrC* and *myrD*, that potentially encode for an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl-coenzyme A (CoA) synthetase and an enoyl-CoA hydratase, respectively, and a relationship between these gene and β-myrcene catabolism is suggested by the authors (Iurescia et al., 1999).

The bioconversion of β-myrcene was also shown when using solubilized enzyme fraction from mycelium lyophilisates of *Pleurotus ostreatus*. Perillene and rosefuran were the products of this reaction, the first one with a fresh citrus-flowery odor and the second with a flowery rose-like note. An intracellular pathway of perillene formation by *Pleurotus ostreatus* starts with the epoxidation of β-myrcene at conjugated double bound, but another pathway is described using soluble enzymes. In this pathway, β-myrcene and its derivatives were transformed into perillene, 6,7-epoxyperillene, 7-hydroxyperillene and rosefuran through the corresponding endoperoxides, concluding that a dioxygenase-type enzyme introduces O_2 at the 1,3-diene moieties of the monoterpene precursors (Krügener et al., 2009).

3.9. Terpinenes

α-Terpinene (1-isopropyl-4-methylcyclohexa-1,3diene) and γ-terpinene (1,4-*p*-menthadiene) are monocyclic terpenes used in pharmaceutical and perfume industries. They are the major compounds of tea tree (*Melaleuca alternifolia*) oil and of other different essential oils, like *Artemisia annua* L. and *Senecio graveolens*, conferring antioxidant properties (Gomes-Carneiro et al., 2005; Pyka; Bober, 2002). Beside the antioxidant capacity, α–terpinene and γ–terpinene have been described as antibacterial, antifungal, anti-

inflammatory, anticancer and acaricidal activity against *Hyalomma marginatum* (Marzec et al., 2010; Cetin et al., 2010). However, the use of these substances in medicine and cosmetics requires care. α-Terpinene, for example, autoxidizes to form allergens, while γ-terpinene is instable and presents cytotoxicity properties (Rudbäck et al., 2012; Krings et al., 2005).

Some biotransformation assays using α–terpinene or γ–terpinene as substrate have been made. In a fermentation process with *Corynesporium cassiicola*, these terpenes were converted into (1*R*,2*R*)-*p*-menth-3-ene-1,2-diol and (1*R*,2*R*)-*p*-menth-4-ene-1,2-diol (Farooq et al., 2004). In another study, Krings et al. (2005) identified a wild strain of the fungus *Stemphyluim botryosum* that was able to grown in high concentrations of γ–terpinene. The authores used this strain in a γ–terpinene biotransformation process and found that the two major products were identified as *p*-cymene-9-ol and *p*-mentha-1,4-dien-9-ol. The last one has never been described as a biotransformation product, due its chemical instability. The authors showed that the enzymatic process involved is the addition of oxygen atom at non activated carbon.

3.10. Others

Besides the monoterpenes previously described, several other have a great potential to be used as the substrate in biotransformation processes, but were insufficiently investigated. One example is camphor ($C_{10}H_{16}O$), a dicyclic terpenic ketone widely distributed in nature. The main source of *d*-camphor ((+)-camphor) is the wood of the tree *Cinnamomum camphora* Ness, *l*-camphor ((-)-camphor) can be found in the essential oil from *Blumea balsamifera*, *Artemisa tridentate* and *Lavandula pedunculata*, whilst *dl*-camphor is mainly present in the oil of *Chrysanthemum sinense* var. *japonicum* (Chizzola et al., 2004; Tabanca et al., 2006; Mighri et al., 2010; Zuzarte et al., 2009). Due to its minty and diffusive aroma, camphor is used in perfuming industrial products, beverages, condiments, baked goods and frozen dairy and it was also described as substrate for biotransformation (Maróstica; Pastore, 2007; Burdock, 2010).

Besides its importance in the flavor industry, camphor acts on bone metabolism inhibiting bone reabsorption, not causing toxic effects, has strong antifungal and antibacterial activity, and therefore, may be useful in the clinical treatment of fungal diseases, particularly dermatophytosis (Zuzarte et al., 2009; Mühlbauer et al., 2003; Tabanca et al., 2001).

On the same approach, fenchone ($C_{10}H_{16}O$) is an irregular bicyclic monoterpene ketone that occurs in many fennel (*Foeniculum vulgare* Mill.) and thuja (*Thuja orientalis*, *Cupressaceae*) oils (Croteau et al., 1980; Díaz-Maroto et al., 2005; Raal et al., 2012). This compound resembles camphor very closely on its properties, as it has a camphoraceous odor, threshold of 510 ppb, and is used to prepare artificial fennel oils, to perfume household products and as a flavor additive in some food products, having an annual consumption of about 10.00 lb (Burdock, 2010). Besides its importance in the industry, fenchone has other applications such as repellent activity against *Aedes aegypti* (Diptera: Culicidae) females, moderate antifungal activity and acute local anesthetic activity (Zuzarte et al., 2009; Mimica-Dukic et al., 2003; Zalachoras er al., 2010. These properties highlight the importance of fenchone and the need for more researches to obtain new derivatives from this terpene.

Few biotechnological studies have been conducted for the application of these and other compounds as substrate, which could be an interesting field of study in order to obtain the derivatives of commercial interest.

4. Conclusion

The use of monoterpenes as the substrate for obtaining new flavor compounds presents a great potential field of research and development. Besides the recognized importance of these substrates, directed both by their sensory profile as well as by their economic interest, bioprocesses in which they are employed can lead to obtaining new natural molecules with high added value and biological or biotechnological potential. Despite this great potential, many efforts should be directed towards the identification of new compounds, new metabolic pathways, suitable microorganisms with potential for this area and also new strategies to overcome the problems related to these processes. In fact, there are yet some challenges related to the biotransformation of terpenes, such as the chemical instability of both substrate and product, the low water solubility of the substrate, the high volatility of both substrate and product, the high toxicity of both substrate and product, the low yields and the high costs related to fermentation processes.

Overall, this chapter showed the importance of some monoterpenes as substrates for the production of new metabolites. It is important to note that many of them remain without any study aiming to evaluate their biological and biotechnological potential, becoming a research field that requires further efforts directed to obtain new natural flavor

compounds and improve their biotechnological production for the food and flavor industry, cosmetics and pharmaceuticals.

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CAPÍTULO 2

ISOLATION AND SCREENING OF ENDOPHYTIC FUNGAL STRAINS FOR NATURAL FLAVOR PRODUCTION

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ABSTRACT

Aromas and fragrances are extensively employed in food, cosmetic and pharmaceutical fields in order to make products more attractive for consumers. The biotechnological production of aroma compounds is a promising alternative to supply the increasing demand for natural products to substitute synthetic additives due to the concern of consumer market on health and nutrition. Using the biotransformation of terpenes, a wide range of value-added products can be obtained from low-cost substrates. Endophytic microorganisms are suitable biocatalysts to be used in this process, especially when isolated from terpene-rich sources. Unripe genipap fruits are an unexploited source of iridoids (monoterpenes) with characteristics of either a colorant or a bioactive compound. The aim of this work was to isolate endophytic fungal strains from unripe genipap fruits and select them according to their monoterpenes biotransformation ability. Fifteen strains were obtained after isolation process and among them, three proved to be able to convert monoterpenes in their oxygenated form. These strains were randomly identified as JV-7, JV-10 and JV-15. From the substrate α-pinene it was possible to obtain verbenol and verbenone, using JV-7 strain, whereas from β-pinene, it was obtained pinocarveol as major compound by JV-15 strain. *R*-limonene and *S*-limonene were converted to dihydrocarveol and limonene-1,2-diol respectively using JV-10 strain. These products are important aroma compounds of industrial application. The monoterpene citronellol was also tested as substrate, however, no product formation was observed.

Keywords: endophytic fungi, monoterpenes, verbenol, verbenone, pinocarveol, limonene-diol, aroma compounds

1. Introduction

Aroma compounds are prepared for several types of market world-wide, mainly to be used as additive in food, beverages, cosmetic and pharmacy (UBIC 2014). Nowadays, there is a tendency to substitute synthetic additives for natural ones, due to the increasing awareness of consumer market of the link between diet and health. In this approach, it is necessary to find new ways to obtain natural flavor compounds (Mapari et al., 2010; Vespermann et al., 2017). The methods for obtaining natural aroma compounds include direct extraction from vegetable sources and biotechnological production. The first case cannot be employed to supply the industrial demand due to the low concentrations of product, dependency on seasonal and climatic features and possible environmental issues caused by extraction (Bicas et al., 2010; Felipe et al., 2017). In contrast, in the biotechnological processes, microbial or enzymes are used as catalysts for the production of flavor from natural raw materials. Usually, these reactions are highly selective and can be considered eco-friendly and sustainable due to the low residues generation (Schrader, 2007).

Biotransformation is a biocatalytic conversion to modify a precursor structurally related to the compound of interest, which occurs in few biochemical reactions (Bhatti et al., 2014). Most of the biotransformation processes use whole cells as biocatalysts, being that microorganisms are the most suitable since they have an enzymatic arsenal to metabolize a great variety of complex organic molecules (Palmerín-Carreño et al., 2015). In order to be economically viable, the substrate to be used in this process must be found in nature and its isolation in high concentration must be easily feasible (Schrader, 2007). One example is the use of lignin-containing agricultural residues, especially cereal bran and sugar-beet pulp, to obtain ferulic acid, the precursor of vanillin (Bicas et al., 2010).

Terpenes are suitable compounds to be employed as substrates in biotransformation processes. They can be found in nature as major constituents of essential oils and their oxyfunctionalised forms are extensively applied in industry as flavorings (Schempp et al., 2017; Molina et al., 2014). They can also be found in residues from fruits processing and pulp and paper industries (Kowalska et al., 2017). In the biotransformation of terpenes employing microorganisms as biocatalysts, the monooxyfunctionalisation reactions occur by the action of cytochrome P450 monooxygenases enzymes under mild conditions, whereas in chemical oxidation process, toxic heavy metals are needed (Schrader, 2007).

One of the major drawbacks in the biotransformation of terpenes is to find an appropriate biocatalyst to be used. Several studies have already been made in order to isolate a fungal, yeast or bacterial strain with ability to produce value-added compounds from mono and sesquiterpenes, although few researches focus on endophytic microorganisms (Rottava et al., 2010; Bier et al., 2011; Molina et al., 2013; Palmerín-Carreño et al., 2015; Bier et al., 2017). Endophytes are microorganisms that grow intra or intercelullarly in higher plants, generally without causing diseases on the host plant. They can produce a wide range of substances with bioactive properties and that may have potential for industrial application in food, agriculture and medicine (Strobel; Daisy, 2003; Pimentel et al., 2011). It is expected that the native strains may have the metabolic pathways to catalyze specific reactions, therefore, if isolated from terpene-rich sources, these endophytic microorganisms may present resistance to this type of compound, and possibly, have potential to be used as biocatalyst in natural flavor production (Pimentel et al., 2011; Molina et al., 2013).

In general, the first step in screening assays is to identify the best source of microorganisms, for example industrial residues, fruits or samples rich in terpenes and even extreme environments (Borges et al., 2009). Once isolated, these microorganisms are incubated with the substrate to be used in the process in order to find out which strains are capable to resist to its toxicity and use the substrate as sole carbon source indicating the existence of a metabolic pathway for substrate degradation and possible accumulation of interesting metabolites (Bicas; Pastore, 2007). The possible products obtained after biotransformation are usually identified and quantified by gas chromatography (gas chromatography coupled with mass spectrometry or gas chromatography with flame ionization detector) (Bier et al., 2011).

Unripe genipap fruit (*Genipa americana* L.) is a underutilized source of monoterpenes from iridoids class. These fruits contain 1-3% of genipin, a colorless compound that, in presence of oxygen, reacts with primary amines and proteins, producing blue/violet pigments (Ramos-de-la-Peña et al., 2016). This reaction is responsible for the blue coloration observed when unripe genipap fruits are cut open and its interior exposed to the air, but the detailed mechanism of blue color formation is still unclear (Brauch, 2016; Buchweitz, 2016). Genipin is also recognized by its biological activities and cross-linker properties, therefore, it is possible its application either as colorant or as bioactive compound (Neri-Numa et al., 2017). The genipin content in genipap is dependent of the ripening stage of the fruits, being that in ripe fruits, the genipin concentration decreases and the glycosylated forms (geniposide

and genipin-1-β-D-gentiobioside) are the major compounds found (Bentes; Mercadante, 2014; Bentes et al., 2015).

The aim of this work was to isolate endophytic microorganisms from unripe fruits of genipap and select them for natural aroma production through biotransformation of the monoterpenes citronellol, *R*-limonene, *S*-limonene, α-pinene and β-pinene.

2. Material and methods

2.1. Chemicals

The standards used as substrates were *R*–(+)–Limonene (Fluka, 98%), *S*–(-)–Limonene (Sigma-Aldrich, 96%), 1*S*–(-)–α-pinene (SAFC, 98%), 1*S*–(-)–β–pinene (SAFC, 97%) and *DL*-Citronellol (Sigma-Aldrich, 95%). For quantification curves and identification of compounds produced, the standards used were verbenol (Sigma, 95%), verbenone (SAFC, 93%), myrtenol (SAFC, 95%), carveol (Sigma-Aldrich, 97%), pinocarveol (Sigma-Aldrich 96%), α-terpineol (SAFC, 96%) and limonene-1,2-diol (Sigma-Aldrich, 97%). Ethyl acetate (P.A. Synth) and *n*-decane (Sigma-Aldrich) were used for samples preparation.

2.2. Microorganisms

The microbial strains used in this work were isolated from unripe fruits of genipap. These fruits were harvested in the months of July and August 2016 inside the University of Campinas (UNICAMP) campus, located in the city of Campinas, São Paulo, Brazil. The voucher specimen of this plant material is deposited at UEC Herbarium – UNICAMP (UEC 11713) (www.splink.org.br).

For endophytic strains isolation, the genipap fruits were manually washed and cleaned, under sterile conditions, with sodium hypochlorite 5% for 5 minutes and ethanol 70% for 1 minute. The fruits were then washed with sterile distillated water and dried under UV light for 15 minutes. After the external sanitization procedure, slices of unripe genipap were transferred to petri plates containing culture media potato-dextrose-agar (KASVI), Sabouraud-dextrose-agar (OXOID) and yeast-malt (YM; in g/L: glucose = 10, bacteriological peptone = 5, malt extract = 3, yeast extract = 3 and agar = 20). All plates were incubated at 30 ºC until the appearance of colonies. The growth strains were transferred to new petri plates containing the same culture-media already described, in order to obtain a pure culture of each microorganism (Dionísio et al., 2010; Abrahão et al., 2014).

The isolated strains were tested for monoterpene biotransformation using *R*-limonene, *S*-limonene, α-pinene, β-pinene or citronellol as substrate.

2.3. Inoculum for monoterpenes biotransformation screening

A piece of agar (approximately 1 cm^2) with a 72 h-old culture of each strain to be tested for biotransformation was transferred to a 250 mL Erlenmeyer flask containing 50 mL of YM liquid media. The material was homogenized under sterile conditions with Ultra-Turrax™ T25 (Ika, Wilmington, NC, USA) until complete disruption of the agar culture. After incubation at 30 ºC and 150 rpm for 72 h, the humid biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman nº1 (Bicas et al., 2008; Bicas et al., 2010; Molina et al., 2015).

2.4. Biotransformation procedure

The fungal humid biomass obtained was divided (approximately 3 g) amongst 250 mL Erlenmeyer flasks containing 50 mL of mineral media (MM; in g/L: $MgSO_4.7H_2O$ = 0.5, $NaNO_3$ = 3, K_2HPO_4 = 1, KCl = 0.5 and Fe_2SO_4 = 0.01). In this culture, 0.5 % (v/v) of the substrate to be tested (*R*-limonene, *S*-limonene, α-pinene, β-pinene or citronellol) was added. The flasks were incubated at 30 ºC and 150 rpm for 96 h. Periodically, 500 µL samples were collected to monitor the consumption of substrate and product formation (Bicas et al 2008; Molina et al., 2015). Blanks of the biotransformation experiments were performed without the fungal strain, to ensure that the product formation is due to action of biocatalysts and not through chemical reactions (Maróstica Jr.; Pastore, 2007).

2.5. Sample analysis

Each sample was extracted (1 minute in Vortex) with the same ethyl acetate volume, containing 1% of *n*-decane as internal standard. After phase separation, the organic fraction was dried over sodium sulphate and 1 µL was injected in a gas chromatograph with a flame ionization detector (GC-FID) HP-7890 (Agilent Technologies, Santa Clara, CA, USA), coupled to a HP-5 column (30 m length x 0.25 mm i.d. x 0.25 µm of film thickness), in split mode (split ratio 1:20). The oven temperature as kept at 80 ºC for 3 minutes, raised at 20 ºC/min until 220 ºC and held for 4 minutes. Temperature of the injector and detector were kept at 250 ºC. Helium was used as carrier gas (2 mL/min). Substrates and products were quantified by external calibration curves, also using *n*-decane as internal standard.

The identification of the volatile compounds produced was performed on a GC-MS system with a gas chromatograph HP-7890 coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, CA, USA) and a HP-5MS column (J&W Scientific, Folsom, California, USA) with 30 m length x 0.25 mm i.d. x 0.25 µm of film thickness. The programming of the gas chromatograph was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 ºC, impact energy of +70 eV and a mass range 35-500 m/z. The compounds identification was made by comparing the spectra with NIST 2008 library over 90% similarity, and comparison with commercial standard (Molina et al., 2015).

3. Results and discussion

3.1. Isolation of endophytic microorganisms

A total of 15 filamentous fungi strains and 2 bacteria were obtained after isolation procedure, however, only the fungal strains were tested for monoterpenes biotransformation. Bacteria strains could be easier to work in biotechnological processes, however, filamentous fungi are considered more robust for this purpose (Bicas et al., 2010). The isolated strains were considered different according to visual analysis of their morphology and coloration after 72 h of incubation in YM agar medium and randomly coded as JV-1 to JV-15.

3.2. Aroma compounds production

All strains of filamentous fungi isolated were submitted to biotransformation process using one of the five monoterpenes as substrates: *R*-limonene, *S*-limonene, α-pinene, β-pinene or citronellol. Among the 15 strains, only 3 presented product formation after 96 hours of incubation for at least 1 substrate, as shown on Table 1.

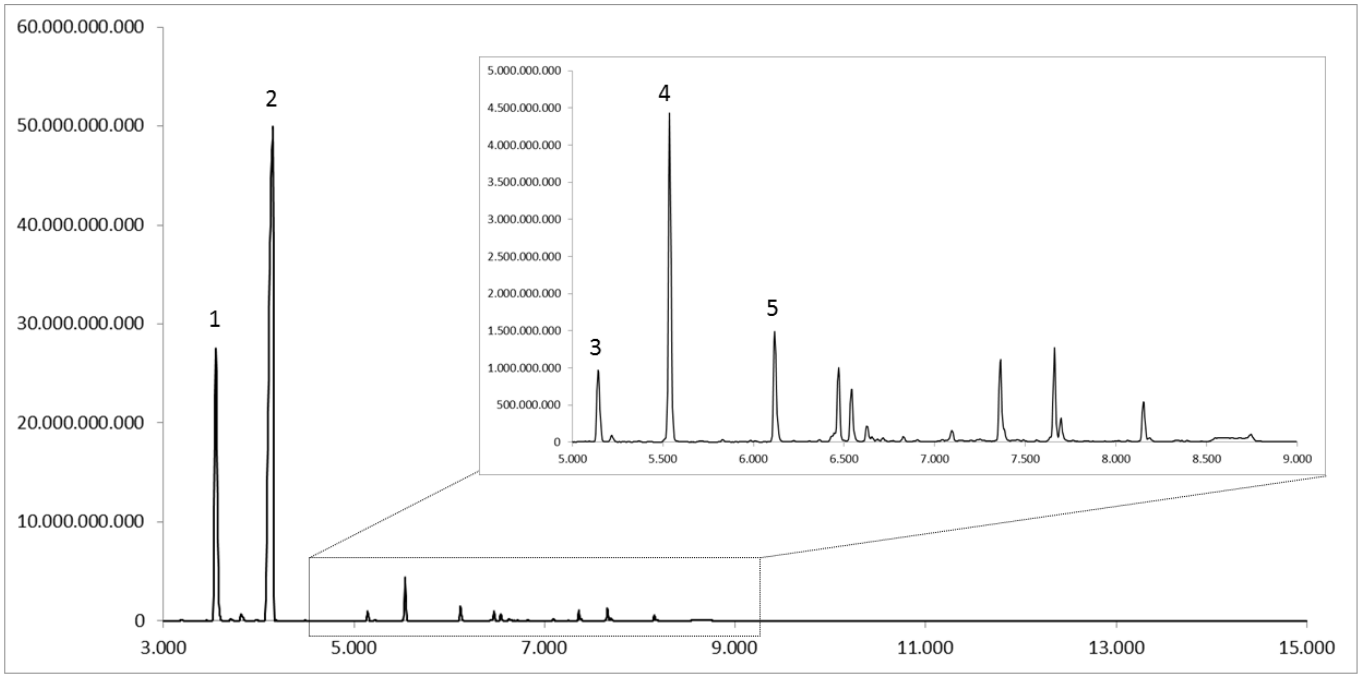
Table 1. Results obtained after biotransformation process (96h) using monoterpenes as substrates and endophytic fungi as biocatalysts.

Strain	***R* –limonene**	***S*-limonene**	**α – pinene**	**β- Pinene**
JV – 7	n.d.	n.d	Verbenol (222.4 ± 2,3 mg/L) and Verbenone (79.47 ±3,6 mg/L)	Pinocarveol (36.7mg/L)
JV – 10	Neodihydrocarveol (n.q.)	1-methyl-4-(1-methylethenil)- 1,2-ciclohexanediol (n.q.)	n.d.	n.d.
JV – 15	n.d.	n.d	Verbenol (88.1 mg/L)	Pinocarveol (64.8 ±0,6 ml/L)

n.d. = not detected/ n.q. = not quantified – LOQ = 20mg/L

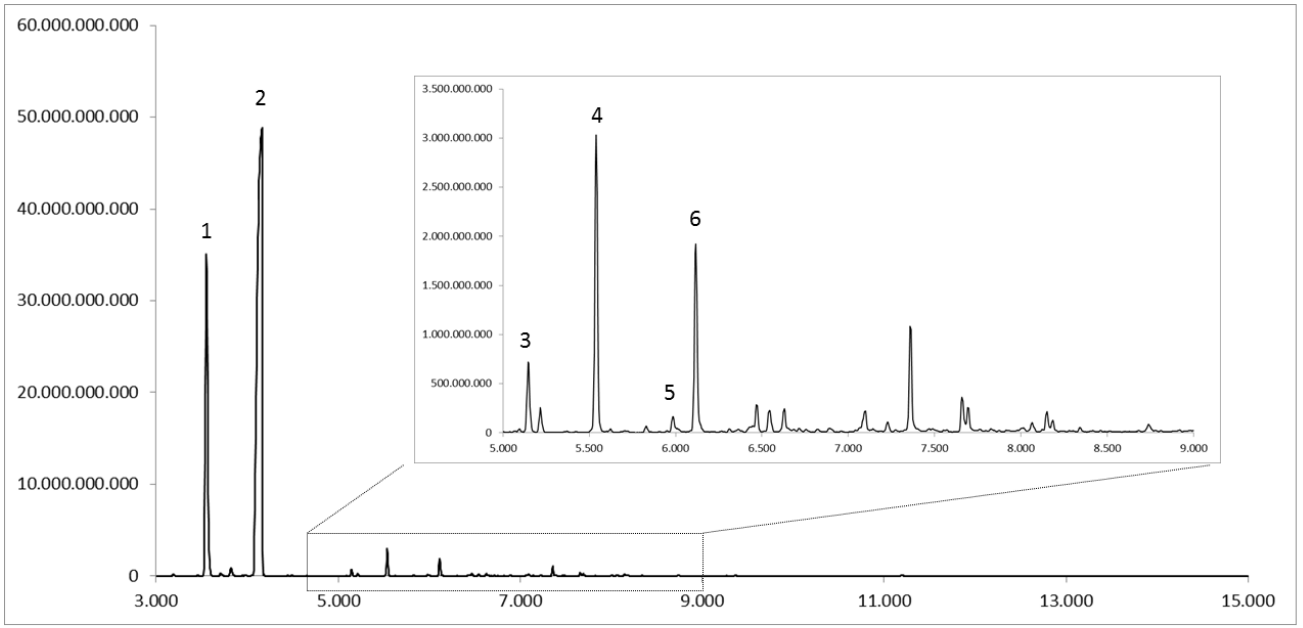
3.3. Biotransformation of α-pinene

Among the fungal strains isolated from unripe genipap fruits, the strains identified as JV-7 and JV-15 proved to be able to use α-pinene as substrate for biotransformation. Figures 1 and 2 provide the chromatographic profile obtained after analysis of 96h-fermentation samples as well as the chemical structures of the main products of biotransformation.



#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	3.551	Alpha pinene	96 %	7785-70-8	C10H16	
2	4.136	Decane	81 %	124-18-5	C10H22	
3	5.14	Alpha pinene epoxide	96 %	1686-14-2	C10H16O	
4	**5.541**	***cis*-verbenol**	**90 %**	**473-67-6**	**C10H16O**	
5	6.118	*cis*-verbenone	98 %	1196-01-6	C10H14O	

Figure 1. Compounds obtained in the biotransformation of α-pinene by JV-7 strain.



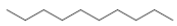
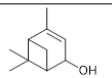
#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	3.556	Alpha pinene	83 %	80-56-8	C10H16	
2	4.149	Decane	81 %	124-18-5	C10H22	
3	4.149	Alpha pinene epoxide	97 %	1686-14-2	C10H16O	
4	**5.541**	***cis*-Verbenol**	**67 %**	**473-67-3**	**C10H16O**	
5	5.99	Myrtenol	95 %	515-00-4	C10H16O	
6	**6.118**	**Verbenone**	**98 %**	**80-57-9**	**C10H14O**	

Figure 2. Compounds obtained in the biotransformation of α-pinene by JV-15 strain.

The monoterpene α-pinene has been used for the biotechnological production of many natural aroma compounds, for example verbenol, verbenone, α-terpineol, isoterpineol, isonovalal, novalic acid and myrtenol (Agrawal; Joseph 2000; Rozenbaum et al., 2006; Krings et al., 2009; Trytek et al., 2015; Siddhardha et al., 2012; Fontanille et al., 2002; Bicas et al., 2008; Linares et al., 2009; Colocousi et al., 1996).

It is interesting to highlight that both strains produced verbenol as major product, but in higher concentrations for strain JV-7 (222.4 mg/L compared to 88.1 mg/L for JV-15 strain). This fact is advantageous for studies of optimization of process conditions, aiming to obtain an isolated compound and also can lower process costs since it facilitates the recovery and purification of verbenol. For the strains JV-15 it was possible to obtain a large number of compounds from α-pinene biotransformation. In this case, the recovery of a specific compound is laborious, however, this mixture can originate a novel aroma perception, which can be pleasant and suitable for food or cosmetic formulation (Bier et al., 2011). Figure 3 shows the kinetics of α-pinene biotransformation by JV-7 strain.

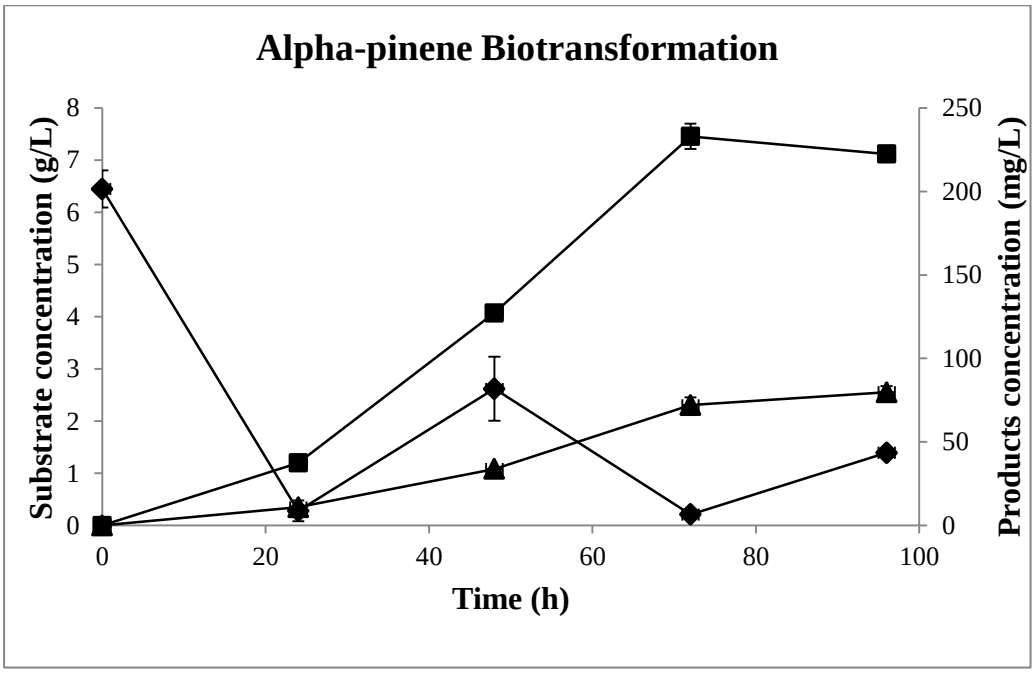


Figure 3. Biotransformation of α-pinene by JV-7 strain. α-Pinene (♦) was added during the process to supplement the losses by evaporation. The products obtained were verbenol (■) and verbenone (▲).

Regarding the major compound obtained, verbenol is a bicyclic monoterpene alcohol recognized by its characteristic fresh pine, ozone aroma with camphor and mint notes.

Its world annual consumption is estimated to be lower than 1kg, with application in baked goods, beverages and candies (Burdock et al., 2010). The formation of this compound occurs by the action of hydroxylases that converts α-pinene to verbenol and then, it can be converted to verbenone by dehydrogenation (Figure 4) (Agrawal; Joseph 2000; Negoi et al., 2017). Verbenone, another interesting compound produced, is used as strawberry and mint flavorings. Besides the application as aroma compounds, these monoterpenoids are also recognized due to its biological activities and for being a precursor for production of Taxol, a drug used in chemotherapy (Molina et al., 2014; Verpermann et al., 2017).

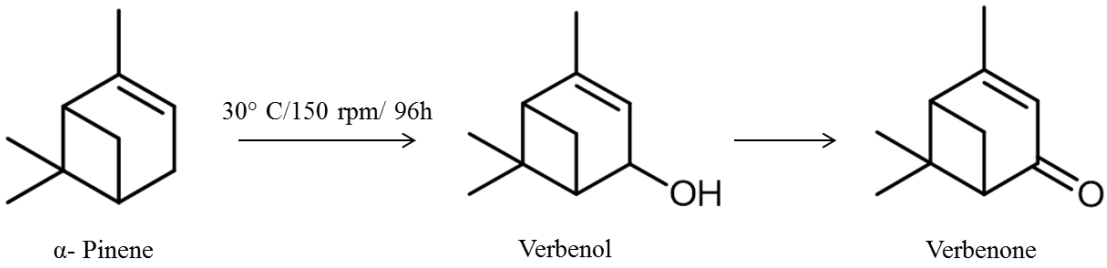


Figure 4. Pathway for α-pinene biotransformation.

Several microorganisms have been reported to use α-pinene to produce verbenol, for example strains of *Aspergillus niger* and *Penicillium digitatum*, *Pseudomonas putida*, *Serratia marcescens* or other isolated strains (Rao et al., 2003; Divyashree et al., 2006; Wright et al., 1986; Trytek et al., 2015).

Verbenol (yield 20-25 %) and verbenone (yield 2-3 %) could be obtained from 0.6% (v/v) of α-pinene using a culture of *A. niger* after 8 h of incubation at 28.5 ºC using (Parshikov and Sutherland, 2014). Agrawal and Joseph (2000) reported a better conversion rate of α-pinene to verbenone, of approximately 16.5 % when *Aspergillus* sp. was incubated for 6 h at pH 6 and with substrate concentration of 0.2 mg/mL. Similarly, immobilized cells of *A. niger* IOC-3913 proved to be able to produce verbenol and verbenone from α-pinene (Rozenbaum et al., 2006).

A strain of yeast identified as *Hormonema* sp. UOFS Y-0067, isolated from pine tree samples, proved to metabolize α-pinene to produce 0.4 g/L of verbenol and 0.3 g/L and verbenone after 72 h of incubation (Van Dyk et al. 1998). In another screening study, Rottava et al. (2010) obtained 31 microorganisms with ability to produce verbenol, all of them isolated from residues from orange juice industry. The highest verbenol concentration obtained was about 125 mg/L. A strain of *Serratia marcescens* was used to produce verbenol, verbenone

and sobrerol from 0.4 % (v/v) of α-pinene and the authors also studied the effects of other media components in the metabolic pathways during biotransformation (Wright et al. 1986).

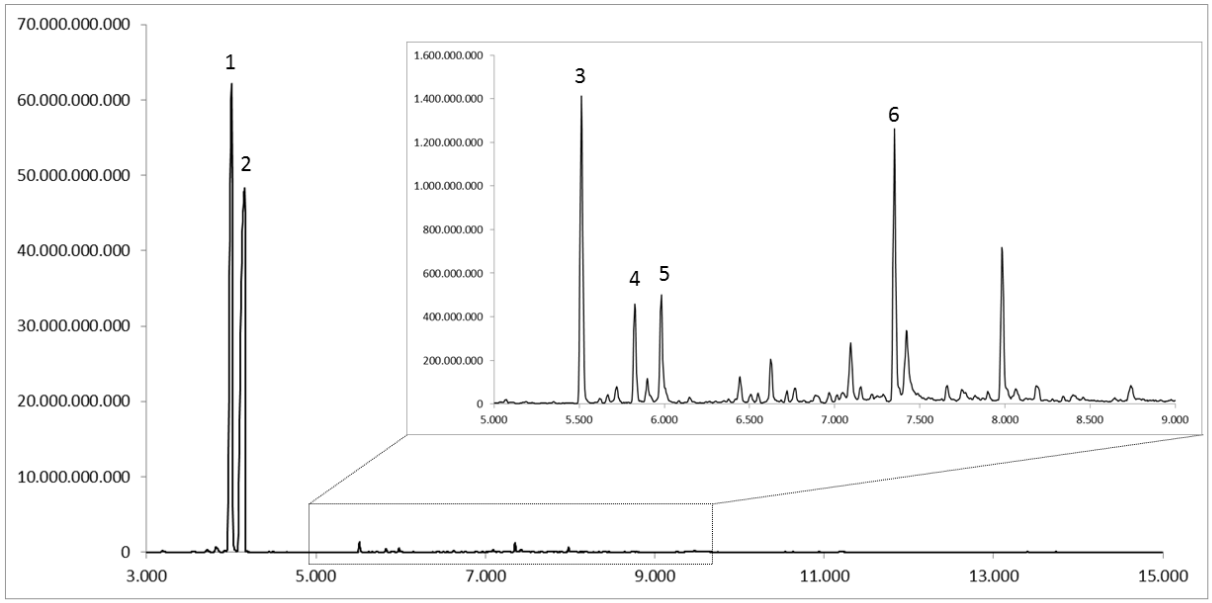
Trytek et al. (2015) isolated a psychotrophic fungus identified as *Chrysosporium pannorum* A-1 and after its incubation at 20 ºC and 150 rpm in a basal medium (malt extract 1 %, peptone 0.5 %, glucose 1 %, and yeast extract 0.5 %) with α-pinene addition, it was possible to obtain 722 mg/L of verbenol. The advantage of using low temperatures in biotransformation is to reduce evaporation and consequently volatile compounds losses during the process, aiming to recover a higher amount of product.

In an attempt to improve α-pinene biotransformation, Agrawal et al. (1999) treated *A. niger* and *Penicillium* spp. with ultraviolet irradiation, colchicine or ethyl methanesulfonate (EMS). The best results were obtained from the UV treated strains, reaching verbenol concentration of 4566 μg/100mL and 3698 μg/100 mL for *A. niger* and *Penicillium* spp., respectively. In a different approach, Rao et al. (2003) used an intergenic hybrid of *A. niger* and *P. digitatum* obtained by protoplast fusion to biotransform α-pinene. The parental *A. niger* strain was able to biotransform α-pinene and produce high concentrations of verbenol and *P. digitatum* to produce high amounts of biomass, therefore, the hybrid showed increased bioconversion efficiency of 60 %, producing 1.08 mg of verbenol from each gram of biomass.

These are some of the studies involving verbenol and verbenone production which demonstrates the potential of natural aroma production via biotechnology (Vespermann et al., 2017).

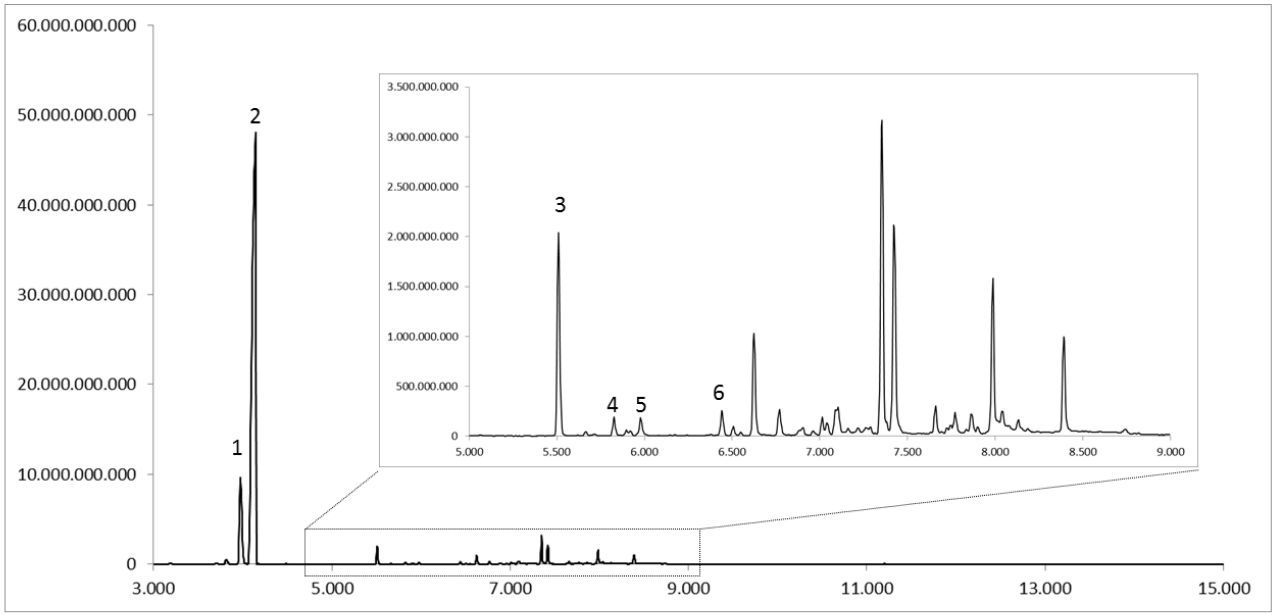
3.4. Biotransformation of β-pinene

In the biotransformation processes employing β-pinene as substrate, the fungal strains identified as JV-7 and JV-15 also presented compounds formation after 96 h (Figures 5 and 6).



#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	3.995	Beta-pinene	86 %	127-91-3	C10H16	
2	4.143	Decane	81 %	124-18-5	C10H22	
3	**5.516**	**L-pinocarveol**	**83 %**	**547-61-5**	**C10H16O**	
4	5.829	Pinocamphone	97 %	547-60-4	C10H16O	
5	5.986	Myrtenol	95 %	515-00-4	C10H16O	
6	7.35	2-Cyclohexen-1-ol, 1 methyl-4-(1-methylethyl)	72 %	29803-82-5	C10H18O	

Figure 5. Compounds obtained in the biotransformation of β-pinene by JV-7 strain.



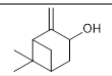
#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	3.979	Beta pinene	91 %	127-91-3	C10H16	
2	4.132	Decane	91 %	124-18-5	C10H22	
3	**5.512**	***trans*-Pinocarveol**	**78 %**	**547-61-5**	**C10H16O**	
4	5.826	Isopinocamphone	97 %	15358-88-0	C10H16O	
5	5.982	Myrtenol	97 %	515-00-4	C10H16O	
6	6.44	*cis*-myrtanol	50 %	51152-12-6	C10H18O	

Figure 6. Compounds obtained in the biotransformation of β-pinene by JV-15 strain.

β-Pinene is usually employed as substrate for production of α-terpineol, terpinolene, borneol, myrtenol, pinocarveol, pinocamphone, and pinocarvone (Toniazzo et al., 2005; Rottava et al., 2010, Rozenbaum et al., 2006; Van Dyk et al., 1998; Yoo; Day, 2002; Lindmark-Henriksson et al., 2004).

In this study, the major product obtained was pinocarveol. This is a viscous light yellow oil, with warm, woody, fennel-like odor, naturally found in grapefruit peel oil, types of ginger, Scotch spearmint oils, pepper, hop oil, myrtle leaf and Roman chamomile (Burdock, 2010). Pinocarveol production occurs by the oxidation of α- or β-pinene and its isomerization leads to the formation of carveol. In some cases, myrtenol is also obtained as a second product in β-pinene biotransformation (Figure 7) (Savithiry et al., 1998).

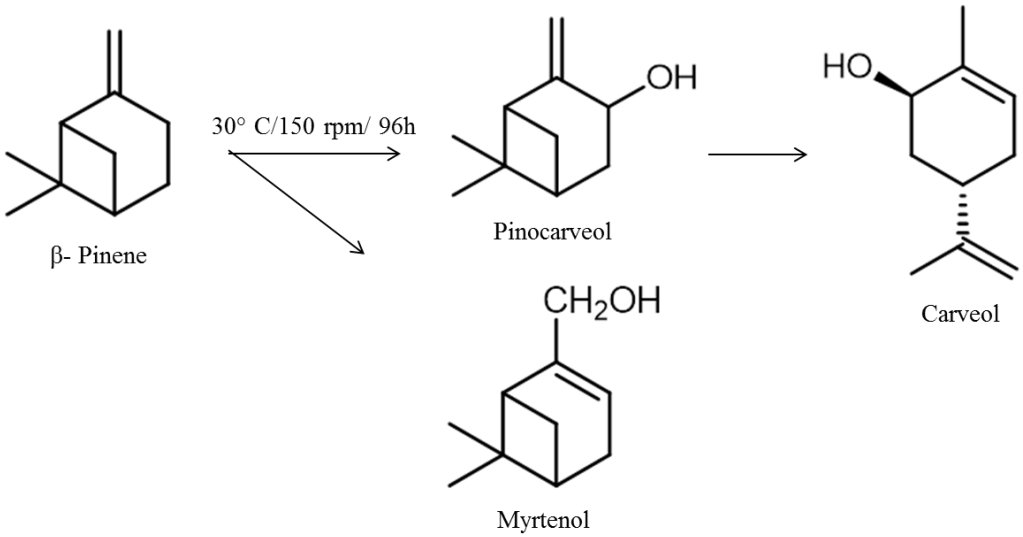


Figure 7. Pathway for β-pinene biotransformation.

Differently from the results of α-pinene biotransformation, in the biotransformation of β-pinene into pinocarveol, the strain JV-15 proved to be more efficient, and it was obtained 64.8 ± 0.6 mg/L of pinocarveol, compared with 36.7 mg/L produced by JV-7 strain (Figure 8).

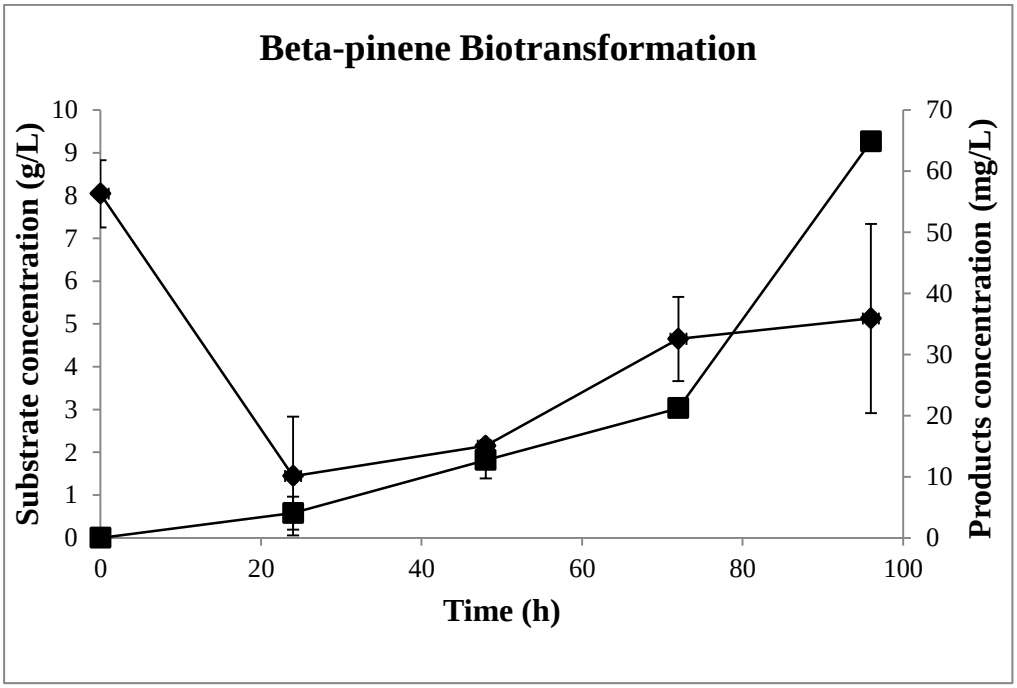


Figure 8. Biotransformation process to obtain pinocarveol (■) from β-pinene (♦) using JV-15 strain.

There are few studies reporting pinocarveol formation from β-pinene biotransformation. Most of the works aims to obtain α-terpineol from this substrate (Toniazzo et al., 2005; Rozenbaum et al., 2006; Rottava et al., 2010; Rottava et al., 2011). In older reports, pinocarveol could be obtained from β-pinene using fungal strains of *Aspergillus niger* NC1M 612 and *Armillariella mellea* (Devi; Bhattacharyya, 1978; Draczyñska et al., 1985).

Savithiry et al. (1998) reported the formation of pinocarveol from both α- and β-pinenes using an aerobic thermophile strain identified as *Bacillus pallidus* BR425 isolated from α-pinene enrichment culture. After 48 h, 5.4 µM (0.8 mg/L) of pinocarveol was obtained along with other oxidized compounds carveol (42 µM = 6 mg/L), carvone (18 µM = 2.7 mg/L) and pinocarvone (6.3 µM = 0.9 mg/L). Using basidiomycete strains, Busmann and Berger (1994) obtained pinocarveol along with verbenol, verbenone and myrtenol from β-pinene biotransformation.

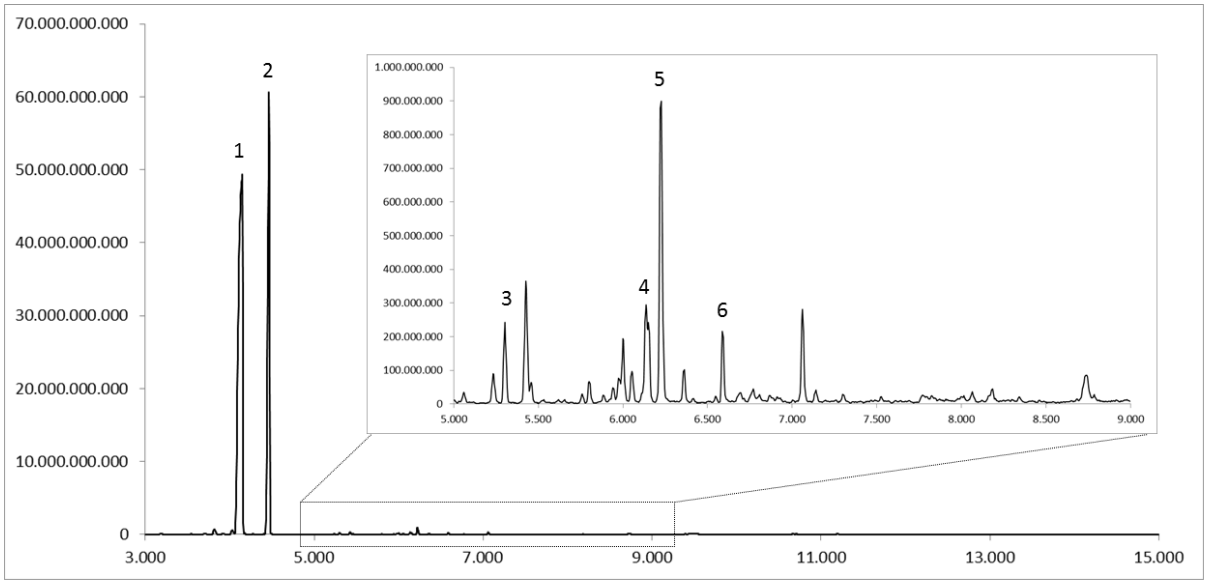
Another study involving production of pinocarveol from β-pinene was performed using plant cell cultures. *Picea abies* cultures were able to produce *trans*-pinocarveol (50%), myrtenol (28%), α-terpineol (13%), pinocamphone (5%) and pinocarvone (0.4%) after 15 days of incubation (Lindmark-Henriksson et al., 2004). The same culture had already been

used in α-pinene biotransformation, producing mainly *trans*-verbenol (35-45%) and verbenone (20-25%) after 4 days (Lindmark-Henriksson et al., 2003).

Although less studied and considered less valuable for industrial purposes, pinocarveol production from β-pinene is economically interesting. Using Molbase database to illustrate this, the reference price of pinocarveol is around US$ 3,923/kg, while β-pinene has reference price of US$ 5/kg (Molbase database, 2015). In this context, it would be a good strategy to invest efforts and resources in β-pinene biotransformation area.

3.5. Biotransformation of *R*-limonene

Only one fungal strain, identified as JV-10 was able to metabolize *R*-limonene as carbon source (Figure 9).



#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	4.115	Decane	81 %	124-18-5	C10H22	
2	4.46	R-limonene	94 %	5989-27-5	C10H16	
3	5.302	trans-*p*-mentha-2,8-dienol	91 %	7212-40-0	C10H16O	
4	6.143	*cis*-carveol	87 %	1197-06-4	C10H16O	
5	**6.226**	**Neodihydrocarveol**	**81 %**	**18675-33-7**	**C10H18O**	
6	6.59	*p*-mentha-1,8-dien-3-one	90 %	16750-82-6	C10H14O	

Figure 9. Compounds obtained in the biotransformation of *R*-limonene by JV- 10 strain.

Limonene is the most important and widespread terpene in nature, being found in more than 300 essential oils in its *R*- and *S*- optically active forms (Burdock, 2010). It has a pleasant lemon-like odor and *R*-limonene is mainly obtained by steam distillation of citrus peels and pulp, by products of juice industry (Maróstica Jr.; Pastore, 2007). It is estimated an annual production of 50 million tons of orange juice, therefore this is an important source of this compound, guaranteeing its availability to be used as flavoring or substrate in biotransformation processes (Aissou et al., 2017). Besides these applications, limonene can also be employed as solvent for resins, inks and rubber (Rottava et al., 2011).

The main limonene degradation pathways have been extensively reviewed and include: (i) oxidation of carbon 7 to perillyl compounds; (ii) ring double bond epoxidation, followed by the corresponding diol formation and its oxidation; (iii) carbon 6 oxidation to form carveol, carvone, dihydrocarveol and dihydrocarvone; (iv) carbon 8 hydroxylation to directly form a-terpineol; (v) oxidation of carbon 3 to form isopiperitenol and isopiperitenone and (vi) 8,9 double bond epoxidation to form limonene-8,9-epoxide (Maróstica Jr.; Pastore, 2007; Duetz et al., 2003).

In this study, the major compound obtained was dihydrocarveol, formed by hydrogenation of carveol (Figure 10). This compound has a spearmint-like odor and can be found in the essential oils *Mentha longifolia*, *Mentha verticillata*, *Artemisia juncea*, spearmint oil and spearmint scotch oil (Burdock, 2010). Its annual consumption is around 1100 lb and the reference price is about US$ 492/g (Molbase database, 2015). This compound was not quantified, only identified in the biotransformation media, however, since this is a high-value product, it is interesting to continue the researches focusing in this product.

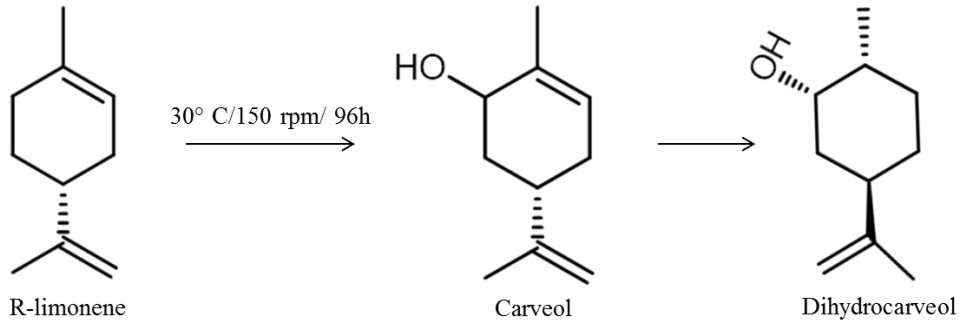


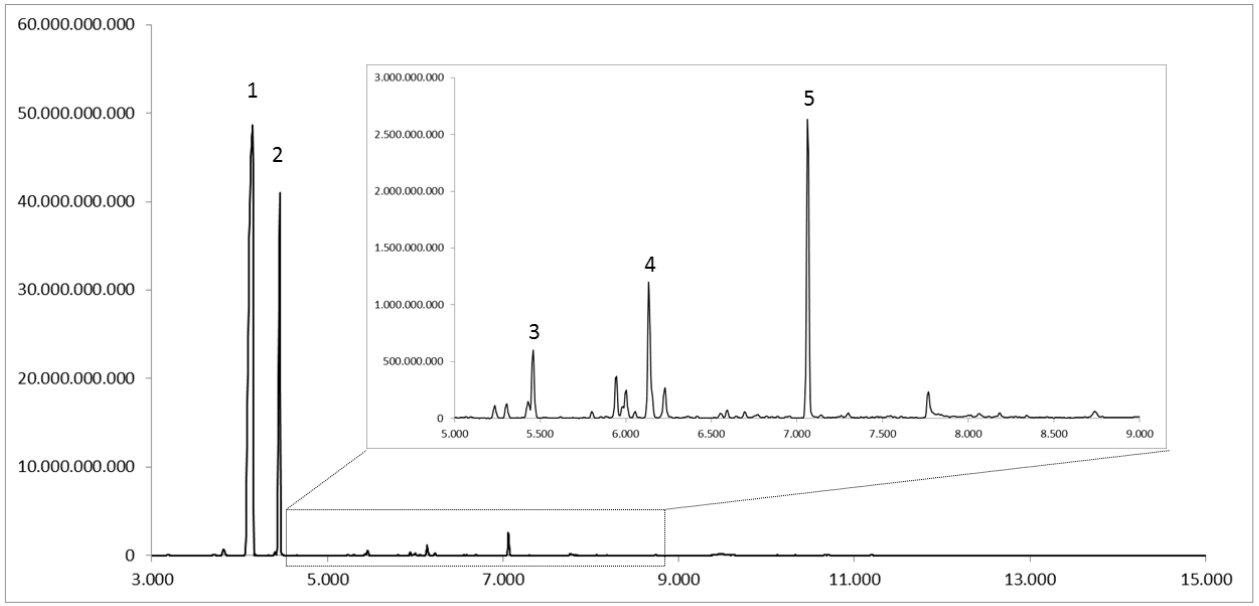
Figure 10. Pathway of dihydrocarveol formation from *R*-limonene.

Although *R*-limonene biotransformation is a well-studied area, there are few reports of dihydrocarveol formation using this substrate. Demyttenaere et al. (2001) found neo-dihydrocarveol as minor components in the biotransformation of *R*-limonene by *Penicillium digitatum*. The author studied the biotransformation of *R*- and *S*- limonene by several strains of fungi and obtained as main metabolites α-terpineol using *Penicillium digitatum* and limonene-diol with the strain *Corynespora cassiicola*. In a screening study conducted by Bier et al. (2011), dihydrocarveol was also identified. In this case, a strain of *Pichia stipites* isolated from reforestation area of the Arauco Forest (Brasil) was employed as biocatalyst.

Due to its wide application in household products, cosmetic and flavor preparations, α-terpineol is the most aimed compound to be produced through biotransformation of limonene. Isolation and screening of strains with ability to grow on limonene and to produce α-terpineol have been performed (Bier et al., 2011; Rottava et al., 2010). The formation of this compound from orange-based residues is advantageous since it reduce costs with substrate addition (Maróstica Jr.; Pastore, 2007; Bier et al., 2017). Another important step is to increase product concentration after fermentation, to facilitate recovery and increase process yields (Bicas et al., 2008; Bicas et al., 2010; Rottava et al., 2011). As limonene is an abundant agro-industrial by-product, more studies with focus in production of other oxygenated compounds besides α-terpineol are required to increase biotransformation value.

3.6. Biotransformation of *S*-limonene

In the biotransformation of S-limonene isomer, again, only the strain identified as JV-10 presented oxygenated compound formation (Figure 11).



#	Retention time	Compound	Similarity	Cas #	Chemical formula	Structure
1	4.135	Decane	81%	124-18-5	C10H22	
2	4.457	S-Limonene	84%	5689-54-8	C10H16	
3	5.458	*trans*-limonene oxide	91%	6909-30-4	C10H16O	
4	6.138	*trans*-carveol	96%	1197-07-5	C10H16O	
5	**7.066**	**1,2-cyclohexanediol, 1-methyl-4-(1-methylethenyl)-**	**86%**	**1946-00-5**	**C10H18O2**	

Figure 11. Compounds obtained in the biotransformation of *S*-limonene by JV- 10 strain.

The S-limonene isomer is the second most abundant monoterpene and is found in oils from *Mentha* species and conifers, while *R*-limonene is present in citrus peel oils (Bauer et al., 2001). There are few studies regarding biotransformation processes using the *S*- isomer.

The metabolic pathways for *S*-limonene biotransformation are similar than for *R*-limonene and using JV-10 strain as biocatalyst, the major compound obtained was 1,2-limonene diol, which can be formed by ring double bond epoxidation, followed by the corresponding diol formation (Figure 12) (Maróstica Jr.; Pastore, 2007). Limonene-1,2-diol has a cool minty aroma and is applied in alcoholics and non-alcoholics beverages, gelatins, chewing gum and candies (Burdock et al., 2010). This compound was not quantified only identified through GC-MS analysis.

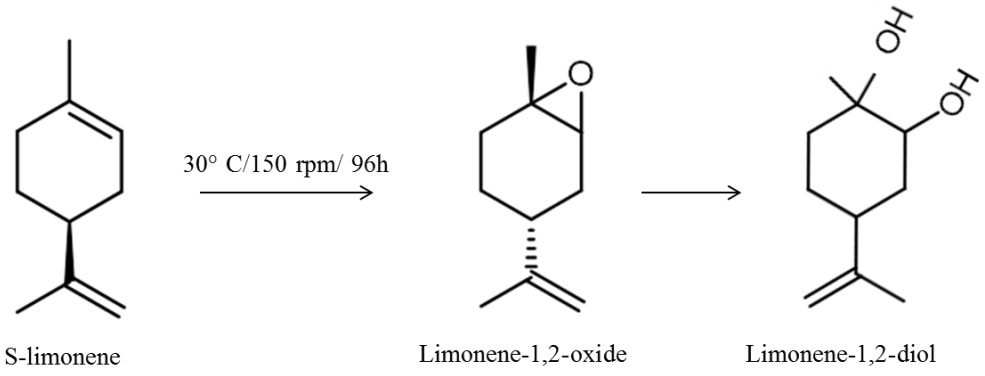


Figure 12. Scheme for biotransformation of *S*-limonene into limonene-1,2-diol.

As already demonstrated by Molina et al. (2015), the use of different isomers *R*- or *S*- limonene may lead to the formation of different compounds using the same biocatalyst, however, there are few studies reporting the bioconversion of both enantiomers of limonene. Braddock and Cadwallader (1995) reported a strain of *Pseudomonas gladioli* able to metabolize *R*- and *S*-limonene and the authors observed a 10-times greater enzymatic activity for *R*-limonene biotransformation into α-terpineol. In a similar approach, a *Sphingobium* sp. strain used both isomers to produce isomers of α-terpineol, showing approximately the same enzymatic efficiency (Bicas et al., 2010).

Demyttenaere et al., 2001 showed that fungal strains of *Penicillium digitatum* and *Corynespora cassiicola* were able to produce α-terpineol and limonene-diol from *R*- and *S*-limonene respectively. In a deeper study with only *Penicillium digitatum*, the same product was obtained from *R*-limonene, however, for *S*-limonene, a mixture of products was observed,

including low amounts of dihydrocarveol (Adams et al., 2003). In this work, neodihydrocarveol was the major product obtained from *R*-limonene, whereas, limonene-diol was obtained from *S*-limonene.

In general, the process for biotransformation of terpenes have been extensively reviewed, showing its potential for obtaining novel aroma compounds and newly isolated strains to be employed as biocatalyst (Duetz et al., 2003).

3.7. Biotransformation of citronellol

Citronellol has already been used in several studies for the production of high-value compounds, such as rose oxide (Demyttenaere et al., 2004; Maróstica Jr.; Pastore, 2006; Pimentel et al., 2012). This compound can be found in traces in diverse vegetable essential oils and may be considered one of the most important fragrances in rose aroma creation (Serra, 2015; Pimentel et al., 2012). Despite being a promising substrate, none of the strains isolated in this work was able to metabolize citronellol to obtain its oxygenated form.

4. Concluding remarks

In this work it was possible to explore the potential of unripe genipap fruits as source of biocatalysts for natural aroma production. Considering the fact that these fruits are rich in genipin, a monoterpene, compounds of the same class (α- and β-pinenes, *R*- and *S*-limonene, and citronellol) were chosen to be employed and single carbon source to be metabolized in oxygenated value-added compounds. Fifteen fungal strains and two bacteria could be isolated from unripe genipap fruits, being that three fungi proved to be able to use at least one substrate for aroma production. The compounds obtained are of great industrial interest to be applied as additives in food and cosmetic and also due to its potential biological activity. With this perspective it is interesting to provide further efforts in this area in order to improve product concentration and obtain higher yields, increasing the potential of natural aroma production through biotechnology.

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CAPÍTULO 3

OPTIMIZATION OF VERBENOL PRODUCTION THROUGH BIOTRANSFORMATION OF α-PINENE

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ABSTRACT

The biotransformation of terpenes is a promising strategy to supply the current demand for natural flavor compounds. α-Pinene can be considered a suitable substrate for this process due to its high availability in the turpentine, a by-product of pulp and paper industries. From this compound, it is possible to obtain verbenol and verbenone, value added products with application in food, beverages and pharmaceuticals. To overcome the drawback of the low yields obtained in biotransformation of terpenes, statistical methods can be employed to better understand the effect of the process parameters and maximize final product concentration. The aim of this work was to perform a central composite design with four variables in order to increase verbenol concentration after α-pinene biotransformation. Under the conditions (22 ºC, 200 rpm, 1.25 % (v/v) of α-pinene and 5 g of fungal biocatalyst/ 50 mL media) it was possible to increase verbenol concentration from 222.4 ± 2 mg/L to 318.4 ± 2.4 mg/L.

Keywords: natural aroma, verbenol, verbenone, biotransformation, response surface methodology, central composite design

1. Introduction

The global market of flavor and fragrances moved approximately US$ 24.5 billion in 2016, with application in food, beverages, cosmetic and pharmacy (Leffingwell; Associates, 2017). Nowadays, most of the aroma compounds are obtained from chemical methods, however, the attraction of the consumer for healthier and more nutritive products leads to an annual grow rate of 9.15 in the market for natural flavors, with a decrease in the market of synthetic compounds (UBIC 2014).

One strategy for natural aroma production is the biotransformation of terpenes. Among these compounds, pinenes have been successfully used as precursor for the synthesis of their oxygenated forms (Bicas et al., 2008; Molina et al., 2014). α-Pinene is one of the most abundant bicyclic monoterpenes, representing 75 to 90% of conifers essential oils (Marmulla; Harder, 2014). It has a characteristic odor of pine, and it has been reported the presence of one or more isomers in at least 400 essential oils, including *Achillea millefolium*, *Artemisia tridentata*, Italian rosemary, wild thyme, French lavender, coriander, cumin, labdanum, neroli, lemon, and *Litsea cubeba* (Burdorck, 2010). To be used as substrate in biotransformation process, α-pinene can be obtained from distillation of turpentine, a low cost by-product of paper and cellulose industry where it can be found in a range of 50 to 70% (Negoi et al., 2017; Verpermann et al., 2017).

Interesting oxygenated compounds can be obtained from α-pinene, such as verbenol, verbenone, α-terpineol, isoterpineol, isonovalal, novalic acid and myrtenol (Agrawal; Joseph 2000; Rozenbaum et al., 2006; Krings et al., 2009; Trytek et al., 2015; Siddhardha et al., 2012; Fontanille et al., 2002; Bicas et al., 2008; Linares et al., 2009; Colocousi et al., 1996). The main routes for α-pinene biotransformation are the epoxidation of the double bond to produce α-pinene epoxide and its conversion to verbenol by the action of hydroxylases. The latter compound can be further converted to verbenone by dehydrogenation (Agrawal; Joseph 2000; Negoi et al., 2017). Verbenol is a valuable food-flavoring compound with fresh pine odor. It is widely used in soft drinks, soups, meats, sausages and ice cream (Burdock, 2010). Verbenone, another α-pinene derivative is in great demand in the food and pharmaceutical industries due to its flavor notes of camphor, strawberry and spearmint flavor and also for being an intermediate in taxol synthesis, a drug used in chemotherapy (Burdock, 2010, Molina et al., 2014; Negoi et al., 2017).

Economically, the advantages of the biotransformation are clear when comparing the reference prices of substrates and products. Verbenol and verbenone are high-value compounds, costing around US$ 1,926/kg and US$ 906/kg respectively, whereas the reference price of α-pinene is about US$ 70/L (Molbase Database, 2015).

Studies involving the microbial biotransformation of α-pinene to produce verbenol have been extensively reported. The oldest one shows a strain of *Aspergillus niger* able to metabolize a mixture of α-pinene isomers (70% *D*-α-pinene and 30% *L*-α-pinene) producing verbenol, verbenone and sobrerol after 8 h of incubation at 28 °C (Prema; Bhattacharyya 1962; Parshikov; Sutherland, 2014). Later, Agrawal and Joseph (2000) obtained a conversion rate of approximately 16.5 % in α-pinene biotransformation by incubation of *A. niger* for 6 h at pH 6, with a substrate concentration of 0.2 mg/mL. Immobilized cells of the same strain were used in the process for obtaining natural verbenol and verbenone (Rozenbaum et al., 2006).

Isolated strains are also being used for biotransformation processes. A strain of yeast identified as *Hormonema* sp. UOFS Y-0067, isolated from pine tree samples, proved to metabolize α-pinene to produce 0.4 g/L of verbenol and 0.3 g/L and verbenone after 72 h of incubation (Van Dyk et al. 1998). Among strains obtained from residues of orange juice industry, 31 microorganisms were able to produce verbenol, reaching a maximum concentration of 125 mg/L (Rottava et al., 2010). Trytek et al., 2015 reported a psychotrophic strain of *Chrysosporium pannorum* isolated from soil samples, to be used in biotransformation processes at low temperatures. This is advantageous to reduce losses due to product evaporation.

α-Pinene and its oxygenated forms have been reported to have potential biological activities, such as antioxidant, anti-inflammatory, antitumor and anti-microbial, therefore, they can also be employed for medical and pharmaceutical purposes, as promising candidate therapeutic agent (Choi et al. 2010; Chen et al. 2015; Scollard et al. 2016; Paduch et al., 2016).

Although promising, the biotechnological production of natural aroma is still under development and improvements to enhance production rate, yields and product recovery are necessary (Bicas et al., 2008). The central composite design (CCD) is a statistical methodology used to analyze the effects and interactions of the independent variables studied. With this technique it is possible to propose a mathematical model where it can be observed

the behavior of the factors studied and identify the optimum process conditions (Rodrigues; Iemma, 2005).

In this approach, the aim of this work is to evaluate four variables (agitation, temperature, substrate concentration and biocatalyst biomass) and analyze their effects in the biotransformation of α-pinene for verbenol production by an endophytic strain, in order to maximize final verbenol concentration.

2. Material and methods

2.1. Microorganisms and chemicals

The fungal strain used in this work was isolated from unripe fruits of genipap (*Genipa americana* L.) and identified as JV-7. The strain was maintained in YM media (in g/L: glucose = 10, bacteriological peptone = 5, malt extract = 3, yeast extract = 3 and agar = 20) at 30 ºC and stored in the same media at 4 ºC.

The compound 1*S*–(-)–α-pinene (SAFC, 98%) was used as substrate and verbenol (Sigma, 95%), verbenone (SAFC, 93%) and myrtenol (SAFC, 95%) were used for quantification curves and identification of compounds. Ethyl acetate (P.A. Synth) and *n*-decane (Sigma-Aldrich) were used for sample preparation.

2.2. Inoculum

A piece of agar (approximately 1 cm²) with a 72h-old culture of the filamentous fungi was transferred to a 250 mL Erlenmeyer flask containing 50 mL of YM media. The material was homogenized under sterile conditions with Ultra-Turrax™ T25 (Ika, Wilmington, NC, USA) until complete disruption of the agar. After incubation at 30 ºC and 150 rpm for 72 h, the biomass could be recovered by vacuum filtration using a Buchner funnel and paper filter Whatman nº 1 (Bicas et al., 2008; Bicas et al., 2010; Molina et al., 2015).

2.3. Biotransformation procedure

The fungal biomass obtained was divided, according to each CCD assay, amongst 250 mL Erlenmeyer flasks containing 50 mL of mineral media (in g/L: $MgSO_4.7H_2O$ = 0.5, $NaNO_3$ = 3, K_2HPO_4 = 1, KCl = 0.5 and Fe_2SO_4 = 0.01). The volume of α-pinene corresponding to each assay was added to the medium. The flasks were incubated at experimental conditions for 120 h. Periodically, 500 µL samples were collected to monitor the

consumption of substrate and product formation (Bicas et al 2008; Molina et al., 2015). Blanks of the biotransformation experiments were performed without the fungal strain, to ensure that the product formation is due to action of biocatalysts and not through chemical reactions (Maróstica Jr.; Pastore, 2007).

2.4. Sample analysis

Each sample was extracted (1 minute in Vortex) with the same ethyl acetate volume, containing 1% of *n*-decane as internal standard. After phase separation, the organic fraction was dried over sodium sulphate and 1 µL was injected to a gas chromatograph with a flame ionization detector (GC-FID) HP-7890 (Agilent Technologies, Santa Clara, CA, USA), coupled to a HP-5 column (30 m length x 0.25 mm i.d. x 0.25 µm of film thickness), in split mode (split ratio 1:20). The oven temperature as kept at 80 ºC for 3 minutes, raised at 20 ºC/min until 220 ºC and held for 4 minutes. Temperature of the injector and detector were kept at 250 ºC. Helium was used as carrier gas (2 mL/min). Substrates and products were quantified by external calibration curves, also using *n*-decane as internal standard.

The identification of the volatile compounds produced was performed on a GC-MS system with a gas chromatograph HP-7890 coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, CA, USA) and a HP-5MS column (J&W Scientific, Folsom, California, USA) with 30 m length x 0.25mm i.d. x 0.25 µm of film thickness. The programming of the gas chromatograph was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 ºC, impact energy of +70 eV and a mass range 35-500 m/z. The compounds identification was made by comparing the spectra with NIST 2008 library over 90 % similarity, and comparison with commercial standard (Molina, 2015).

2.5. Optimization experiments

In order to identify the best process conditions for biotransformation of α-pinene into verbenol, a central composite design was performed, with 4 independents variables (2^4), 4 repetitions in the central point and 8 axial points (total of 28 experiments) (Rodrigues and Iemma, 2005). Considering the factor that most affect the fermentative process, the variables substrate concentration, agitation, mass of fungal biocatalyst and temperature were selected to be evaluated (Tables 1 and 2). The dependent variables were verbenol concentration at different fermentation times (0, 24, 48, 72, 96 and 120h), however, only the results of 120h

were used for further analysis. Through response surface methodology, it was possible to identify the best process conditions to improve verbenol concentration.

Table 1. Variables and levels evaluated in the central composite design 2^4.

	Variable	Units	Level				
			-2	**-1**	**0**	**+1**	**+2**
X_1	Biomass concentration	g/50mL	1	2	3	4	5
X_2	Substrate concentration	%	0.25	0.5	0.75	1	1.25
X_3	Agitation	rpm	50	100	150	200	250
X_4	Temperature	ºC	22	26	30	34	38

For experimental condition validation, 3 experiments in the condition with highest verbenol concentration were performed. In this case, these conditions were: fungal biomass 5 g/50 mL (coded value = 2), substrate concentration of 2 % (v/v) (coded value = 2), agitation of 200 rpm (coded value = 1) and temperature of 22 ºC (coded value = -2). The results obtained in these experiments were compared with verbenol concentration predicted by the mathematical model obtained after statistical analysis.

2.6. Data analysis

The results and the response surface methodology were analyzed using the Statistica 12 software (StatSoft Inc, Oklahoma, USA). A significance level of 10% ($p< 0.1$) was considered for the central composite design, which is acceptable for biotechnological processes (Rodrigues; Iemma, 2005).

Table 2. Experiments of the central composite design and the results obtained for verbenol concentration (mg/L).

Assay	Biomass (X_1)	Substrate concentration (X_2)	Agitation (X_3)	Temperature (X_4)	Verbenol concentration (mg/L)					
					0h	24h	48h	72h	96h	120h
1	-1	-1	-1	-1	0	0	0	19.93	32.90	47.96
2	+1	-1	-1	-1	0	0	0	27.25	57.24	109.33
3	-1	+1	-1	-1	0	0	21.70	21.97	46.21	74.86
4	+1	+1	-1	-1	0	0	33.22	84.42	100.42	114.63
5	-1	-1	+1	-1	0	0	19.92	53.53	86.12	117.90
6	+1	-1	+1	-1	0	0	30.62	108.89	237.38	253.41
7	-1	+1	+1	-1	0	0	18.66	47.06	135.34	177.95
8	+1	+1	+1	-1	0	0	30.64	91.72	165.67	170.33
9	-1	-1	-1	+1	0	0	12.76	25.38	37.75	56.83
10	+1	-1	-1	+1	0	0	16.83	44.54	63.79	104.47
11	-1	+1	-1	+1	0	0	16.99	28.64	54.61	74.33
12	+1	+1	-1	+1	0	0	32.60	44.40	77.47	120.91
13	-1	-1	+1	+1	0	0	21.70	16.64	24.61	35.89
14	+1	-1	+1	+1	0	0	19.64	21.09	36.60	49.81
15	-1	+1	+1	+1	0	0	10.45	15.16	23.00	33.46
16	+1	+1	+1	+1	0	0	23.21	23.77	35.57	55.92
17	-2	0	0	0	0	0	0	16.03	22.79	27.49
18	+2	0	0	0	0	0	17.68	54.12	95.10	146.38
19	0	-2	0	0	0	0	0	0	0	9.85
20	0	+2	0	0	0	0	21.81	40.12	74.94	167.31
21	0	0	-2	0	0	0	0	5.80	12.34	16.91
22	0	0	+2	0	0	0	52.64	123.93	197.69	218.09
23	0	0	0	-2	0	0	26.81	85.29	191.78	198.71
24	0	0	0	+2	0	0	10.64	19.95	20.34	22.06
25	0	0	0	0	0	0	0	22.80	37.54	61.32
26	0	0	0	0	0	0	18.35	20.98	26.29	59.17
27	0	0	0	0	0	0	18.35	26.41	40.09	57.92
28	0	0	0	0	0	0	16.75	39.73	71.84	51.68

3. Results and discussion

3.1. Central composite design

In order to understand how process parameters affect verbenol production, the variables fungal biomass, substrate concentration, agitation and temperature were evaluated in a central composite design 2^4, with 4 repetitions at the central point and 8 axial points, totaling 28 experiments. The results obtained from 0 to 120 h of fermentation for each experiment in the central composite design are provided in Table 2. The independent variable chosen to be used as response (Y) in the statistical analysis was only the verbenol concentration at 120 h. As it can be observed, the experiments performed in the central point showed a good reproducibility, with an average verbenol concentration of 57.52 ± 2.9 mg/L. After statistical analysis, it was possible to obtain the regression coefficients for the parameters (Table 3).

Table 3. Regression coefficients of the model parameters for the response verbenol concentration (120h).

Parameters	RC	SE	t(13)	p –value
Mean	**57.52**	**19.65**	**2.93**	**0.0118**
Linear				
X_1	**24.89**	**8.02**	**3.10**	**0.0084**
X_2	**15.07**	**8.02**	**1.88**	**0.0828**
X_3	**24.74**	**8.02**	**3.08**	**0.0087**
X_4	**-37.00**	**8.02**	**-4.61**	**0.0005**
Quadratic				
X_1^2	7.19	8.02	0.90	0.3864
X_2^2	7.60	8.02	0.95	0.3605
X_3^2	14.83	8.02	1.85	0.1073
X_4^2	13.05	8.02	1.63	0.1276
Interaction				
X_1X_2	-9.83	9.82	-1.00	0.3353
X_1X_3	-1.94	9.82	-0.20	0.8462
X_1X_4	-6.15	9.82	-0.63	0.5420
X_2X_3	-5.34	9,82	-0.54	0.5958
X_2X_4	1.78	9.82	0.18	0.8591
X_3X_4	**-34.64**	**9.82**	**-3.53**	**0.0037**

RC = regression coefficients; SE = standard error; X_1= Biomass (g/50mL); X_2=Substrate concentration (%); X_3= Agitation (rpm); X_4=Temperature (ºC). Parameters in bold are statistically significant for the model (p <0.1).

Using only the significant parameters, it was possible to verify the validity of the model through an analysis of variance (ANOVA), which is shown on Table 4.

Table 4. ANOVA of the quadratic model.

Variation source	**SS**	**df**	**MS**	**F calculated**	***p*-value**
Regression	87067.6	5	17413.52	12.58	0.001360
Residue	30456.9	22	1384.41		
Total	117524.6	27			
$R^2 = 0.74$				$F_{0,9\ (5,22)} = 2.13$ F_{calc}:$F_{listed} = 5.91$	

SS = Sum of squares; df = degrees of freedom; SM = mean square; R^2 = coefficient of determination

The ANOVA table shows that the quadratic model adjusted for the process responses was satisfactory. One of the parameters that indicate that fitted equations are predictive is the F calculated value, which must be higher than F listed value (Khuri and Cornell, 1996). In this case, it was approximately 6 times higher than the respective listed value and the *p*-value was lower than 0.001. The value of R^2 was lower than the ideal (>0.8), however, it is still acceptable for biological systems (Rodrigues and Iemma, 2005). With these data, it was possible to obtain a mathematical model that predicts verbenol concentration (Equation 1). The regression coefficients which composes the model are adjusted with only significant parameters.

$$Y = 94.10 + 24.89X_1 + 15.07X_2 + 24.74X_3 - 37X_4 - 34.64X_3X_4 \ (Eq.1)$$

Where Y is the dependent variable verbenol concentration in mg/L, and X are the coded independent variables (X_1 = fungal biomass, X_2 = substrate concentration, X_3 = agitation and X_4 = temperature). This validated model enables the plotting of response surfaces and contour plots (Fig. 1 and 2).

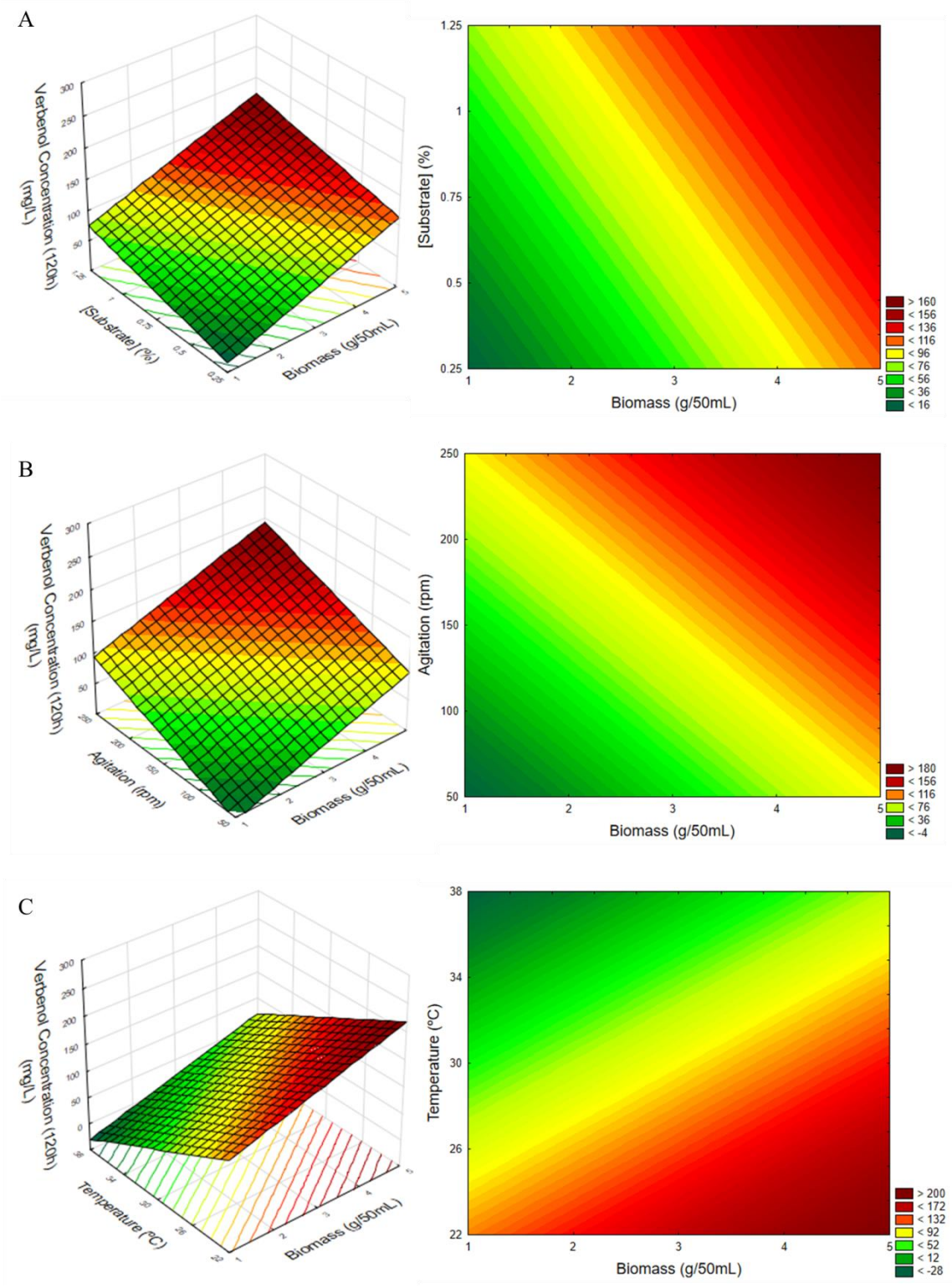

Figure 1. Contour plots and response surfaces obtained after statistical analysis of optimization experiments.

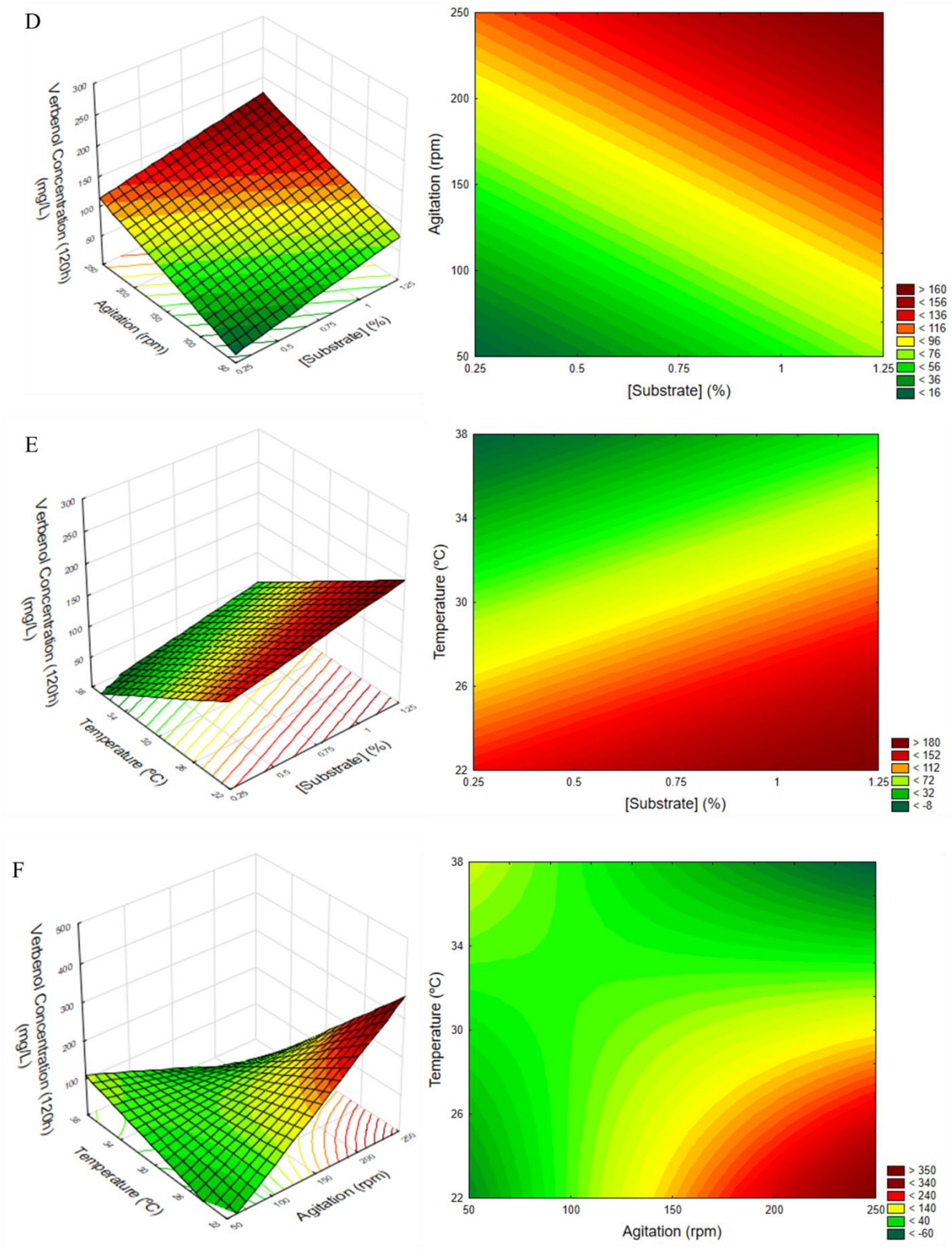

Figure 2. Contour plots and response surfaces obtained after statistical analysis of optimization experiments.

The optimization of natural aroma production using response surface methodology has already been described in literature. Bicas et al. (2008) studied α-terpineol production from *R*-limonene using *Fusarium oxyxporum* 152b as biocatalyst and obtained 2.4 g/L of product under optimized conditions. The same monoterpene alcohol was the focus in other two optimization studies: the first used experimental designs to increase α-terpineol concentration from both *R*-limonene and β-pinene biotransformation using previously isolated strains. The best conditions found were substrate concentration of 1.75 % and mass of inoculum of 2 g (Rottava et al., 2011). The second shows the highest concentration of α-terpineol ever described in biotransformation process, obtaining about 500 g/L of product under optimized conditions. The authors employed a Plackett-Burman design followed by a central composite design to evaluate process conditions (Molina, 2014).

This work applied a similar approach described by Rottava et al (2011) and used a newly isolated strain in a central composite design in order to improve biotransformation processes. As it can be observed from Table 3, only the linear parameters of the factors studies were significant in verbenol production process, and for this reason, the response surfaces graphs are also linear. From the response surfaces and contour plots (Figure 1), it was possible to obtain the optimal conditions for biotransformation process to occur.

As shown in Figures 1A, 1B and 1C, the biocatalyst biomass to be added in the biotransformation process should be approximately 5 g/50 mL or higher in order to maximize verbenol formation. The inoculum size is an important factor in industrial fermentation processes due to the fact that age and density of inoculum affects the duration of lag phase, specific growth rate, biomass yield and the quality of product, which impacts in the production costs (Sen; Swaminathan, 2004). Some authors have already described that the use of mycelium concentrates in fungal experiments might enhance the process yield (Bicas et al., 2008).

Regarding substrate addition, Figures 1A, 2D and 2E show that the concentration of substrate should be maintained in the higher level. This is an interesting result since both substrate and products usually presents high cytotoxicity and high volatility and the monoterpenes may increase fungal membranes fluidity, leading to loss of its integrity (Onken; Berger, 1999).

As demonstrated in Figures 1B, 2D and 2F, the agitation had a positive effect in verbenol formation. High agitation rates increases cell-substrate contact, however, it also

might enhance the loss of substrate and product and the occurrence of side reactions. For this reason, the experimental validation of optimized conditions was not performed in the higher level (250 rpm), but in a lower agitation speed, of 200 rpm.

Another factor that greatly affects biotransformation reactions is the temperature. The use of high temperatures increases process energy costs and also causes microbial growth inhibition and enzyme denaturation (Bicas et al., 2008). Figures 1C, 2E and 2F show that this parameter had a negative effect in α-pinene biotransformation. As reported by Trytek et al. (2015), it is advantageous the use low temperatures in biotransformation aiming to reduce evaporation and consequently volatile compounds losses during the process, aiming to recover a higher amount of product

To summarize, in this study, 5 g/L of biomass (codified value: +2), 1.25 % (v/v) of substrate (codified value: +2), agitation of 200 rpm (codified value: +1) and temperature of 22 °C (codified value: -2) were the conditions to maximize verbenol production through biotransformation of α-pinene. As it can be observed, the verbenol concentration still can be increased if another range of the parameters were studied, and with fermentation time longer than 120h, therefore, further studies are required to explore the potential of natural aroma production.

3.2. Experimental validation

The experimental validation of the optimization study was performed with three experiments in the conditions with best results for verbenol concentration. Table 5 and 6 summarizes the parameters used and the results obtained after 120h of process.

Table 5. Verbenol concentration obtained in the validation experiments.

	Verbenol concentration (mg/L)							
	0h	24h	48h	72h	96h	120h	Mean (120h)	SD
V_1	0	0	28.79	95.97	249.65	316.07		
V_2	0	0	22.71	75.71	266.50	322.02	318.40	2.42
V_3	0	0	17.86	59.53	219.85	317.10		

SD = standard deviation

From the mathematical model described in equation 1, it was possible to obtain the predicted verbenol concentration to be reached in each condition studied. Table 6 shows the comparison of experimental and predicted values for validation experiments.

Table 6. Experimental validation under the optimized conditions for the production of verbenol from the biotransformation of α-pinene.

	Parameter	Unit	Codified value	Real value	EV	PD	RD
X_1	Biomass	g/50mL	2	5	318.4	342.05	-7.43
X_2	[Substrate]	%	2	1.25			
X_3	Agitation	rpm	1	200			
X_4	Temperature	°C	-2	22			

EV (Experimental values) = Mean values obtained in optimal conditions from contour curves analyses, in mg/L; PD = Predicted values by the model, in mg/L; RD (Relative deviation) = [(experimental value – predicted value)/experimental value] x 100

A relative deviation of approximately 7.5% was obtained between experimental and predicted results. This value is acceptable the optimization of biotechnological process regarding biological systems, therefore, the conditions employed for optimization of verbenol production by the biotransformation of α-pinene are validated (Rodrigues; Iemma, 2005). With the experiments from the central composite design and through response surface methodology, it was possible to identify the best process conditions for biotransformation of α-pinene and increase verbenol concentration in around 1.4-fold, from 222.4 ± 2 mg/L initially obtained in screening assays to 318.4 ± 2.4 mg/L.

4. Concluding remarks

In this work, we show that the use of statistical tools proved to be an interesting approach to evaluate the process variables in natural aroma production through biotechnology, aiming to increase final product concentration. The parameters of incubation (agitation and temperature), substrate concentration and amount of fungal biocatalysts greatly affect verbenol production by the biotransformation of α-pinene and through response surface analysis and proposal of a mathematical model, it was easier to identify the best process condition. Using the conditions: 5 g/L of biomass, 1.25 % of substrate (v/v), agitation of 200 rpm and temperature of 22 °C, it was possible to achieve a 1.4-fold increase in verbenol concentration, from 222.4 ± 2 mg/L to 318.4 ± 2.4 mg/L. This improvement is advantageous since it can reduce costs in the industrial production of this compound using biotechnology.

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CAPÍTULO 4

OPTIMIZATION OF PINOCARVEOL PRODUCTION BY THE BIOTRANSFORMATION OF β-PINENE

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ABSTRACT

Pinenes represent 75-90% of conifers essential oil and can be found as major compounds of turpentine, a residue from pulp and paper industry. By the biotransformation of β-pinene, one of the products obtained is pinocarveol. It has a woody, slightly piney odor, and can be found in grapefruit peel oil, bilberries, types of ginger, among others and its applications include baked goods, frozen dairy, non-alcoholic beverages, soft candy and puddins. One of the main limitations of industrial application of biotransformation of terpenes is the low yields that may be obtained during fermentation, therefore, the use of statistical methods and optimization designs are valuable tools to increase product concentration. The aim of this work was to employ a central composite design - CCD (2^4) to evaluate the influence of the variables substrate concentration, amount of biocatalyst biomass, agitation and temperature of incubation in the production of pinocarveol through biotransformation of β-pinene, using an endophytic fungus isolated from genipap (*Genipa americana* L.) as biocatalyst. After analysis through response surface methodology, it was possible to identify that substrate concentration of 1 % (v/v), biomass concentration of 5 g/50 mL, agitation of 150 rpm and temperature of 30 ºC, were the best conditions for pinocarveol formation. With these experiments, it was possible to increase pinocarveol concentration from 64.8 ± 0.6 to 141.32 ± 2.6 mg/L.

Keywords: biotransformation, natural aroma, pinocarveol, β-pinene, optimization

1. Introduction

Industrial biotechnology has emerged in the past years as a suitable alternative for obtaining natural products, especially aroma compounds to be used as food additives (Schempp et al., 2017). Flavors and fragrances were firstly obtained by its extraction from plant biomass and the identification of the main constituents of these extracts allowed the synthetic production of flavors. Nowadays, an efficient, sustainable and eco-friendly way to supply the demand for natural flavors is the biotransformation of terpenes using microbial cells (Carroll et al., 2016; de Carvalho; da Fonseca, 2006).

It is expected that the biotechnologically produced flavors could substitute the synthetic and the nature identical labeled products, since they can be obtained in a controlled environment and at lower cost. However, the success of implementation of biotechnology depends on the control of the whole production chain, including the use of low cost adequate substrate, study of the fermentation process conditions, environmental regulations and also product protection (UBIC 2014; Leffingwell; Leffingwell, 2015).

Pinenes are bicyclic hydrocarbon monoterpenes that represents around 75-90 % of conifers essential oils and can be found in high concentration in turpentine, a residue from paper and pulp industry (Vespermann et al., 2017). Each year, thousands of tons of these monoterpenes are discarded due to its low value and chemical instability, therefore, these by-products can be employed as low cost substrate in the biotransformation process (Berger, 2009). The oxyfunctionalised compounds obtained from pinenes include verbenol, verbenone, α-terpineol, pinocarveol, carveol, pinocamphone, myrtenol and isonovalal and several studies have been focusing in obtaining these compounds using microorganisms (Agrawal; Joseph 2000; Rozenbaum et al., 2006; Lindmark-Henriksson et al., 2004; Trytek et al., 2015; Siddhardha et al., 2012; Fontanille et al., 2002; Bicas et al., 2008; Linares et al., 2009).

β-Pinene has a characteristic turpentine odor with a dry, woody or resinous aroma. It is used as additive in candies, beverages, baked goods and frozen dairy (Burdock, 2010). This compound is usually found in lower percentages than α-pinene, and this fact might corroborate to the fact that few studies explore its potential application as substrate and also its biological activities (Vespermann et al., 2017). The main routes for β-pinene metabolism include its isomerization to limonene followed by oxidation to carveol and carvone or direct oxidation to pinocarveol or pinocarvone, which can be further isomerized to carveol or carvone respectively (Savithiry et al., 1998).

Pinocarveol has a woody, balsamic, slightly piney, fennel-like odor, and at concentration of 25 ppm, its characteristic taste is camphoraceous, woody with cooling minty under notes (Burdock, 2010). This is one of the less studied product obtained from β-pinene, due to its low application as flavoring, however, from economic point of view, its production is advantageous. The reference price of pinocarveol is around US$ 3,923/kg, while β-pinene has reference price of US$ 5/kg (Molbase database, 2015). In this context, it would be a good strategy to invest efforts and resources to better understand β-pinene biotransformation.

Several parameters can influence the fermentation process using β-pinene as substrate. One of them is the low solubility and high toxicity of both substrate and products (Vespermann et al., 2017). Agitation and temperature must be well controlled to reduce process energy costs and to avoid substrate and product losses by evaporation. They also affect yields and product titer due to its influence in the optimal activity of the biocatalyst (Bicas et al., 2008). The process conditions can be optimized using classical methods, by varying the parameters one-at-a-time and maintaining the other variables constant, however, this methodology is time-consuming and the interaction between the factors is not considered (Sen et al., 2004). The central composite design is a statistical method to analyze the effects and interactions of the variables of a biotechnological process. With this methodology, it is possible to propose a mathematical model to establish their optimal values in order to maximize the desirable response (Rodrigues; Iemma, 2005).

As few researches reported the biotransformation of the isomer β-pinene, and considering the importance of increasing product concentration for industrial application, the aim of this work was to evaluate the effects of process parameters in the production of pinocarveol from β-pinene using a fungal biocatalyst isolated from a Brazilian fruit. Using a central composite design with four variables and through response surface methodology it was possible to identify the best conditions to maximize pinocarveol concentration.

2. Material and Methods

2.1. Microorganisms and chemicals

The fungal strain used is this work was isolated from unripe fruits of genipap (*Genipa americana* L.) and identified as JV-15. The strain was maintained in YM media (in g/L: glucose = 10, bacteriological peptone = 5, malt extract = 3, yeast extract = 3 and agar = 20) at 30 ºC and stored in the same media at 4 ºC.

The standard 1*S*–(-)–β–pinene (SAFC, 97%) was used as substrate and for quantification curves and identification of compounds produced, the standards used were myrtenol (SAFC, 95%), carveol (Sigma-Aldrich, 97%), pinocarveol (Sigma-Aldrich 96%). Ethyl acetate (P.A. Synth) and *n*-decane (Sigma-Aldrich) were used for sample preparation.

2.2. Inoculum

A piece of agar (approximately 1 cm²) with a 72h-old culture of the filamentous fungi was transferred to a 250 mL Erlenmeyer flask containing 50 mL of YM media. The material was homogenized under sterile conditions with Ultra-Turrax™ T25 (Ika, Wilmington, NC, USA) until complete disruption of the agar. After incubation at 30 ºC and 150 rpm for 72 h, the biomass could be recovered by vacuum filtration using a Buchner funnel and paper filter Whatman nº 1 (Bicas et al., 2008; Bicas et al., 2010; Molina et al., 2015).

2.3. Biotransformation procedure

The fungal biomass obtained was divided according to each assay, amongst 250 mL Erlenmeyer flasks containing 50mL of mineral media (MM; in g/L: $MgSO_4.7H_2O$ = 0.5, $NaNO_3$ = 3, K_2HPO_4 = 1, KCl = 0.5 and Fe_2SO_4 = 0.01). The volume of β-pinene corresponding to each assay was added to the medium. The flasks were incubated at experimental conditions for 120 h. Periodically, 500 µL samples were collected to monitor the consumption of substrate and product formation (Bicas et al 2008; Molina et al., 2015). Blanks of the biotransformation experiments were performed without the fungal strain, to ensure that the product formation is due to action of biocatalysts and not through chemical reactions (Maróstica Jr.; Pastore, 2007).

2.4. Sample analysis

Each sample was extracted (1 minute in Vortex) with the same volume of ethyl acetate, containing 1% of *n*-decane as internal standard. After phase separation, the organic fraction was dried over sodium sulphate and 1 µL was injected to a gas chromatograph with a flame ionization detector (GC-FID) HP-7890 (Agilent Technologies, Santa Clara, CA, USA), coupled to a HP-5 column (30 m length x 0.25 mm i.d. x 0.25 µm of film thickness), in split mode (split ratio 1:20). The oven temperature as kept at 80 ºC for 3 minutes, raised at 20 ºC/min until 220 ºC and held for 4 minutes. Temperature of the injector and detector were

kept at 250 ºC. Helium was used as carrier gas (2 mL/min). Substrates and products were quantified by external calibration curves, also using *n*-decane as internal standard.

The identification of the volatile compounds produced was performed on a GC-MS system with a gas chromatograph HP-7890 coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, CA, USA) and a HP-5MS column (J&W Scientific, Folsom, California, USA) with 30 m length x 0.25mm i.d. x 0.25 µm of film thickness. The programming of the gas chromatograph was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 ºC, impact energy of +70 eV and a mass range 35-500 m/z. The compounds identification was made by comparing the spectra with NIST 2008 library over 90% similarity, and comparison with commercial standard (Molina et al., 2015).

2.5. Optimization experiments

Aiming to identify the best process conditions for pinocarveol formation by the biotransformation of β-pinene, it was performed a central composite design with 4 independents variables (2^4) with 4 repetitions in the central point and 8 axial points, totaling 28 experiments (Rodrigues; Iemma, 2005). The independent variables substrate concentration, agitation, mass of fungal biocatalyst (humid biomass) and temperature were chosen due to the fact that these are parameters that most affects fermentation process, and the levels and experiments performed are shown on Tables 1 and 2. The dependent variables were pinocarveol concentration at different fermentation times (0, 24, 48, 72, 96 and 120h), however, only the results of 120h were used for further analysis. Through response surface methodology, it was possible to identify the best process conditions to improve pinocarveol concentration.

Table 1: Variables and levels evaluated in the central composite design 2^4

	Variable	**Units**	**Level**				
			-2	**-1**	**0**	**+1**	**+2**
X_1	Biomass	g/50mL	1	2	3	4	5
X_2	Substrate concentration	%	0.25	0.5	0.75	1	1.25
X_3	Agitation	rpm	50	100	150	200	250
X_4	Temperature	ºC	22	26	30	34	38

For experimental condition validation, 3 experiments in the condition with highest pinocarveol concentration were performed. In this case, these conditions were: fungal biomass

5 g/50mL (coded value = 2), substrate concentration of 1 % (v/v) (coded value = 1), agitation of 150 rpm (coded value = 0) and temperature of 30 ºC (coded value = 0). The results obtained in these experiments were compared with pinocarveol concentration predicted by the mathematical model obtained after statistical analysis.

2.6. Data analysis

The results and the response surface methodology were analyzed using the Statistica 12 software (StatSoft Inc, Oklahoma, USA). A significance level of 10% ($p< 0.1$) was considered for the central composite design, which is adequate to biotechnological processes (Rodrigues; Iemma, 2005).

Table 2: Experiments of the central composite design and the results obtained for pinocarveol concentration (mg/L).

Assay	Biomass (X_1)	Substrate concentration (X_2)	Agitation (X_3)	Temperature (X_4)	Pinocarveol concentration (mg/L)					
					0h	24h	48h	72h	96h	120h
1	-1	-1	-1	-1	0	0	16.48	24.42	26.03	30.00
2	+1	-1	-1	-1	0	0	22.44	24.84	34.67	37.73
3	-1	+1	-1	-1	0	0	17.14	22.34	40.46	54.80
4	+1	+1	-1	-1	0	0	27.94	42.75	61.68	83.97
5	-1	-1	+1	-1	0	0	24.44	49.24	53.06	61.02
6	+1	-1	+1	-1	0	0	31.94	68.98	94.87	103.05
7	-1	+1	+1	-1	0	0	24.45	44.01	65.34	50.69
8	+1	+1	+1	-1	0	23.93	60.96	73.66	71.07	95.21
9	-1	-1	-1	+1	0	0	22.13	33.46	49.09	66.84
10	+1	-1	-1	+1	0	0	24.08	29.03	35.51	55.01
11	-1	+1	-1	+1	0	0	26.64	34.14	57.14	69.90
12	+1	+1	-1	+1	0	0	0	26.96	44.03	57.85
13	-1	-1	+1	+1	0	0	19.35	28.84	17.51	24.73
14	+1	-1	+1	+1	0	0	26.64	38.56	42.85	54.87
15	-1	+1	+1	+1	0	0	33.19	49.91	56.09	57.00
16	+1	+1	+1	+1	0	0	37.05	68.10	79.66	79.42
17	-2	0	0	0	0	0	18.17	26.59	36.92	41.26
18	+2	0	0	0	0	0	36.36	67.07	111.02	144.60
19	0	-2	0	0	0	0	0	0	0	16.54
20	0	+2	0	0	0	0	44.24	72.67	81.92	106.90
21	0	0	-2	0	0	0	0	44.69	59.88	65.88
22	0	0	+2	0	0	20.09	32.79	29.68	26.66	48.51
23	0	0	0	-2	0	15.66	35.72	59.18	60.16	68.17
24	0	0	0	+2	0	0	24.25	28.52	35.57	47.09
25	0	0	0	0	0	0	32.16	60.08	69.91	91.83
26	0	0	0	0	0	0	44.76	52.96	67.63	93.75
27	0	0	0	0	0	0	45.16	60.45	68.68	86.53
28	0	0	0	0	0	0	33.68	51.72	77.89	96.65

3. Results and discussion

3.1. Central composite design

A central composite design with the four independent variables agitation, temperature, biocatalyst biomass and substrate concentration was performed in order to elucidate their effects on β-pinene biotransformation and optimize pinocarveol production. As described above, the biocatalyst used was a newly isolated fungal strain, obtained from unripe genipap (*Genipa americana* L.).

The results obtained in each assay of the experimental design are shown on Table 2. It was possible to observe a good reproducibility in the central points (assays 25-28), with an average pinocarveol concentration of 92.2 ± 3 mg/L in 120 h of fermentation. The statistical analyses were performed only with the results from 120 h, since the highest pinocarveol was obtained in these assays. Table 3 provides the regression coefficients for each parameter, highlighting the significant ones.

Table 3. Regression coefficients of the model parameters for the response pinocarveol concentration (120h)

Parameters	RC	SE	t(13)	p -value
Mean	**92.19**	**9.55**	**9.65**	**0.000000**
Linear				
X_1	**14.95**	**3.90**	**3.83**	**0.002067**
X_2	**12.35**	**3.90**	**3.17**	**0.007428**
X_3	1.46	3.90	0.38	0.713178
X_4	-3.88	3.90	-0.99	0.338344
Quadratic				
X_1^2	-0.81	3.90	-0.21	0.838107
X_2^2	**-8.61**	**3.90**	**-2.21**	**0.045680**
X_3^2	**-9.75**	**3.90**	**-2.50**	**0.026600**
X_4^2	**-9.64**	**3.90**	**-2.47**	**0.028024**
Interaction				
X_1X_2	1.00	4.77	0.21	0.837338
X_1X_3	7.88	4.77	1.65	0.122740
X_1X_4	-5.92	4.77	-1.24	0.236738
X_2X_3	-2.39	4.77	-0.50	0.624544
X_2X_4	0.62	4.77	0.13	0.899231
X_3X_4	**-8.57**	**4.77**	**-1.79**	**0.096083**

RC = regression coefficients; SE = standard error; X_1= Biomass (g/50mL); X_2=Substrate concentration (%); X_3= Agitation (rpm); X_4=Temperature (ºC). Parameters in bold are statistically significant for the model (p <0.1).

The validity of the model was verified through analysis of variance (ANOVA) considering only the statistically significant variables (p<0.1) (Table 4).

Table 4. ANOVA of the quadratic model

Variation source	**SS**	**df**	**MS**	**F calculated**	***p*-value**
Regression	14793.3	6	2465.55	7.57	0.000565
Residue	6839.0	21	325.67		
Total	21632.3	27			
R^2 = 0.7				$F_{0,9\ (6,21)}$ = 2.08 F_{calc}:F_{listed} = 3.64	

SS = Sum of squares; df = degrees of freedom; SM = mean square; R^2 = coefficient of determination

One of the parameters that indicate that fitted equations are predictive is the F calculated value, which must be higher than F listed value (Khuri and Cornell, 1996). In this case, it was approximately 3.5 times higher than the respective listed value and the *p*-value was lower than 0.001. The value of R^2 was lower than the ideal (>0.8), however, it is still in the limit acceptable for biological systems (Rodrigues; Iemma, 2005), therefore, from the ANOVA table it was possible to conclude that the quadratic model adjusted for the process responses was satisfactory.

With these data, it was possible to obtain a mathematical model that predicts pinocarveol concentration (Equation 1). The regression coefficients which compose the model are adjusted with only significant parameters.

$$Y = 90.97 + 29.90X_1 + 24.69X_2 - 16.82X_2^2 - 19.09X_3^2 - 18.87X_4^2 - 17.13X_3X_4 \quad (Eq.1)$$

Where Y is the dependent variable pinocarveol concentration in mg/L, and X are the coded independent variables (X_1 = fungal biomass, X_2 = substrate concentration, X_3 = agitation and X_4 = temperature). This validated model was used in the plotting of response surfaces and contour plots to verify the optimum conditions for pinocarveol formation (Fig. 1).

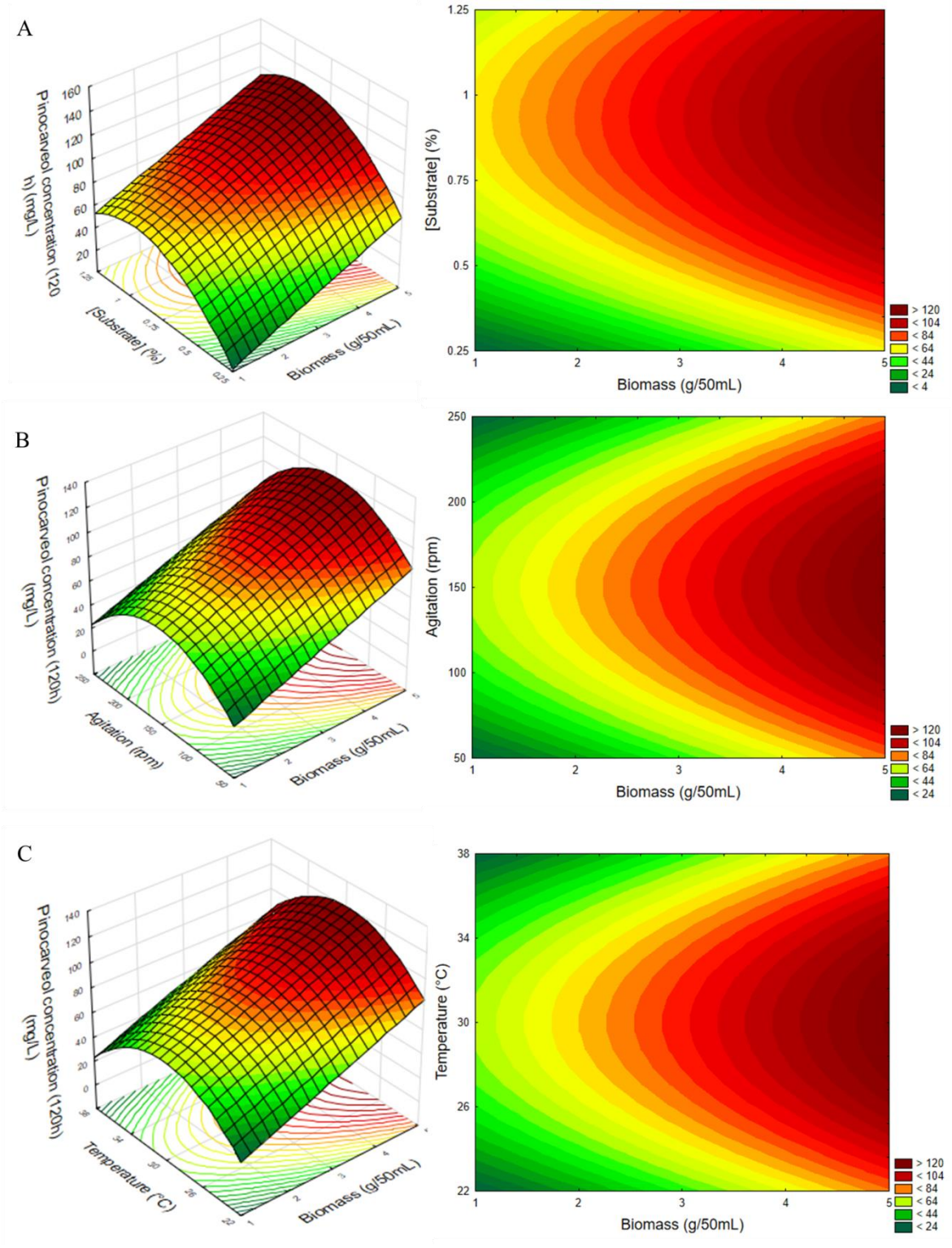

Figure 1. Contour plots and response surfaces obtained after statistical analysis of optimization experiments.

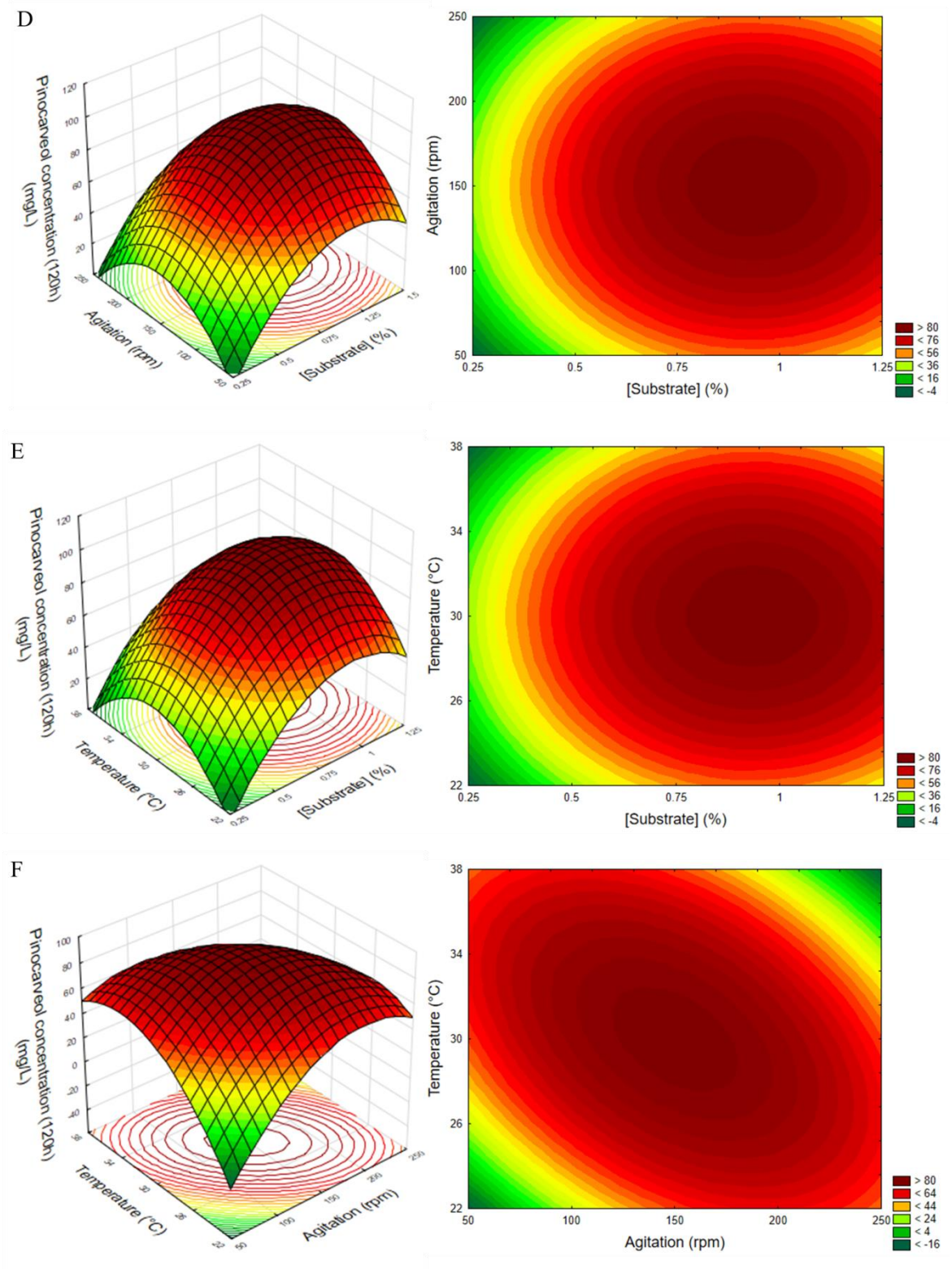

Figure 2. Contour plots and response surfaces obtained after statistical analysis of optimization experiments.

Studies aiming to optimize the biotransformation process using β-pinene as substrate have already been reported. Rottava et al. (2011) used a central composite design

and response surface methodology to evaluate the process parameters substrate concentration, substrate to ethanol ratio and biomass of inoculum in the biotransformation of *R*-limonene and β-pinene into α-terpineol. It is interesting to highlight that in these processes, the biocatalysts used were *Aspergillus* sp. isolated from orchards and industries of citric fruits in Brazil. The best process conditions reached were substrate concentration of 1.75 %, mass of inoculum of 2 g, and substrate to ethanol volume ratio of 1:1, being obtained 1,700 mg/L and 770 mg/L of α-terpineol from R-limonene and β-pinene respectively.

In a similar approach, Bicas et al., 2008 studied α-terpineol production from *R*-limonene using *Fusarium oxyxporum* 152b as biocatalyst and obtained 2.4 g/L of product under optimized conditions: 0.5 % of *R*-limonene in pure distilled water as culture media, inoculum/media ratio of 0.25 (w/w), 26 ºC and 240 rpm. In general, there are few reports of optimization of natural aroma compounds production through biotechnology.

In this work, all four variables studies had significant effect in pinocarveol formation from β-pinene (Table 3). The optimal values for each parameter could be obtained by the analysis of the response surfaces and contour plots (Figure 1).

As shown in Figures 1A, 1B and 1C, the biomass of biocatalyst to be added in the biotransformation process should be 5 g/50 mL or higher in order to maximize pinocarveol formation. In other optimization studies, some authors described that the use of mycelium concentrates in fungal experiments might enhance the process yield, as well as that age and density of inoculum affects the duration of lag phase, specific growth rate, biomass yield and the quality of product, which impacts in the production costs (Sen; Swaminathan, 2004; Bicas et al., 2008). Substrate concentration is another factor that highly influences in biotransformation due to its high cytotoxicity and volatility and may cause loss of fungal cell membrane integrity (Onken; Berger, 1999). Besides these drawbacks, figures 1A, 2D and 2E show that the concentration of substrate should be maintained in approximately 1 % (v/v).

Regarding overall industrial processes, agitation and temperature are the parameters that most affects production costs. As demonstrated in Figures 1B, 2D and 2F, the agitation should be maintained in the central point, with an optimum agitation value of 150 rpm. Similar results were observed for temperature (Figures 1C, 2E and 2F), being 30 ºC the optimal temperature for this process. It is advantageous that the biotechnological process for natural aroma production occurs in mild conditions in order to avoid loss of substrate and

product due to evaporation, the occurrence of side reactions and also microbial growth inhibition and enzyme denaturation (Bicas et al., 2008).

To summarize, in this study, 5 g/L of biomass (codified value: +2), 1% (v/v) of substrate (codified value: +1), agitation of 150 rpm (codified value: 0) and temperature of 30 °C (codified value: 0) were considered the optimal values to increase final pinocarveol concentration after 120 h.

3.2. Experiments validation

The best conditions for pinocarveol production were experimentally validated in triplicates. Table 5 and 6 summarizes the parameters used and the results obtained after 120h of process.

Table 5. Experimental validation under the optimized conditions for the production of pinocarveol from the biotransformation of β-pinene

	Pinocarveol concentration (mg/L)							
	0h	24h	48h	72h	96h	120h	Mean (120h)	SD
V_1	0	15.60	27.14	40.89	68.28	145.20		
V_2	0	9.90	20.18	38.02	56.86	138.23	141.32	2.59
V_3	0	11.86	19.01	43.01	54.28	140.52		

SD = standard deviation

The results observed in these experiments could be compared with predicted pinocarveol concentration obtained from the mathematical model described in Equation 1 (Table 6).

Table 6. Comparison of experimental data with predicted values for pinocarveol concentration under optimized conditions

	Parameter	Unit	Codified value	Real value	EV	PD	RD
X_1	Biomass	g/50mL	2	5			
X_2	[Substrate]	%	1	1	141.32	158.64	-12.25
X_3	Agitation	rpm	0	150			
X_4	Temperature	°C	0	30			

EV (Experimental values) = Mean values obtained in optimal conditions from contour curves analyses, in mg/L; PD = Predicted values by the model, in mg/L; RD (Relative deviation) = [(experimental value – predicted value)/experimental value] x 100

A relative deviation of approximately 12.5% was obtained between experimental and predicted results. This value is a little higher than the acceptable for optimization processes, however, this value can be justified since the biotransformation of β-pinene is a biotechnological process regarding biological systems. In addition, it was possible to increase pinocarveol concentration from 64.8 ± 0.6 ml/L initially obtained in strain screening assays to 141.32 ± 2.6 mg/L under optimized conditions, representing a 2-fold increase in pinocarveol concentration.

4. Concluding remarks

In this work, it was studied the biotransformation of β-pinene for pinocarveol production. Using a central composite design with four variables, it was possible to elucidate how the process parameters agitation, temperature, substrate concentration and biomass of biocatalyst affect pinocarveol production and to establish a mathematical model to predict product concentration to be obtained. After response surface analysis and identification of the best conditions, it was possible to obtain a 2-fold increase in pinocarveol concentration in the process employing an endophytic fungus isolated from genipap. These achievements are important to support development of natural aroma production and to demonstrate the potential of using wild strains in biotechnology.

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DISCUSSÃO GERAL

A produção de aromas naturais através da biotransformação de terpenos é uma área em expansão, e muitos pesquisadores têm focado na obtenção de novos biocatalisadores para este processo (Rottava et al., 2010; Molina, et al., 2013; Palmerín-Carrenõ et al., 2015; Bier et al., 2017).

O capítulo 2 deste trabalho relata o isolamento e seleção de três linhagens de fungos filamentosos capazes de metabolizar monoterpenos para a produção de compostos de maior valor agregado. As linhagens identificadas como JV-7 e JV-15 apresentaram resultados positivos para a biotransformação de α- e β-pinenos. Estes monoterpenos bicíclicos representam de 75 a 90% dos óleos essenciais de coníferas e também são amplamente encontrados resíduos da indústria de papel e celulose (Vespermann et al., 2017). Embora ambas as linhagens tenham sido capazes de biotransformar os dois substratos, melhores resultados foram obtidos para a biotransformação de α-pineno utilizando-se a linhagem JV-7 e para a biotransformação de β-pineno utilizando-se a linhagem JV-15.

Para a biotransformação de α-pineno pelo fungo JV-7, os principais produtos obtidos foram verbenol (222,4 mg/L) e verbenona (79,47 mg/L), formados a partir da hidroxilação de α-pineno a verbenol e desidrogenação de verbenol a verbenona (Negoi et al., 2017). A produção destes compostos por micro-organismos isolados já havia sido reportada na literatura, como por exemplo, Rottava e colaboradores (2010) demostraram a capacidade de 31 linhagens isoladas de resíduos industriais para a biotransformação de α-pineno em verbenol. De modo semelhante, uma levedura identificada como *Hormonema* sp. foi isolada de amostras de coníferas e produziu 0,4 g/L de verbenol e 0,3 g/L de verbenona a partir de α-pineno (Van Dyk et al., 1998).

No caso da biotransformação de β-pineno pela linhagem JV-15, o produto obtido em maior concentração foi o pinocarveol (64,8 mg/L), formado pela oxidação de α- e β-pinenos (Savithiry et al., 1998). Este é um dos poucos trabalhos descrevendo a produção de pinocarveol utilizando-se micro-organismos como biocatalisadores, sendo que a maioria dos artigos relacionados à biotransformação de β-pineno tem como objetivo a produção de α-terpineol (Toniazzo et al., 2005; Rottava et al., 2011).

A linhagem JV-10 mostrou-se capaz de metabolizar os isômeros ópticos *R*- e *S*-limoneno. Este substrato é um monoterpeno monocíclico encontrado em mais de 300 óleos

essenciais e obtidos principalmente em cascas de frutas cítricas, resíduos das indústrias de processamento de frutas e produção de sucos (Maróstica Jr; Pastore, 2007). Na biotransformação de *R*-limoneno, o composto majoritário formado foi o dihydrocarveol, obtido a partir da hidrogenação do carveol (Maróstica Jr; Pastore, 2007). Novamente, existem poucos trabalhos na literatura reportando a formação de dihidrocarveol a partir de *R*-limoneno, sendo que este composto é geralmente obtido como produtos secundários (Demyttenaere et al., 2001; Bier et al., 2011). No caso deste trabalho, não foi possível a correta quantificação do composto formado devido ao baixo limite de quantificação (LDQ = 20 mg/L).

Para a biotransformação de *S*-limoneno, o produto obtido em maior concentração foi o limoneno-1,2-diol. A formação desde composto ocorre pela epoxidação da dupla ligação do anel, seguida pela formação do diol correspondente (Maróstica Jr; Pastore, 2007). Este mesmo composto foi obtido em um estudo publicado por Molina e colaboradores (2015), no qual é realizado um comparativo da biotransformação dos isômeros *R*- e *S*-limoneno. Esta é uma abordagem interessante, pois avalia o comportamento de um mesmo biocatalisador em processos utilizando substratos diferentes e que levam a formação de compostos distintos. Esse mesmo tipo de estudo poderá ser realizado futuramente com os micro-organismos isolados neste trabalho, uma vez que as três linhagens obtidas foram capazes de biotransformar dois dos substratos testados.

A fim de conhecer melhor o processo de biotransformação de pinenos e avaliar as condições de processo para a formação de compostos de aroma, dois delineamentos compostos centrais rotacionais (DCCR) foram aplicados. Para fins de comparação, as mesmas variáveis e níveis foram selecionados para serem estudados (Capítulos 3 e 4). O uso de ferramentas estatísticas para otimização da produção de aromas naturais através de biotransformação já está descrito na literatura, porém utilizando-se principalmente *R*-limoneno como substrato para a obtenção de maiores concentrações de α-terpineol (Bicas et al., 2008; Rottava et al., 2011; Molina et al., 2014).

O primeiro DCCR empregado neste estudo (Capítulo 3) teve como objetivo maximizar a concentração de verbenol na biotransformação de α-pineno pela linhagem JV-7. As análises estatísticas mostraram que apenas os parâmetros lineares do modelo que prediz a produção verbenol foram significativos. Além disso, as curvas de contorno e superfícies de resposta mostraram que a concentração de verbenol tende a aumentar com o aumento dos

níveis das variáveis: concentração de substrato, concentração de biomassa de catalisador e agitação e com a diminuição da temperatura do processo. A utilização de baixas temperaturas na produção de aromas é vantajosa, devido a diminuição dos custos energéticos do processo e das perdas de produto e substrato por evaporação (Trytek et al, 2015). A região ótima para a produção de verbenol não foi atingida neste estudo, portanto novos níveis deverão ser avaliados futuramente para definição das melhores condições de processo. Apesar disto, este trabalho atingiu um aumento de 1,4 vezes na concentração de verbenol produzida, de 222,4 mg/L para 318,4 mg/L.

O segundo DCCR visou a otimização da produção de pinocarveol pela biotransformação de β-pineno pela linhagem JV-15 (Capítulo 4). Neste caso, a região ótima para a produção de pinocarveol foi identificada através das análises de superfícies de resposta e curvas de contorno obtidas após os experimentos: concentração de biocatalisador 5 g/50 mL, concentração de substrato de 1% (v/v), agitação de 150 rpm e 30 °C. Nestas condições, foi possível a duplicação da concentração de pinocarveol previamente obtida, de 64,8 mg/L para 141,32 mg/L. A biotransformação de β-pineno utilizando-se uma linhagem de *Aspergillus* sp. já havia sido reportada, porém com o objetivo de se aumentar a concentração de α-terpineol produzida. Rottava e colaboradores (2011) alcançaram a concentração de 770 mg/L de α-terpineol utilizando 1,75% de substrato, 2g de massa de inóculo e uma razão substrato:etanol de 1:1. Este é o primeiro trabalho descrevendo a utilização de métodos estatísticos para a otimização da produção de pinocarveol pela biotransformação de β-pineno.

De acordo com os resultados obtidos nos três capítulos práticos deste trabalho, foi possível demonstrar o potencial de se utilizar linhagens isoladas de fontes ricas em monoterpenos para a produção de aromas naturais e que o emprego de métodos estatísticos é uma promissora alternativa para a obtenção de maiores concentrações de produto, auxiliando nos futuros desenvolvimentos na área de produção destes aditivos.

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CONCLUSÃO GERAL

Visando explorar o potencial de frutos típicos da flora brasileira para a prospecção de biocatalisadores a serem utilizados na produção de aromas naturais pela biotransformação de terpenos, quinze linhagens de fungos endofíticos foram isoladas de frutos verdes de jenipapo (*Genipa americana* L.) Estas foram testadas para biotransformação dos monoterpenos *R*-limoneno, *S*-limoneno, α- pineno, β-pineno ou citronelol, sendo que três linhagens foram capazes de metabolizar pelo menos um substrato para a produção de seus compostos oxigenados. A linhagem identificada como JV-7 mostrou-se capaz de biotransformar α-pineno produzindo verbenol (222,4 ± 2,3 mg/L) e verbenona (79,47 ± 3,6 mg/L), enquanto que para a linhagem JV-15, foi possível a obtenção de pinocarveol (64,8 ± 0,6 mg/L) através da biotransformação de β-pineno. A linhagem JV-10 mostrou se capaz de utilizar os dois isômeros *R*- e *S*- limoneno para a produção de neodihidrocarveol e limoneno-1,2-diol respectivamente.

Devido a potencial aplicação dos compostos formados, a linhagem JV-7 foi utilizada como biocatalisador no estudo da otimização da produção de verbenol a partir biotransformação de α-pineno. Nos níveis avaliados neste estudo, as melhores condições identificadas foram agitação de 200 rpm, temperatura de 22 ºC, concentração de substrato igual a 1,25 % (v/v) e 5 g de biomassa de biocatalisador em 50mL de meio. Nessas condições foi possível um aumento de aproximadamente 1,4 vezes a concentração final de verbenol após 120 horas de processo, de 222,4 ± 2 mg/L para 318,4 ± 2.4 mg/L. Outros níveis ainda poderiam ser avaliados com o intuito de se encontrar as condições ótimas de processo. Na otimização da biotransformação de β-pineno para produção de pinocarveol pela linhagem JV-15, as condições ótimas foram: concentração de substrato de 1% (v/v), biomassa fúngica 5 g/50 mL de meio, agitação de 150 rpm e 30 ºC. Desta forma, a concentração de pinocarveol previamente obtida em estudos de seleção (64,8 ± 0,6 mg/L) pode ser elevada para 141,32 ± 2.6 mg/L.

Apesar de ainda serem concentrações relativamente baixas, estes aumentos já podem reduzir os custos e aumentar o potencial para a produção de aromas naturais, e demonstram que a utilização de modelos matemáticos pode auxiliar no desenvolvimento de novos processos biotecnológicos para a produção de aromas naturais.

Este trabalho atingiu o objetivo proposto de demostrar o potencial de micro-organismos naturalmente encontrados em fontes ricas em terpenos 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 devem ser realizados. As linhagens de fungos endofíticos obtidas no estudo de isolamento e seleção de biocatalisadores deverão ser identificadas em nível de gênero e espécie, possibilitado um melhor entendimento do metabolismo dos micro-organismos e comparação com trabalhos que já utilizam estas linhagens em bioprocessos. Além disso, o processo de produção de verbenol pela biotransformação de α-pineno pode ser melhorado, através da avaliação de outros níveis dos parâmetros estudados neste trabalho. Finalmente, a recuperação e purificação dos compostos voláteis produzidos através da biotransformação de terpenos ainda é uma área pouco explorada, porém com muito impacto no processo global de obtenção de compostos de aroma e que, portanto, deve ser melhor estudada.

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ANEXOS

DECLARAÇÃO

Campinas, setembro de 2017

Declaramos que estamos cientes da Lei da Biodiversidade, Lei nº 13.123/2015, em vigor a partir de 17 de novembro de 2015, que revoga a Medida Provisória nº 2.186-16/2001 e estabelece novas regras para acesso ao patrimônio genético, acesso ao conhecimento tradicional associado e repartição de benefícios, sendo necessário o registro das atividades de acesso através de cadastro eletrônico no Sistema Nacional de Gestão do Patrimônio Genético – SISGen (Art. 12 da Lei nº 13.123/2015).

Entretanto, o funcionamento deste cadastro depende da regulamentação da Lei nº 13.123/2015, que está sendo conduzido pela Casa Civil da Presidência da República e portanto, não pode ser realizado até o fechamento deste material.

Desta forma, nos comprometemos a regularizar o cadastro do projeto de Tese de Doutorado intitulado "Prospecting endophytic biocatalysts for bioflavors production" após a disponibilização do cadastro pelo CGen, respeitando o prazo de um ano a ser contado a partir da data de disponibilização, conforme o Art. 38 da Lei nº 13.123/2015.

Sem mais,

Marina Gabriel Pessôa

Marina Gabriel Pessôa

Gláucia Maria Pastore