GUSTAVO MOLINA

BIOTECHNOLOGICAL PRODUCTION OF AROMA COMPOUNDS BY THE BIOTRANSFORMATION OF TERPENES

PRODUÇÃO BIOTECNOLÓGICA DE COMPOSTOS DE AROMA POR BIOTRANSFORMAÇÃO DE TERPENOS

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BIOTECHNOLOGICAL PRODUCTION OF AROMA COMPOUNDS BY THE BIOTRANSFORMATION OF TERPENES

Orientadora: Prof[a]. Dr[a]. Gláucia Maria Pastore

PRODUÇÃO BIOTECNOLÓGICA DE COMPOSTOS DE AROMA POR BIOTRANSFORMAÇÃO DE TERPENOS

Tese de Doutorado apresentada à Faculdade de Engenharia de Alimentos – Unicamp para obtenção do título de Doutor em Ciência de Alimentos.

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Este exemplar corresponde à versão final da tese defendida pelo aluno Gustavo Molina e orientada pela Profa. Dra. Gláucia Maria Pastore.

Assinatura do Orientador

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Abstract

This work aimed to study the biotechnological production of aroma compounds from the biotransformation of terpenes, analyzing potential biocatalysts, biochemical processes and optimization of process conditions involved in the production of natural flavors for industrial applications. Initially, the practical work was directed to a series of studies with the fungal strain *Fusarium oxysporum* 152B, aiming to enhance the knowledge obtained in recent advances achieved by our research group in the characterization and optimization of the biotransformation of *R*-(+)-limonene into α-terpineol. Thus, the results were promising, showing the great potential and versatility of this fungus to the specific area, and this biocatalyst was able to produce new metabolites from substrates *S*-(–)-limonene, α-pinene, γ-terpinene and linalool. In the sequence, this study aimed at assessing the bioconversion of *S*-(–)-limonene into limonene-1,2-diol in greater detail. Thus, chapters 3, 4 and 5 were designed to a better characterization of this pathway. The production of this compound under non-optimized conditions, reached 1.2 gL^{-1}, and the continuation of this pathway has been detected when this product was subsequently metabolized to 1-hydroxy-2-oxolimonene, suggesting that the fungus *Fusarium oxysporum* 152B might have a limonene degradation pathway only recently discovered. The practical work also conducted an extensive optimization of the production of limonene-1,2-diol by means of a sequential strategy. Based on statistical analyzes, the production of this compound from the bioconversion of *S*-(–)-limonene by the fungus *Fusarium oxysporum* 152B reached 3.7 gL^{-1}, using pH 6.5, 5 gL^{-1} of substrate at 28 ° C and 250 rpm agitation. Moreover, this is the first description of the use of agroindustrial residue known as cassava wastewater, for the production of this fungal biomass, as well as one of the highest concentration of biotechnological limonene-1,2-diol reported in the specific literature. In addition, the research conducted aimed to perform a series of comparisons between practical conditions involved in the biotransformation of *R*-(+)-limonene into α-terpineol, and the bioconversion of *S*-(–)-limonene into limonene-1,2-diol, for the same strain. Additionally, this is the first study that reported the ultrastructural differences along the bioconversion process of these substrates by means of scanning and transmission electron microscopy. Finally, this work evaluated the optimization of the production of α-terpineol from the biotransformation of limonene using the biocatalyst recognized as *Sphingobium* sp. After the practical work and statistical analysis, it was observed that

the best conditions for developing this process were pH 7.0, concentration of limonene 350 g L^{-1}, agitation at 200 rpm and 28 ^{o}C. Accordingly, the production of the monoterpe alcohol reached 500 g.L^{-1}, which can be considered as the highest concentration of α-terpineol already reported in the literature for biotechnological processes.

Este trabalho de doutorado teve como principal objetivo estudar a produção biotecnológica de compostos de aroma a partir da biotransformação de terpenos, analisando potenciais biocatalisadores, processos bioquímicos diferenciados e a otimização das condições de processo envolvidas na produção destes aromas naturais para possíveis aplicações industriais. Inicialmente, o trabalho prático foi direcionado para uma série de estudos com o fungo *Fusarium oxysporum* 152B, visando complementar os recentes avanços alcançados pelo grupo de pesquisa na caracterização e otimização da bioconversão de *R*-(+)-limoneno em α-terpineol. Desta forma, os resultados foram promissores, demonstrando o grande potencial e versatilidade deste fungo para a área, sendo que este biocatalisador foi capaz de produzir diferentes metabólitos a partir dos substratos *S*-(–)-limoneno, α-pineno, γ-terpineno e linalol. Na sequência, o trabalho visou avaliar a bioconversão de *S*-(–)-limoneno para limoneno-1,2-diol em maiores detalhes. A produção de limoneno-1,2-diol, em condições não otimizadas, chegou a 1,2 $g.L^{-1}$, sendo que foi detectada a continuação desta via metabólica quando este produto foi posteriormente metabolizado a 1-hidroxi-2-oxolimoneno, sugerindo que o fungo *Fusarium oxysporum* 152B possui uma via de degradação de limoneno recentemente descoberta. O trabalho prático visou também a otimização da produção de limoneno-1,2-diol por meio de uma estratégia sequencial de experimentos. Com base nas análises estatísticas, a produção deste composto a partir da bioconversão de *S*-(–)-limoneno pelo fungo *Fusarium oxysporum* 152B chegou a 3,7 $g.L^{-1}$, utilizando pH 6,5, 5 $g.L^{-1}$ de substrato, a 28 ^{o}C e 250 rpm de agitação. Além disso, esta foi a primeira descrição da utilização do resíduo agroindustrial conhecido como manipueira, originada ao longo do processamento da mandioca, para a produção de biomassa deste fungo, bem como uma das maiores concentrações de limoneno-1,2-diol reportadas na literatura específica. Além disso, o trabalho conduzido visou realizar uma série de comparações práticas entre a biotransformação de *R*-(+)-limoneno a α-terpineol e a bioconversão de *S*-(–)-limoneno a limoneno-1,2-diol, pela mesma linhagem. Adicionalmente, este foi o primeiro trabalho que analisou as diferenças ultraestruturais causadas no biocatalisador ao longo do processo de bioconversão destes substratos, por meio de microscopia eletrônica de varredura e transmissão. Finalmente, foi estudada a otimização da produção de α-terpineol, a partir da biotransformação de limoneno, utilizando o biocatalisador reconhecido como *Sphingobium* sp. Após o trabalho prático

e análise estatística dos dados, observou-se que as melhores condições para desenvolvimento deste processo foram pH 7,0, concentração de limoneno de 350 $g.L^{-1}$, agitação de 200 rpm e 28 ^{o}C. Nestas condições, a produção deste álcool monoterpênico chegou a 500 $g.L^{-1}$, que pode ser considerada como a maior concentração de α-terpineol já relatada na bibliografia de processos biotecnológicos.

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Apresentação Geral

Esta tese de Doutorado em Ciência de Alimentos apresenta diversos experimentos desenvolvidos no Laboratório de Bioaromas, do Departamento de Ciência de Alimentos da Faculdade de Engenharia de Alimentos de Universidade Estadual de Campinas (Unicamp), entre os meses de Agosto de 2010 e Dezembro de 2013, sob orientação da Prof[a]. Dr[a]. Gláucia Maria Pastore e com financiamento da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (bolsa de estudo) e da Fundação de Amparo a Pesquisa do Estado de São Paulo (projetos de pesquisa n° 08/59000-1 e n° 11/50687-7). Parte do trabalho foi desenvolvido no Laboratoire de Génie Chimie et Biochimie da Polytech'Clermont-Ferrand, Université Blaise Pascal, na cidade de Clermont-Ferrand (França), entre os meses de agosto de 2011 e maio de 2012, sob supervisão do Prof. Dr. Christian Larroche e com apoio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (bolsa de Doutorado Sanduíche).

O trabalho foi estruturado em seis capítulos, apresentados na forma de artigos científicos, abordando os seguintes temas:

- Capítulo 1: Estudo bibliográfico sobre os tópicos abordados na presente pesquisa (produção de compostos de aroma por via biotecnológica);
- Capítulo 2: Avaliação do potencial do fungo *Fusarium oxysporum* na biotransformação de diversos mono-, sesqui- e tetraterpenos e descrição das vias metabólicas;
- Capítulo 3: Caracterização da bioconversão de *S*-(–)-limoneno em limoneno-1,2-diol pelo fungo *Fusarium oxysporum*;
- Capítulo 4: Aproveitamento de resíduos agroindustriais e otimização da produção de limoneno-1,2-diol;
- Capítulo 5: Estudo comparativo da produção de α-terpineol e limoneno-1,2-diol utilizando os isômeros do limoneno como substratos;
- Capítulo 6: Otimização da produção de α-terpineol em elevadas concentrações pelo biocatalisador *Sphingobium* sp.

Objetivo Geral

O principal objetivo deste trabalho foi estudar a produção biotecnológica de compostos de aroma a partir da biotransformação de terpenos, visando o desenvolvimento e adaptação de processos para a obtenção de bioaromas de interesse industrial.

Objetivos Específicos

Parte 1: Revisão bibliográfica

Realizar uma ampla revisão bibliográfica sobre o tema específico da tese, abordando substratos e produtos de interesse para a área de obtenção de aromas biotecnológicos.

Parte 2: Potencial do biocatalisador *Fusarium oxysporum*

Estudar o potencial desta linhagem em metabolizar diversos mono-, sesqui- e tetraterpenos. Identificar e quantificar os produtos obtidos, bem como estudar as vias metabólicas utilizadas no processo de biotransformação.

Parte 3: Caracterização da biotransformação de *S*-(–)-limoneno

Caracterizar o processo de bioconversão de *S*-(–)-limoneno a limoneno-1,2-diol, avaliando a influência de variáveis como idade de inóculo, indução de células, presença de co-solventes e adição sequencial de substrato no processo de interesse.

Parte 4: Otimização da produção de biomassa e limoneno-1,2-diol

Utilização de diversos resíduos agroindustriais para a produção de biomassa, visando o desenvolvimento de processo sustentável e econômico. Otimização da produção de biomassa utilizando manipueira como meio de cultura alternativo. Utilização da biomassa produzida em ensaios de biotransformação. Otimização do processo de produção de limoneno-1,2-diol.

Parte 5: Estudo comparativo com isômeros de limoneno

Realizar um estudo comparativo com base na biotransformação de *R*-(+)-

limoneno a α-terpineol e *S*-(–)-limoneno a limoneno-1,2-diol, utilizando o mesmo biocatalisador *Fusarium oxysporum*. Realizar a análise ultraestrutural do biocatalisador durante o processo de bioconversão de substratos terpênicos por meio de microscopia eletrônica de varredura e transmissão.

Parte 6: Otimização da produção de α-terpineol

Realizar a otimização da produção de α-terpineol a partir de limoneno pela linhagem *Sphingobium* sp., visando a produção de elevadas concentrações deste produto.

1. Aromas naturais: A importância da pesquisa e aplicação industrial

O consumo de alimentos e bebidas está diretamente relacionado ao estímulo dos sentidos humanos. O sabor específico de um alimento é uma resposta integrada às sensações do gosto e do aroma. O gosto é atribuído aos compostos não voláteis presentes nos alimentos, tais como açúcares, sais e ácidos, determinando os cinco gostos básicos, conhecidos como doce, salgado, amargo, ácido e *umami*. Enquanto isso, o aroma é uma mistura complexa de substâncias voláteis, representantes de diversas classes químicas que apresentam diferentes propriedades físico-químicas (Franco, 2003). A sensação de um aroma é desencadeada por uma mistura complexa de moléculas de diversas classes químicas, que ocorrem em pequenas concentrações, detectadas pelas células receptoras específicas do epitélio olfatório, localizadas na cavidade nasal (Berger, 1995).

Os compostos de aroma são moléculas de baixa massa molecular (em geral menores que 400 Da) e, apesar de não representarem nenhuma função nutritiva ao alimento, estes compostos são frequentemente utilizados como aditivos na indústria de alimentos, bebidas, perfumes e cosméticos, para realçar, reforçar e melhorar o odor do produto, e mesmo sabor, como o caso dos alimentos (Berger, 1995).

Estes compostos podem ser extraídos empiricamente de fontes vegetais ou animais, mas também podem ser produzidos por mecanismos químicos ou biológicos. Estes compostos não apresentam uma função química específica, podendo ser hidrocarbonetos, alcoóis, cetonas, aldeídos, ácidos, ésteres ou lactonas (ácidos cíclicos), éteres, etc (Bicas, 2009). Além de estarem presentes em pequenas concentrações na composição das matrizes alimentícias (ppm ou ppb), os aromas possuem uma grande diversidade de polaridades, solubilidade, volatilidade, temperatura e pH de estabilidade (Berger, 1995).

As rotas naturais para a produção de aromas podem ser divididas em basicamente três métodos: i) conversão de precursores naturais pela biocatálise, iii) síntese *"de novo"* e iii) isolamento a partir de fontes vegetais e animais. Enquanto isso, os aromas produzidos por métodos químicos são considerados como "idênticos ao natural" e, por serem menos visados pelos consumidores, impulsionaram as pesquisas na área da biotecnologia (Serra et al., 2005). A legislação européia define que os aromas são considerados "naturais" quando são obtidos a partir de fontes naturais, por processos físicos ou fermentativos. Enquanto isso, a legislação brasileira (ANVISA, Resolução nº 104, de 14 de maio de 1999) define "aromas naturais" como aqueles obtidos por métodos físicos, microbiológicos ou enzimáticos a partir de

matérias-primas aromatizantes ou aromas naturais, semelhante às legislações norte-americana (FDA: Code of Federal Regulations, 21CFR101.22) e européia (Council Directive 88/388/EEC) (Chiappini, 2008; Demyttenaere, 2001). Desta forma, a biotecnologia surge como uma importante ferramenta, permitindo estudos para obtenção de compostos de aroma naturais que atendam às exigências dos consumidores (Serra et al., 2005), pois atualmente observa-se a preferência pelo consumo de alimentos que contenham ingredientes naturais em sua formulação, em substituição aos aditivos sintéticos. Esta diferenciação de mercado ocasiona a agregação de valor aos produtos que utilizam os aromas produzidos biotecnologicamente (Bicas et al., 2009).

A aplicação de aromas abrange diversas indústrias, como a de alimentos, farmacêutica e de cosméticos. Os alimentos submetidos às operações de processamento como a colheita prematura, tratamentos físicos, aumento do tempo de estocagem e de vida de prateleira, podem sofrer perdas no aroma, o que requer sua suplementação. Desta forma, o mercado de aromas está em ascensão, buscando aromas que possam ser produzidos biotecnologicamente a partir de fontes alternativas e que supram a demanda do mercado (Krings e Berger, 1998).

Dados de Krings e Berger (1998) demonstram que, no início da década de 90, aproximadamente 80% dos aromas consumidos mundialmente pela indústria foram produzidos por via química. Entretanto, em torno de 70% dos aromas utilizados pela indústria de alimentos na Alemanha eram naturais, seguindo uma tendência atribuída ao apelo dos alimentos naturais, com maior valor nutricional e potencial à saúde humana.

Em 1994, a indústria de aromas e fragrâncias rendeu um montante estimado em aproximadamente 9,7 bilhões de dólares (Somogyi, 1996). Até esta época, em torno de 6.400 voláteis naturais e 10.000 fragrâncias sintéticas eram conhecidos, entretanto, apenas em torno de 400 compostos de aroma foram comercializados em escala superior a 1 tonelada por ano (Krings e Berger, 1998). Outros dados mostram que no ano de 2006, a indústria de aromas e fragrâncias apresentou um faturamento estimado em torno de US$ 12.6 bi (IAL Consultants, 2007), e subiu para US$ 21.8 bi em 2011 (Leffingwell and Associates, 2011).

A diferença entre o valor de um aroma natural e um aroma produzido por síntese química apresenta uma discrepância significativa como, por exemplo, a vanilina sintética, comercializada por US$ 12 Kg^{-1} enquanto o preço da vanilina extraída dos favos de baunilha podem chegar a US$ 4.000 Kg^{-1} (Feron et al., 1996).

Os dados mais recentes são escassos, mas o desenvolvimento da biotecnologia implicará em vantagens adicionais à indústria de aromas, como a independência da agricultura e condições de produção, clima, uso de agrotóxicos, fertilizantes, doenças e as questões sócio-

políticas envolvidas. Além disso, poderá fornecer a possibilidade de elevação de escala dos produtos empregando-se a engenharia genética, regulação de metabolismo e recuperação de produtos homogêneos e bem-definidos (Krings e Berger, 1998).

2. Terpenos: Substratos ideais para a produção de novos compostos

Os terpenos são metabólitos secundários de plantas, produzidos, em parte, para defesa contra micro-organismos e insetos. Contém estrutura carbônica formada por resíduos de isopreno (C_5H_8), podendo ser classificados quanto ao número de carbonos presentes, como monoterpenóides (dez carbonos), sesquiterpenóides (quinze carbonos), diterpenóides (vinte carbonos), triterpenóides (trinta carbonos) e tetraterpenóides ou carotenóides (quarenta carbonos). Estes compostos são encontrados em grande quantidade em óleos essenciais, cujo aroma é atribuído principalmente aos mono e sesquiterpenos (os terpenos mais voláteis) presentes em sua composição (De Carvalho e Da Fonseca, 2006). Na maioria dos casos, o odor característico de frutas, ervas e especiarias é resultante de terpenos voláteis (Krings et al., 2006).

Uma possibilidade para a produção de aromas naturais é a utilização de terpenos, pois diversas características os tornam substratos ideais para os processos biotecnológicos. Do ponto de vista econômico são interessantes, pois ocorrem largamente na natureza, como o caso do limoneno e α-pineno, que podem ser encontrados por um preço baixo e acessível, por serem rejeitos industriais gerados em escala industrial com valor comercial reduzido (Yoo et al., 2001). Além disso, são bons materiais de partida para a síntese de produtos químicos refinados devido a sua similaridade estrutural com os produtos resultantes (Bicas et al., 2009) e representam um substrato ideal para processos biotecnológicos, sendo intensamente estudados em experimentos de conversão microbiana (Maróstica Jr. e Pastore, 2007a).

A crescente demanda por produtos naturais e funcionais tem intensificado a produção científica e tecnológica nesta área. A produção de compostos de aroma está intimamente relacionada aos avanços nas pesquisas e processos de biocatálise e biotransformação, devendo-se cada vez mais buscar métodos simples e econômicos aliados a alta produtividade e praticidade.

3. *Fusarium oxysporum* 152B: Biocatalisador potente e versátil

A capacidade do fungo *Fusarium oxysporum* 152B em biotransformar o *R*-(+)-limoneno já foi bem elucidada anteriormente por outros pesquisadores. Inicialmente, a produção de α-terpineol a partir do limoneno foi estudada utilizando manipueira, um resíduo

líquido do processamento de mandioca, como meio alternativo de cultivo do fungo *Fusarium oxysporum*. Os autores constataram que a biotransformação de *R*-(+)-limoneno resultou em 450 mg.L^{-1} de *R*-(+)-α-terpineol (Maróstica Jr. e Pastore, 2007b).

Na sequência, Bicas et al. (2008) realizaram a otimização das condições envolvidas na produção de *R*-(+)-α-terpineol a partir de *R*-(+)-limoneno pelo fungo *Fusarium oxysporum* 152B. Primeiramente, empregou-se a matriz de Placket-Burman para definir as variáveis impactantes no processo, como a composição do meio, presença de cosubstrato, condições de cultivo e ainda a relação entre inóculo e meio de cultivo. A partir de então, o resultado foi otimizado através da metodologia de superfície de resposta. Após o extensivo trabalho, os autores obtiveram um significativo aumento na produção de *R*-(+)-α-terpineol, chegando a 2,4 g.L^{-1}.

Recentemente, aliando os conhecimentos obtidos até o momento sobre o potencial do fungo, Bicas e colaboradores (2010) propuseram um processo de coprodução de lipase e *R*-(+)-α-terpineol. Repetindo as condições do processo de biotransformação, os autores atingiram uma produção de 4 g.L^{-1} após 48 horas de processo, sendo que este valor é um dos maiores descritos até o momento em processos de biotransformação que usam fungo como catalisador. Estes resultados sugerem um grande potencial desta linhagem na biotransformação de terpenos, tendo em vista que foram coletados resultados substanciais até o presente momento.

Apesar destes estudos serem de grande importância, as principais características do fungo *Fusarium oxysporum* na biotransformação de outros terpenos ainda não foi descrita em grandes detalhes. Esta linhagem tem um grande potencial na produção de bioaromas, o que requer maiores esforços para elucidar possíveis novas vias metabólicas, utilizando outros substratos terpênicos no processo, além de estudar novas possibilidades de aplicação dos produtos obtidos. Além disso, a produção de α-terpineol em bioreator deve ser estudada, pois a elevação de escala de produção deste álcool monoterpênico pode fornecer dados concretos para uma possível aplicação industrial.

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Natural flavors from biotechnology: Recent developments and industrial perspectives

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ABSTRACT

Natural flavor compounds obtained by biotechnological processes nowadays play an increasing role in the food, cosmetic, chemical and pharmaceutical industries, due to an increasing demand of the consumer for natural food additives. Due to this fact, extensive research has been devoted to the biotechnological production of flavor compounds during the past decade, because the compounds obtained by biotechnological process are considered "natural". Biotechnological production is an attractive alternative for the production of flavors, 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". Additionally, there are some compounds that may be produced exclusively *via* biotechnology. The biotechnological production of aroma compounds may be performed in two basic ways: through *de novo* synthesis or by biotransformations, both being possible with genetically modified organisms. *De novo* synthesis refers to the production of complex substances from simple molecules through complex metabolic pathways. In the literature, a great diversity of compounds have been related, *e.g.* ketones (butanone, methyl ketones), alcohols (ethanol, 1-octen-3-ol), aldehydes (acetaldehyde), esters (ethyl hexanoate, ethyl acetate), organic acids (butyric acid, caproic acid), pyrazines, and others, and the main classes of compounds will be described in this chapter. On the other hand, biotransformations are single reactions catalyzed enzymatically to result in a product structurally similar to the substrate molecule. The biotransformation using terpenes as substrate, *e.g.* limonene, citronellol and pinenes (α- and β), are the common procedures to obtain aroma compounds with high added value, *e.g*. *R*-(+)-α-terpineol with a floral and typically lilac odour, and *L-cis*-rose oxide, that has an impacting fruity flavor. The aromatic description of the aroma compounds obtained by the biotechnological processes and the microorganisms employed will be described in this

chapter. Moreover, this chapter reviews the main biotechnological processes employed for the production of bioflavors by means of *de novo* synthesis and biotransformations. Thus, the main groups of compounds obtained by both methods are described and the latest advances in the production of natural flavors, discussed.

Keywords: Biotransformation, Bioconversion, *De novo* synthesis, Terpenes.

1. **Introduction**

Flavors and fragrances have a wide application in the food, feed, cosmetic, chemical and pharmaceutical sectors (Vandamme, 2003). Nowadays, the total market for flavors and fragrances is estimated at US$18 billion, with eight major global companies sharing 60% of the world market (Guentert, 2007). The largest markets are in Europe (36%) and North America (32%), followed by the Asian Pacific region (26%) (Schwab et al., 2008).

Many flavor compounds on the market are still produced via chemical synthesis or via extraction from plants. However, due to the high cost or lack of availability of natural flavor extracts, most commercial flavorants are “nature-identical”, which means that they are the chemical equivalent of natural flavors but are chemically synthesized (Schwab et al., 2008). On the other hand, chemical synthesis often results in environmentally unfriendly production processes and an undesirable racemic mixture of compounds. Thus, due increasing health and nutrition conscious lifestyles, the consumer has developed a “chemophobia”-attitude towards to synthetic compounds (Cheetham, 1993; Cheetham, 1997; Krings & Berger, 1998).

In this sense, the growing market for flavored and fragranced products requires novel strategies for aroma chemical production (Krings & Berger, 1998), and a rapid switch towards the bio-production and use of flavor compounds from biotechnological origin (bioflavors) has been observed.

The production of bioflavors has usually been undertaken by direct recovery from nature, although many disadvantages are encountered, such as (i) low concentrations of the product of interest, which increases the extraction and purification procedures; (ii) dependency on seasonal, climatic and political features; and (iii) possible ecological problems involved with the extraction (Bicas et al., 2009). On the other hand, biotechnological production is an attractive alternative for the production of flavors, since it occurs at mild conditions, presents high regio- and enantio-selectivity, does not generate toxic waste and the products obtained may be labeled as "natural". In this sense, biotechnology is an interesting approach for the production of bioflavors, since these compounds are defined as "natural" or

"naturally produced" flavors (Demyttenaere, 2001; Bicas et al., 2009). 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).

The bio-route for flavor synthesis is based on *de novo* microbial processes (fermentation) or on bioconversions of natural precursors with microbial cells or enzymes (biotransformation). In general, microorganisms are capable of producing an amazingly broad array of flavor compounds, by *de novo* synthesis. However, production levels are very poor and thus constitute a limit for industrial exploitation. For this reason, biotechnologists have focused on bioconversion processes that offer more economic advantages (Feron & Waché, 2006).

Several studies have been conducted to make bio-processes more economically viable. Berger (2009) recently discussed the latest advances in the bio-production of flavor compounds. For the author, progress is expected from the toolbox of genetic engineering which is likely to help in identifying metabolic bottlenecks and in creating novel high-yielding strains. Bioengineering, in a complementary way, provides promising technical options, such as improved substrate dosage, gas-phase or two-phase reactions and *in situ* product recovery. Thus, the use of agro-industrial residues, *e.g.* cassava bagasse, cassava wastewater and coffee husk, are proposed and studied by some research groups as substrates for biotechnological processes (Bicas et al., 2009).

The focus of this chapter is based on the current state of art of bioflavor synthesis, with emphasis in *de novo* synthesis and biotransformation to produce flavor compounds.

2. **Production of natural flavors by *de novo* synthesis**

As mentioned before, *de novo* synthesis comprises the production of complex substances from simple molecules through complex metabolic pathways (Bicas et al., 2009), and the production of chemically quite different volatile flavors, such as short-chain alcohols, esters aldehydes, ketones, methylketones and acids as well as pyrazines and lactones that could be formed concurrently (Krings & Berger, 1998).

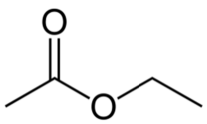
The main groups of bioflavor compounds obtained by means of *de novo* synthesis will be discussed below. Some examples of processes to obtain aroma compounds are detailed in Table 1.

Table 1. Examples of aroma compounds produced by *de novo* synthesis. The structures of the compounds cited are shown in Figure 1.

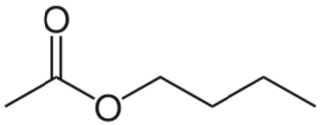
Product	Aroma description	Strain	Process information[a]	Ref.[b]
Esters				
Ethyl acetate	Fruity, solvent, and brandy, balsamic[A,B,I]	*Kluyveromyces marxianus*	C=138 mg.L^{-1} (whey fermentation)	1
Ethyl hexanoate	Apple, fruity, sweetish, banana, aniseed, strawberry, green apple[A,B,C,I]	*Neurospora* sp. ATCC 46892	C= 180 mg.L^{-1} (pregelatinized rice with 5% malt broth)	2
Lactones				
γ-Decalactone	Peach and fruity	*Sporobolomyces odorus*	C = 0.5 mg.L^{-1} (standard medium)	3
		Sporobolomyces odorus	C = 1.6 mg.L^{-1}	4
		Sporidiobolus salmonicolor CCRC 21975	C = 131.8 mg.L^{-1} (ricinolei acid)	5
		Yarrowia lipolytica	C = 10 g.L^{-1} (castor oil)	6
δ-Decalactone	Coconut and peach	*Ceratocystismoniliformis*	*SQ* - addition of glycerol	7
γ-Octalactone	Coconut	*Polyporus durus*	C = 7 mg.L^{-1}	8
cis-6-Dodecen-4-olide	Peach	*Fusarium poae*	C = 2 mg.L^{-1}	9
6-penthyl-α-pyrone	Coconut	*Trichoderma viride and T. harzianum*	C = 17 0mg.L^{-1} (potato dextrose agar)	10
Alcohols				
1-octen-3-ol	Mushroom	*Neurospora* sp.	C= 20 mg.L^{-1} (Malt extract - 5 days)	11
2-Phenylethanol	Rose, sweetish, Perfumed[A]	*Geotrichum fragrans*	C=22.6 mg.L^{-1}(cassava wastewater + fructose+ yeast extract)	12

		Saccharomyces cerevisiae	C= 20-27 mg.L^{-1} (beer)	13
		Pichia fermentans	C= 453.1 mg.L^{-1} (sucrose + yeast extract + phenylalanine)	14
Isoamyl alcohol	Fusel oil, whiskey-characteristic, pungent[G]	*Neurospora* sp. T.	C=57.2 mg.L^{-1} (malt extract)	15
Aldehydes				
Benzaldehyde	Almond	*Several Basidiomycetes strains*	C < 1mg.L^{-1}	16
		Ischnoderma benzoinum	C = 6–50 mg.L^{-1}	17
Acetaldehyde	Leafy-green	*Zymomonas mobilis* mutant	C ~ 4 g.L^{-1} (from glucose and high aeration)	18
Vanilin	Vanilla	*Escherichia coli*	*SQ* - from glucose	19
		Schizosaccharomyces pombe	C = 65 mg.L^{-1}	20
		Saccharomyces cerevisiae	C = 45 mg.L^{-1}	20
Acids				
Citric acid	Sour	*Aspergillus niger*	*SQ*	21
Methylbutyric acid	Pinneaple	*Acetobacter sp.*	*SQ*	22
Carboxilic acids	Fruity	*Geotrichum klebahnii*	*SQ*	23
Sulfur compounds				
2-mercaptoethanol	potato	*Hanseniaspora uvarum*	C=480 µg.L^{-1} (grape must)	24
Pyrazines				
Tetramethylpyrazine	Musty, fermented, sharp	*Corynebacterium glutamicum*	3 g.L^{-1} (5 days with aminoacids)	20

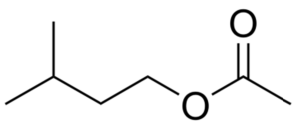
[a] C: concentration obtained (production), SQ: Small quantities, [b] References: 1. Dragone et al., 2009; 2. Yamauchi et al., 1989; 3. Tahara et al., 1972; 4. Tahara et al., 1973; 5. Lee et al., 1998; 6. Vandamme, 2003; 7. Lanza et al., 1976; 8. Gatfield, 1986; 9. Sarris & Latrasse, 1985; 10. Welsh et al., 1989; 11. Pastore et al., 1994; 12. Damasceno et al., 2003; 13. Kobayashi et al., 2008; 14. Huang et al, 2001; 15. Yamauchi et al., 1991; 16. Lomascollo et al., 1999; 17.Krings et al., 1995; 18. Li & Frost, 1998; 19. Welsh et al., 1989; 20. Hansen et al., 2009; 21. Armstrong et al., 1989; 22. Vandamme & Soetaert, 2002; 23. Janssens et al., 1992; 24. Moreira et al., 2008.



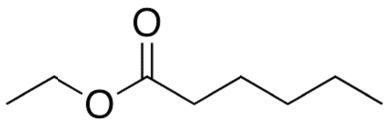
Ethyl acetate



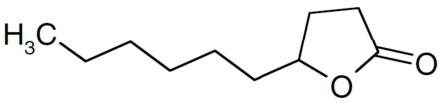
Butyl acetate



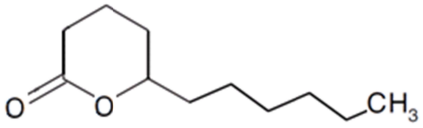
Isoamyl acetate



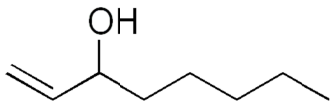
Ethyl hexanoate



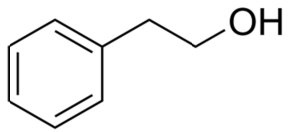
γ-Decalactone



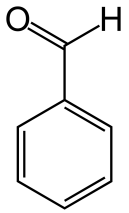
δ-Decalactone



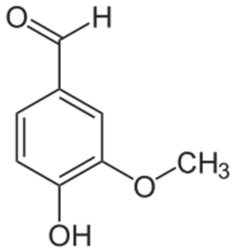
1-Octen-3-ol



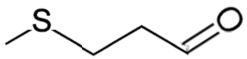
2-Phenylethanol



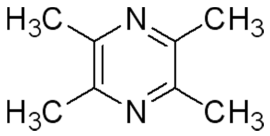
Benzaldehyde



Vanillin



Methional



Tetramethylpyrazine

Figure 1. Structures of some products obtained by *de novo* synthesis.

2.1. Esters

Ethyl acetate is a fruity smelling liquid with a brandy note, and is the most common ester in fruits (Bauer et al., 2001). This ester, for example, has a significant effect on the organoleptic characteristics of distillates, and could be produced by different microorganisms, *e.g. Staphylococcus* (Talon et al., 1998), *Saccharomyces cerevisiae* (Brown & Hammond, 2003; Carrau et al., 2010; Kobayashi et al., 2008; Sumby et al., 2010; Valero et al., 2002), *Geotrichum fragans* (Damasceno et al., 2003), *Kluyveromyces marxianus* (Dragone et al. 2009, Medeiros et al., 2000; Medeiros et al., 2001), *Ceratocystis fimbriata* (Soares et al., 2000), *Ceratocysis moniliformis* (Bluemke & Schrader, 2001), *Neurospora* sp. (Brigido, 2000), *Hanseniaspora guilliermondii* 11104 and *Pichia anomala* (Rojas et al., 2003).

Butyl acetate has a strong, fruity odor; burning and then sweet taste reminiscent of pineapple. It occurs in many fruits and is a constituent of apple (Bauer et al., 2001). The bio-production was reported by Medeiros and co-workers (2000) using *Kluyveromyces marxianus* in cassava bagasse.

Another important compound is isoamyl acetate. This compound has a fruity, banana, sweet, fragrant, powerful odor with a bittersweet taste reminiscent of pear with an aroma threshold of 2 to 43 ppb. Its uses comprise alcoholic beverages, gelatins, puddings, baked goods, hard candy, chewing gum, nonalcoholic beverages, confectionary, frosting, soft candy, frozen dairy, sweet sauce in a range of 19-112 ppm. It occurs naturally in some fruits like apple, apricot, banana, grape, berries, melon, papaya, peach, pear, pineapple and products *e.g.* vinegar, wheat and rye bread, cheeses, butter, alcoholic and non-alcoholic (Burdock & Fenaroli, 2010). Thus, the biotechnological production was reported using *Saccharomyces cerevisiae* (Brown & Hammond, 2003; Kłosowski & Czuprynyski, 2006, Kobayashi et al., 2008), *Staphylococcus* (Talon et al., 1998), *Ceratocystis fimbriata* (Soares et al., 2000) and *Ceratocysis moniliformis* (Bluemke & Schrader, 2001).

Ethyl butyrate occurs in fruits and alcoholic beverages, but also in other foods like cheese. It has a fruity odor, reminiscent of pineapples (Bauer et al., 2001). *Pseudomonas fragi* produced ethyl butyrate when grown in skimmed milk at 15°C and *Kluyveromyces marxianus* was able to produce it in cassava bagasse (Medeiros et al., 2000) as well as a strain of *Neurospora* sp in different culture medium (Brigido , 2000).

Although ethyl hexanoate is found naturally in fruits *e.g* strawberry, kiwi fruit, apple, pineapple, and products like strawberry jam, cheeses, whiskies, grape wines, passion fruit juice, culture medium with *Neurospora* sp. produces this ester (Pastore et al., 1994; Yamauchi et al., 1991; Yoshizawa et al., 1988). Pastore and co-workers (1994) obtained 59 $mg.L^{-1}$ of

ethyl hexanoate using malt extract broth as the culture medium after 3-4 days of fermentation while Yamauchi et al. (1989), using pregelatinized rice impregnated with 5% malt broth as solid culture medium, obtained around 180 $mg.L^{-1}$.

Strains of *Pseudomonas fragi* (Cormier et al., 1991) *Ceratocystis frimbriata* (Christen et al. 1997), *Saccharomyces cerevisiae* (Kłosowski & Czuprynyski, 2006; Kobayashi et al., 2008; Sumby et al., 2010), *Corynebacterium* sp., *Arthrobacter globiformis* and *Serratia marcescens* can also produce this compound.

2.1.1. Lactones

The biogeneration of volatile lactones has proved to be industrially successful (Cardillo et al., 1990) and some examples include γ-nonalactone, γ-decalactone and δ-decalactone (annual consumption estimated is approximately 16.5 metric tons), γ-undecalactone and β-methyl-γ-octalactone (whiskey lactone), and others (Surburg & Pantem, 2006).

γ-Decalactone has a pleasant, fruity, peach-like odor with an aroma threshold between 1 to 11 ppb. This lactone is used in perfumery for heavy, fruity flower odors and in aroma compositions, particularly peach flavors (Burdock & Fenaroli, 2010). The annual potential market for natural γ-decalactone is estimated at 10 tons (Vandamme, 2003). Tahara and co-workers (1972, 1973) reported the production of this compound, when the yeast *Sporobolomyces odorus* (currently known as *Sporidiobolus salmonicolor*) was grown on standard medium. Although produced in low yields ($0.5mg.L^{-1}$) the culture medium displayed an intense fruity and peach odor. One of the most promising industrial processes described yields over 10 $g.L^{-1}$ of γ-decalactone by supplying castor oil to the medium, which is rich in ricinoleic acid (12-hydroxy-C18:1), using yeasts such as *Sporidiobolus salmonicolor* and *Yarrowia lipolytica* (Vandamme, 2003).

A coconut flavored lactone, 6-pentyl-α-pyrone, was produced by a soil fungus, *Trichoderma viride*, reaching 170 $mg.L^{-1}$ (Welsh et al., 1989). An integrated fermentation process was proposed, with the aim of continuously removing the lactone by pervaporation over a selective membrane, since it inhibits growth upon accumulation in the broth (Häusler & Münch, 1997; Bluemke & Schrader, 2001).

The production of an intense fruity and pleasant odor was achieved when *Polyporus durus*, a wood rotting fungus, was grown on synthetic medium, due the production of γ-lactones. γ-Octalactone was identified as the main component at a concentration of 7 $mg.L^{-1}$

(Gatfield, 1986). This compound has a fruity and coconut-like odor and is used both in aroma compositions and in heavy blossom perfumes (Surburg & Panten, 2006).

Another lactone identified as *cis*-6-dodecen-4-olide (2 $mg.L^{-1}$) with a peach-like aroma, was produced when *Fusarium poae* was grown on a solid malt medium until sporulation (Sarris & Latrasse, 1985). This product is naturally found in peach, mushroom and dairy products and is applied mainly in baked goods and milk products and has a threshold value lower than 0.5 ppm (Burdock & Fenaroli, 2010).

2.2. Alcohols

1-Octen-3-ol has a powerful, sweet, earthy odor with a strong, herbaceous note reminiscent of lavender–lavandin, rose and hay. This alcohol is found in lavender oil and is an important component of mushrooms. It is used in lavender compositions and in mushroom aromas (Bauer et al*.,* 2001). In studies involving strains of *Neurospora* sp in malt extract medium this alcohol was produced (Yamauchi et al., 1991; Brigido, 2000; Pastore et al. 1994), using *Penicillium camemberti* in glucose enriched by salts and linoleic acid after 5 days of fermentation (Husson et al., 2002).

Phenylethyl alcohol (2-phenylethanol) has a characteristic rose-like odor and an initially slightly bitter taste, then sweet and reminiscent of peach and it is the main component of rose oils obtained from rose blossoms (Burdock & Fenaroli, 2010). It is used frequently and in large amounts in perfumes, cosmetics and food (Bauer et al., 2001, Etschmann et al., 2002). The threshold values are of the order of 0.015 ppb to 3.5 ppm. This compound is naturally found in over 200 foods and beverages including apple, many berries, raisin, melon, papaya, asparagus, cabbage, potato, mentha oils, cinnamon, ginger, breads, butter, saffron, mustard, many cheeses, butter, beverages nuts, oats, honey, mushroom, etc. (Burdock & Fenaroli, 2010).

Although the essential oil of rose can contain up to 60% of phenylethyl alcohol, it is too valuable to be used as a source of natural 2-phenylethanol for food flavorings. Many microorganisms, especially yeasts, are capable of producing 2-phenylethanol by normal metabolism (*de novo* synthesis), but the final concentration of the flavor in the culture broth generally remains very low (Etschmann et al., 2002), for example with *Geotrichum fragans* (Damasceno et al., 2003) and *Kluyveromyces marxianus* (Fabre et al*.,* 1998). However, a concentration as high as 453.1 $mg.L^{-1}$ after 16 hours of fermentation by *Pichia fermentans,* was reported by Huang et al. (2001).

Isoamyl alcohol has a fusel oil, whisky character with a pungent odor and repulsive taste with an aroma threshold value of 250 ppb to 4.1 ppm, and its uses comprise alcoholic beverages, gelatins, puddings, baked goods, hard candy, chewing gum, nonalcoholic beverages, frozen dairy and soft candy. This alcohol occurs in vinegar, cheeses, butter, cognac, rum, whiskies, cider, sherry, grape wines, arctic bramble, olive, gin, quince, sake, buckwheat (Burdock & Fenaroli, 2010). The bioproduction of isoamyl alcohol was reported by Kobayashi et al. (2008), Brown & Hammond (2003), Yamauchi et al. (1991), Brigido (2000) and Pastore et al. (1994) using *Neurospora* sp.

2.3. Acids e Aldehydes

Among the aldehydes, benzaldehyde (almond aroma) and vanillin (see topic 3.6) are the most important and widely used by the food industry. Benzaldehyde is used in aroma compositions due its bitter almond odor and is applied as a starting material for a large number of aliphatic fragrance and flavor materials, and possesses a sweet, floral and spice-like odor (Burdock & Fenaroli, 2010). It naturally occurs in many products such as bitter almond, peach, apricot kernel, cheeses, black tea and others (Surburg & Panten, 2006). This compound can be obtained in natural form, by extraction and distillation from botanical sources, or synthesized from benzyl chloride.

Natural benzaldehyde is used as an ingredient in cherry and other natural fruit flavors and has a market of ~20 tons per year and a price of ~240 €/kg. Benzaldehyde obtained from natural cinnamaldehyde can be purchased for 100 €/kg with an estimated market of more than 100 tons per year (Feron & Waché, 2006). The screening of several white-rot fungi has shown that numerous species are able to synthesize benzaldehyde through *de novo*, such as *Pleurotus sapidus*, *Polyporus* sp., and others (Lomascolo et al., 1999). Berger et al. (1987) studied the formation of a methoxy benzaldehyde in *Ischnoderma benzoicum*.

Methylbutyric acid can be naturally found in the composition of several oils, such as peppermint and spearmint, and fruits (*e.g.* as apricot, apple, grapes, papaya, pineapple and others). This acid has an aroma threshold between 10 to 60 ppb, with notes of fermented pineapple fruity and lingonberry (Burdock & Fenaroli, 2010). It was reported that *Acetobacter* strains are able to synthesise methylbutyric acid. Besides its industrial relevance as a flavor, this acid can be a valuable precursor of common flavor esters (Vandamme & Soetart, 2002).

2.5. Other compounds

2.5.1 Terpenes

Terpenes are the most abundant group in nature responsible for the characteristic odors of essential oils, and are a preferable substrate for bioconversion studies (Janssens et al. 1992). In studies involving *de novo* synthesis, terpenes are present only in a few works.

Drawert et al. 1978 related the use by *Kluyveromyces lactis* in the production of citronellol, linalool and geraniol. Bluemke & Schrader (2001) reported a production of citronellol and geraniol by *Ceratocystis moniliformis* in a 5 liter bioreactor.

2.5.2 Sulfur compounds

Methional is important for the typical taste of cheeses, especially cheddar (Reineccius, 2002). Another example, is methylthioacetate, that been used in baked goods, imitation dairy, chewing gum, meat products, condiments, relishes, nonalcoholic beverages, frozen dairy, snack foods, soft candy, hard candy, soups (Burdock & Fenaroli, 2010) and lager beer, at a concentration between 4 and 31ppb (Reineccius, 2002).

Strains of *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae* are capable of producing sulphur compounds such as methional and 2-methyltetrahydrothiophen-3-one, 2-mercaptoethanol, *cis*-2-methyltetrahydro-thiophen-3-ol, *trans*-2-methyltetrahydro-thiophen-3-ol and 3-mercapto-1-propanol (Moreira et al., 2005; Moreira et al., 2008). Other volatile thiols, in particular 4-mercapto-4-methylpentan-2-one, 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate, are some of the most potent aroma compounds of wine. At optimal concentrations in wine, these compounds impart flavors of passionfruit, grapefruit, gooseberry, blackcurrant, lychee and guava (Swiegers et al., 2007).

2.5.3. Pyrazines

Tetramethylpyrazine has a musty, fermented, coffee odor and is present in several cheeses (swiss, camembert, gruyère), roasted peanuts, soy products, beans, mushroom, cocoa and coffee products. This compound has an aroma threshold varying from 1 to 10 ppm and is of great industrial importance in beverages, baked goods, meat products and frozen dairy products (Burdock & Fenaroli, 2010). A few microorganisms are also able to produce this pyrazine, *i.e. Bacillus subtilis* grown on a sucrose asparagine medium (Kosuge & Kamiya, 1962). The same compound was also evidenced during the fermentation of cocoa beans by the action of microorganisms (Reineccius et al., 1972). One strain of *Corynebacterium*

glutamicum was able to produce 3 $g.L^{-1}$ of tetramethypyrazine in a 5-day fermentation from amino acids (*e.g.* leucine, isoleucine and valine) (Demain et al., 1967).

Several pyrazines such as 2,5-dimethylpyrazine (characteristic earthy potato-like odor), 2,6-dimethylpyrazine (nutty and coffee-like odor) or trimethylpyrazine were produced by strains of *Bacillus cereus*. The latter has a baked potato or roasted nut aroma and is of great commercial importance (Burdock & Fenaroli, 2010). The concentrations of these pyrazines did not reach more than 4 $mg.L^{-1}$ and the production depended on the temperature and the culture medium (Demyttenaere et al., 2002; Feron & Waché, 2006).

3. **Production of natural flavors by biotransformation**

Biotransformations, comprise single reactions catalyzed enzymatically resulting in a product structurally similar to the substrate molecule. In some cases, the precursors employed can be considered inexpensive and readily available, such as fatty or amino acids, and can be converted to more highly valued flavors (Krings & Berger, 1998). The main substrates used in biotransformation processes are described as follows, and some examples of the processes employed to obtain aroma compounds by biotransformation are detailed in Table 2.

3.1. Monoterpenes alcohols

α-Terpineol is one of the most commercially important monoterpene alcohols for the flavor industry. Its annual consumption is estimated to be approximately 9.2 tons, which represents an individual intake of 17.2μg/Kg/day in the USA. It has a lilac odor and sweet smell reminiscent of peach, with an aroma threshold of 280 to 350 ppb. The reported food usages for α-terpineol, usually in a range of 10-20ppm, are: baked goods, chewing gum, condiments, dairy products, candies beverages and others (Burdock & Fenaroli, 2010). It is also applied for the formulation of soaps and cosmetics (Surburg & Panten, 2006). Although it occurs in a large number of essential oils, such as Cupressaceae, Pinaceae, lavandin and many other leaves, herbs and flowers, and may be produced by fractional distillation of pine oils, this alcohol is commonly synthesized by acid hydration of α-pinene or turpentine followed by partial dehydration (Burdock & Fenaroli, 2010; Surburg & Panten, 2006). However, one of the most recent tendencies is the production of natural α-terpineol production by the biotranformation of limonene, α-pinene or β-pinene (Bicas et al., 2008). One of the most viable processes described so far yields almost $130g.L^{-1}$ using limonene as substrate in a biphasic medium (Bicas et al., 2010b).

Table 2. Main examples of aroma compounds produced by biotransformation processes. The structures of the compounds cited are shown in Figure 2.

Product	Precursor	Biocatalyst	Process information[a]	Ref.[b]
Monoterpene alcohols				
α-Terpineol	Limonene	*Penicillium* sp.	C = 3.45 g L^{-1} (8 days)	1
		Penicillium digitatum	C = 3.2 g L^{-1} (96h), sequential substrate feedings	2
		Fusarium oxysporum	C ~ 4 g L^{-1} (48h), frozen biomass	3
		Sphingingobium sp.	C ~ 130 g L^{-1} (96h), aqueous-organic biphasic medium	4
	β-pinene	*Aspergillus* sp.	C = 770 mg L^{-1} (7 days)	5
Carveol	Limonene	*Aspergillus niger*	Y = 18% (6h)	6
		Rhodococcus opacus	Y = 94-97% (2.5h)	7
		Cellulosimicrobium cellulans	C ~ 2 g L^{-1} (11h)	8
Perillyl alcohol	Limonene	*Mortierella minutissima*	C = 258.1 mg.L^{-1} (48h), 15°C, H_2O_2 oxygenated culture	9
		Recombinant *E. coli*	C ~ 12 mg.g^{-1} dry cell (96-120h)	10
		Recombinant *Pseudomonas putida*	C = 6.8 g.L^{-1} (75h), 2L bioreactor	11
Verbenol	α-Pinene	Unidentified yeast	C = 125.6 mg.L^{-1} (8 days)	12
		Aspergillus sp./*Penicillium* sp.	C ~ 150 mg.L^{-1} (1.08mg/g biomass) (6h)	13
		Pseudomonas putida	Y = 35% (6h)	6
		Hormonema sp.	C = 400 mg.L^{-1} (96h)	14
Myrtenol	α-Pinene	*Bacillus palidus*	C ~ 23 mg.L^{-1} (24h)	15
	β-pinene	*Bacillus palidus*	C ~ 17 mg.L^{-1} (24h)	15
Terpene ketones				
Carvone	Limonene	*Rhodococcus opacus*	Aerated two phase bioreactor	16
		Pseudomonas aeruginosa	625 mg.L^{-1} (13 days)	17
		Rhodococcus globerulus	C = 43,5 mg.L^{-1} (27h), 2.5 mL-scale	7

		Carveol	*Rhodococcus erythropolis*	Up to ~150 $g.L^{-1}$ (1.03 M) (167h), 65 mL column reactor	18
	Verbenone	α-Pinene	*Aspergillus niger*	C = ~35 $mg.L^{-1}$ (6-72h)	19
			Hormonema sp.	300 $mg.L^{-1}$ (96h)	14
	Nootkatone	Valencene	*Pleurotus sapidus* (lyophilized)	C = 320 $mg.L^{-1}$ (24h), P = 13 $mg.L^{-1}/h$	20
Monoterpene acids					
	Perillic acid	Limonene	*Pseudomonas putida*	C = 31 $g.L^{-1}$ (7 days), *in situ* product recovery	21
Monoterpene ethers					
	Rose oxide	Citronellol	*Penicillium* sp.	C = 73.4 $mg.L^{-1}$ (5 days)	22
	Linalool oxide	Linalool	*Corynespora cassiicola*	C = 357 $mg.L^{-1}$ (3 days), P = 120 mg/L^{-1} /day	23
Carotenoids					
	β-Ionone	β-Carotene	*I. benzoinum*	C = 8 (in mol %) (12 h)	24
Vanillin					
	Vanillin	Ferulic acid	*Amycolatopsis* sp.	C = 11.5 $g.L^{-1}$ (conversion rate of 77.8%, after 32 h)	25
	Vanillin	Ferulic acid	*Streptomyces setonii*	C = 13.9 $g.L^{-1}$ (yield of 75%, after 17 hours)	26

[a] C: concentration obtained (production), P: productivity, Y: yield. [b] References: 1. Rottava et al., 2010a; 2. Tan et al., 1998; 3. Bicas et al., 2010a; 4. Bicas et al., 2010b; 5. Rottava, *in press*; 6. Divyashree et al., 2006; 7. Duetz, et al., 2001; 8. Wang et al., 2009; 9. Trytek et al., 2009; 10. Cheong & Oriel, 2000; 11. van Beilen et al., 2005; 12. Rottava et al., 2010b; 13. Rao et al., 2003; 14. van Dyk et al., 1998; 15. Savithiry et al., 1998; 16. De Carvalho & Da Fonseca, 2003; 17. Acosta et al., 1996; 18. De Carvalho et al.; 2005; 19. Agrawal & Joseph, 2000a and Rozenbaum et al., 2006; 20. Fraatz et al., 2009; 21. Mirata et al., 2009; 22. Maróstica Jr. & Pastore, 2006; 23. Zorn et al., 2003; 24. Mirata et al., 2008; 25. Rabenhorst & Hopp, 2000; 26. Muheim et al., 2001

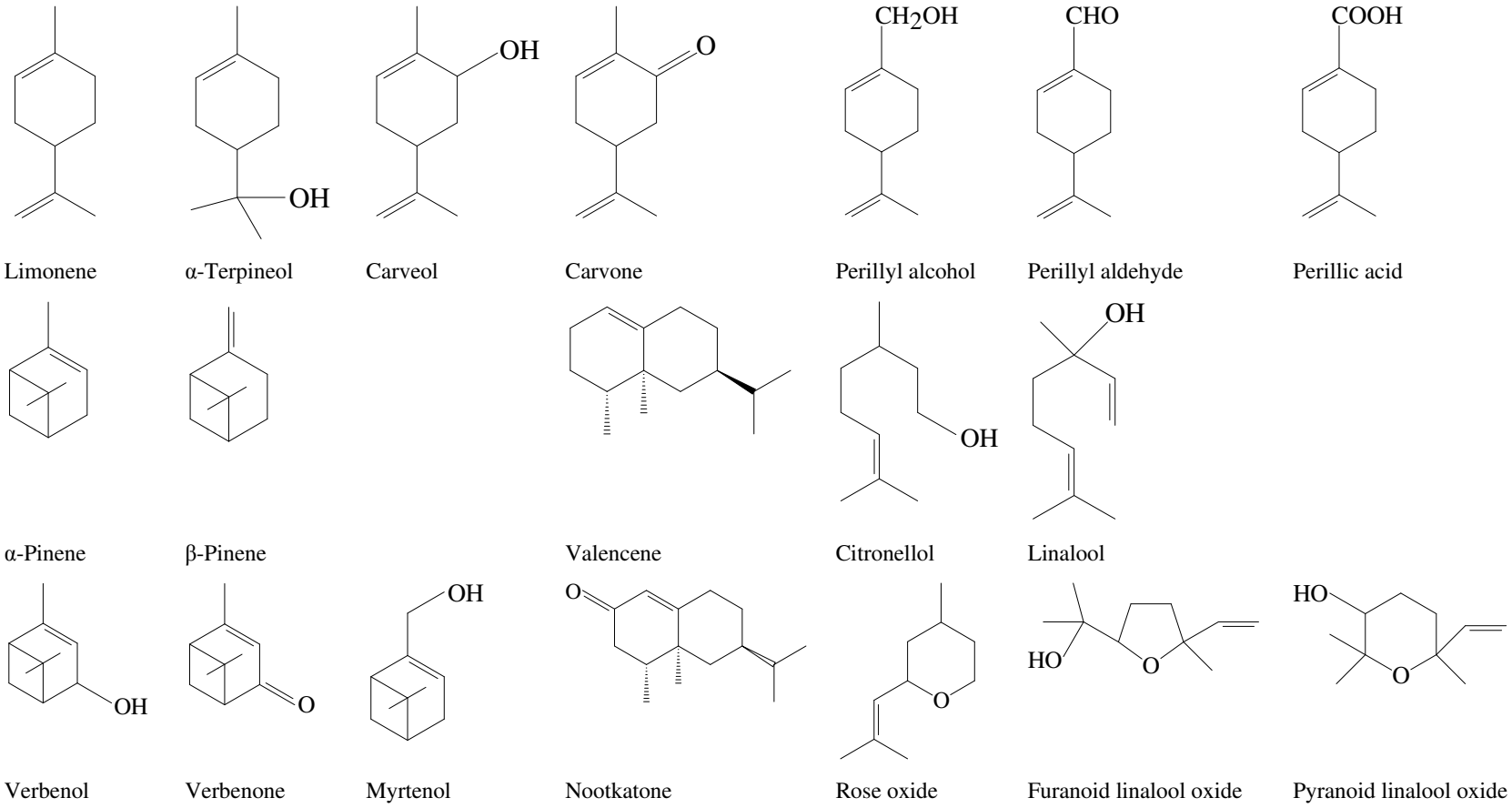


Figure 2. Substrates and products of microbial biotransformation processes.

Carveol, together with carvone (Figure 2), is another important flavor compound. It has a spearmint-like odor used in baked goods, chewing gums, frozen dairy, gelatin, puddings, beverages and candies, in a concentration close to 220 ppm. Its annual consumption is around 1.2 tons, representing an individual intake of 2.3 μg/Kg/day in the USA. Carveol may be extracted from caraway seeds, spearmint, orange juice, mango, eucalyptus oil, where it is present in small amounts, sometimes esterified, but it can also be prepared from the oxidation of limonene (Burdock & Fenaroli, 2010). Biotransformation processes for producing natural carveol as the major product have already been reported using limonene as the substrate and *Aspergillus niger* (Divyashree et al., 2006) or *Rhodococcus opacus* (Duetz et al., 2001) as biocatalysts. One of the highest yields of carveol described so far is 13.4 mM (~2g.L^{-1}) using the bacteria *Cellulosimicrobium cellulans* (Wang et al., 2009).

Perillyl alcohol is one of the most prominent and desirable monoterpene alcohols due to its potential in the treatment of cancers (Koroch et al., 2007). It has a green-type aroma descriptor, also described as sweet, fatty and woody resembling linalool (floral) and terpineol, with a threshold of 7 ppm (Burdock & Fenaroli, 2010). This alcohol is found in small concentrations in essential oils, such as lavander and bergamot (*S*-isomer), caraway (*R*-isomer), gingergrass (*R*- and *S*-), citrus peel, berries, peppermint, hop, cardamom and others. The reported uses of perillyl alcohol are baked goods, frozen dairy, gelatine, pudding, beverages and candies in a concentration of 1-2 ppm. The *S*- form may be obtained starting from β-pinene by reducing perillyl aldehyde with zinc dust and acetic acid, followed by saponification of the acetate (Burdock & Fenaroli, 2010). The biotechnological production of perillyl alcohol starting from limonene is also feasible. Microbial biotransformations for the production of perillyl alcohol as the major product have already been reported using limonene as the substrate from the microorganisms *Aspergillus niger* (Menéndez et al., 2002), *Mortierella minutissima* (Trytek et al., 2009), *Bacillus stearothermophilus* (Chang & Oriel, 1994) or a recombinant *Escherichia coli* (Cheong & Oriel, 2000). The highest perillyl alcohol production was found for a recombinant *Pseudomonas putida* cultivated in biphasic medium. After 75h, a concentration of 6.8 grams per liter of organic phase was reached (van Beilen et al., 2005).

Menthol is one of the most economically important monoterpene alcohols, with an annual consumption of c.a. 90 metric tons in USA. Its main uses are in cigarettes, cosmetics, toothpastes, chewing gum, candies, and medicines (Burdock & Fenaroli, 2010; Surburg & Panten, 2006). 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, with (–)-menthol the isomer that occurs most widely in nature (peppermint and other mint oils). This compound has a mint-like odor and exerts a cooling sensation when in contact with the skin and mucosal surfaces, which is one of its most attractive attributes for industry and may confer analgesic properties (Koroch et al., 2007). Its aroma threshold is between 950 ppb to 2.5ppm (Burdock & Fenaroli, 2010). Although the major portion of the world wide demand is supplied by *Mentha* oils, several processes exist for the synthesis of (–)-menthol. Commercially important routes for the synthesis start from thymol (hydrogenation) (Burdock & Fenaroli, 2010; Surburg & Panten, 2006) or from m-cresol from petrochemical origin (van der Schaft, 2007). In terms of biotechnological processes involving menthol, the references are limited. It may be a precursor in biotransformation processes (see Bicas et al., 2009 for references) and can also be prepared by the biotransformation of menthyl acetate by plant cell and organ cultures (Scragg, 2007). Fungal biotransformation of citronellol to menthol has also been reported (see Noma & Asakawa, 2010 for references).

Verbenol (Figure 2) is a bicyclic monoterpene alcohol with a fresh pine, ozone odor (Burdock & Fenaroli, 2010), also described as having camphor and mint flavor notes (Rottava et al., 2010b). It has been used (concentration of ~1 ppm) in baked goods, beverages, frozen dairy, gelatine, pudding and candies, but its annual consumption is estimated to be lower than 1 Kg. Verbenol is found, for example, in spearmint oil, juniper berry, myrtle and mango and may be prepared by chemical synthesis from (–)-α-pinene (Burdock & Fenarioli, 2010). Microbial biotransformations of α-pinene to verbenol as the major product have also been reported. The microorganisms capable of doing a such conversion were: a hybrid (protoplast fusion) from *Aspergillus niger*. and *Penicillium digitatum* (Rao et al., 2003), *Pseudomonas putida* (Divyashree et al., 2006), *Serratia marcescens* (Wright et al., 1986) or unidentified microbial strains (Rottava et al., 2010b).

Another bicyclic monoterpene alcohol, myrtenol (Figure 2), has a camphoraceous, minty, medicinal, woody odor, with an aroma threshold of 7 ppb. Its reported usages are confection, frosting, gelatin, pudding, milk products and beverages, commonly in a concentration of 5 ppm (Burdock & Fenaroli, 2010). Myrtenol is also present in non-food products, where it is used as an ingredient in decorative cosmetics, fine fragrances, shampoos, soaps household cleaners and detergents. However, its use worldwide is lower than 0.1 metric tonnes per year (Bhatia et al., 2008). Many berries, citrus fruits and herbs contain myrtenol. The synthesis of myrtenol is usually performed with α-pinene, SeO_2 and ethanol (Burdock &

Fenaroli, 2010). The biotransformation of α- and β-pinenes with *Bacillus pallidus* yields myrtenol in low quantities, together with other major products (Savithiry et al., 1998).

3.2. Terpene ketones

Carvone (Figure 2) is a very important monoterpene ketone for the flavor industry. *S*-(+)-Carvone is the main component of caraway oil and dill, with an odor resembling these herbs. The other isomer (*R*-(–)-carvone) occurs at high concentrations (70-80 %) in spearmint oil, and is also the major component responsible for its aroma. The aroma threshold for carvone is in the range of 6.7-820 ppb, for *S*-(+)-carvone, and 2.7-600 ppb for the *R*-(–)-isomer. They have a high annual consumption (almost 90 tons), with reported uses in many products (food, beverages and oral hygiene products) in a usual range of 30-200 ppm (Surburg & Panten, 2006; Burdock & Fenaroli, 2010). Several applications have been reported for carvone. Its use as a fragrance and flavor, potato sprouting inhibitor, antimicrobial agent, building block, biochemical environmental indicator and in medical applications increase the interest in this ketone (De Carvalho & Da Fonseca, 2006). Carvone was traditionally isolated by fractional distillation of caraway or spearmint oils, but the chemical synthesis from limonene has been the preferred method (Surburg & Panten, 2006). The use of the nitrosyl chloride method for obtaining this ketone is one of the most commonly used procedures, although the biotechnological techniques are becoming important (Bicas et al., 2009). The biotransformation of limonene to carvone has already been described for *Rhodococcus opacus* (de Carvalho & da Fonseca, 2003). Carveol has also been used as a substrate using the bacteria *Rhodococcus globerus* (Duetz, et al., 2001) or *R. erythropolis* (de Carvalho et al.; 2005) as biocatalysts. Concentrations up to 150 $g.L^{-1}$ have been achieved in small scale column reactors.

Menthone is another important monocyclic monoterpene ketone. With a characteristic mint-like odor and an annual consumption of c.a. 23 metric tons in USA, it is used for synthetic peppermint oils and bases and is also applied in chewing gums, gelatine and baked goods. (–)-Menthone can be obtained by distillation of dementholized cornmint oil or by oxidation of (–)-menthol (Burdock & Fenaroli, 2010; Surburg & Panten, 2006). Regarding biotechnological processes, (–)-menthone may be prepared by the biotransformation of (+)-neomenthol using plant cells (Scragg, 2007). As for menthol, microbial processes involving menthone are also limited (Noma & Asakawa, 2010).

Verbenone (Figure 2) is a bicyclic monoterpene ketone representing the impact compound of rosemary oil (Schrader, 2007), where it is usually present in trace amounts,

from 0-0.4 to 0.7-2.5%, depending on the source (Surburg & Panten, 2006). This compound, which has flavor notes of camphor and mint, is considered the major constituent of strawberry, raspberry, dill, rosemary or spearmint favor mixtures, and, therefore, verbenone has great demand in the food industry. It is currently obtained from pine and eucalyptus and has a market value of US$ 3000/kg (Agrawal & Joseph, 2000). In biotransformation processes, this ketone is formed as the major product when using *Aspergillus niger* (Agrawal & Joseph, 2000) or the yeast *Hormonema* sp. (Van Dyk et al., 1998). However, in the latter case, high amounts of verbenol are also produced.

The ketone nootkatone will be the only sesquiterpene ketone described in this chapter, due to its high demand for the fragrance, food, cosmetics, and pharmaceutical industries (Fraatz et al., 2009). It is present in the oils of citrus fruits (bergamot, lemon, lime, orange and tangerine), especially in grapefruit peel and juice, where nootkatone is one of the characteristic components of the aroma. The aroma threshold for nootkatone is 170 to 800 ppb and its taste characteristics (grapefruit, citrus, orange and bitter) are observed for concentrations above 20 ppm. Its estimated annual consumption is around 83 Kg and it is used for flavoring beverages. The synthesis of nootkatone is performed by oxidation of valencene (Surburg & Panten, 2006; Burdock & Fenaroli, 2010). This might be performed by microorganisms or their enzymes in biotransformation processes. One of the highest yields was described from the basidiomycete *Pleurotus sapidus* (Fraatz et al., 2009).

3.3. Terpene aldehydes and acids

Perillyl aldehyde (Figure 2) is a monocyclic monoterpene aldehyde with a carbon skeleton similar to its reduced counterpart, perillyl alcohol. Perillyl aldehyde has a threshold value of 30 to 62 ppb and a powerful fatty-spicy, oily, herbaceous odor. It is used in beverages, baked goods, frozen dairy, puddings, candies etc. in a concentration of 2-10 ppm, corresponding to an annual consumption of ~25 Kg. The natural sources of perillyl aldehyde are the essential oils of fruits (*e.g.* citrus, guava), berries (*e.g.* bilberries, blackberry), herbs and others (e.g. gingergrass, lavender, spearmint, caraway seed) (Burdock & Fenaroli, 2010). In biotransoformation processes, perillyl aldehyde may be prepared from limonene using *Mortierella minutissima* (Trytek et al., 2009), but it is usually an intermediate for the production of perillic acid (Speelmans et al., 1998) and, thus, it accumulates only in minor amounts. In fact, high production of perillic acid from limonene, via perillyl aldehyde, has been achieved with *Pseudomonas putida* (Mirata et al., 2009). However, the precursors

perillyl aldehyde and perillyl alcohol, are the industrially more interesting targets (Speelmans et al., 1998).

The sesquiterpene aldehyde α-sinensal has a potent sweet orange aroma, with a very low odor threshold (< 0.1 ppb). It is present in different citrus oils and may be prepared from the biotransformation of farnesene, but the yields obtained were too low (Krings et al., 2006; Schrader, 2007). One alternative may be the use of *trans*-nerolidol as starting product (Hrdlicka et al., 2004).

3.4. Monoterpene ethers

One of the most interesting monoterpene ethers for the flavor and fragrance industries is rose oxide. This compound occurs in two isomeric forms (*cis* and *trans*), which are found in small amounts in the oils of rose (Bulgarian) and geranium. Rose oxide has a powerful distinctive geranium note and is used in perfume compositions for the formulation of rose, geranium and all many other floral fragrances. Baked goods, dairy products, pudding and candies also contain 1-3 ppm of citral (Surburg & Panten, 2006; Burdock & Fenaroli, 2010). Rose oxide is usually prepared from citronellol through a photosensitized oxidation with singlet oxygen or by means of microbial processes (Bicas et al. 2009). The biotransformation of citronellol to rose oxide is also performed by *Aspergillus niger* (Demyttenaere et al., 2004) and *Penicillium* sp. (Maróstica Jr. & Pastore, 2006), but the reported yields were low for this aroma compound.

The oxidation of linalool may produce linalool oxide, another important ether aroma compound, with a sweet woody, earthy-flower, bergamot-like odor, found in many fruits, coffee, hop, lavender and other natural sources. Its reported aroma threshold is about 320 ppb and it is used in perfumery to confer, for example, lavender notes (Surburg & Panten, 2006; Burdock & Fenaroli, 2010). Linalool furanoid and pyranoid oxides may be prepared by the oxidation of linalool (Demyttenaere et al., 2001). The highest productivity was reported for *Corynespora cassiicola* (Mirata et al., 2008).

3.5. Carotenoid-derived aroma compounds

There are many biological effects which are attributed to the presence of carotenoids. One of them is the metabolism of carotenoids to retinoids (Krinsky, 1994), that is the main dietary source of vitamin A. More recently, protective effects of β-carotene and other carotenoids against serious disorders such as cancer, heart disease and degenerative eye disease have been recognized, and have stimulated intensive research into the role of

carotenoids as antioxidants and as regulators of the immune response system (Ziegler, 1991; Zorn et al., 2003).

Besides their biological importance, the carotenoids are important precursors of a variety of compounds: the C_{20}-retinoids, the C_{15}-phytohormones, and the C_9- to C_{13}-aromas (see Figure 3). Among the last type, C_{13}-carotenoid-derived compounds (norterpenoids/norisoprenoids) such as ionones and damascones, constitute an essential aroma compound in tea, grapes, roses, tobacco, and wine (Rodriguez-Bustamante & Sanchez, 2007), and are formed via enzymatic oxidation and photo-oxidation of the various carotenoids found in plants, flowers and fruits (Enzell, 1985; Wu et al., 1999; Winterhalter & Rouseff, 2001). Although carotenoid-derived aroma compounds are ubiquitous constituents in plant derived aromas, very little is known about their biogeneration (Winterhalter & Rouseff, 2001).

The occurrence of norisoprenoids in natural sources is restricted to trace amounts, and extraction has turned out to be tedious and costly (Zorn et al., 2003). In this sense, the bio-production of volatile compounds derived from carotenoids is gaining more attention by the flavor and fragrance industries, because it represents a feasible alternative to chemical synthesis and offers the production of enantiomerically pure molecules which can be labeled as "natural." To date, biotransformation of carotenoids has been reported using cells (plant-cultured cells, fungi, bacteria and yeasts) and pure enzymes (see Bicas et al., 2009 for references).

3.5.1. Norisoprenoids

As discussed before, the norisoprenoids are important flavor compounds derived from carotenoids. These compounds have not only been detected in leaf products, such as tobacco, tea, and mate, but also in many essential oils, fruits (grapes, passionfruit, starfruit, quince, apple, nectarine), vegetables (tomato, melon), spices (saffron, red pepper), as well as additional sources such as wine, rum, coffee, oak wood, honey, seaweeds *etc* (Winterhalter & Rouseff, 2001).

In general three steps are required to generate an aroma compound from the parent carotenoid (i) the initial dioxygenase cleavage, (ii) subsequent enzymatic transformations of the initial cleavage product giving rise to polar intermediates (aroma precursors), and (iii) acid-catalyzed conversions of the non-volatile precursors into the aroma-active form (Krings & Berger, 1998).

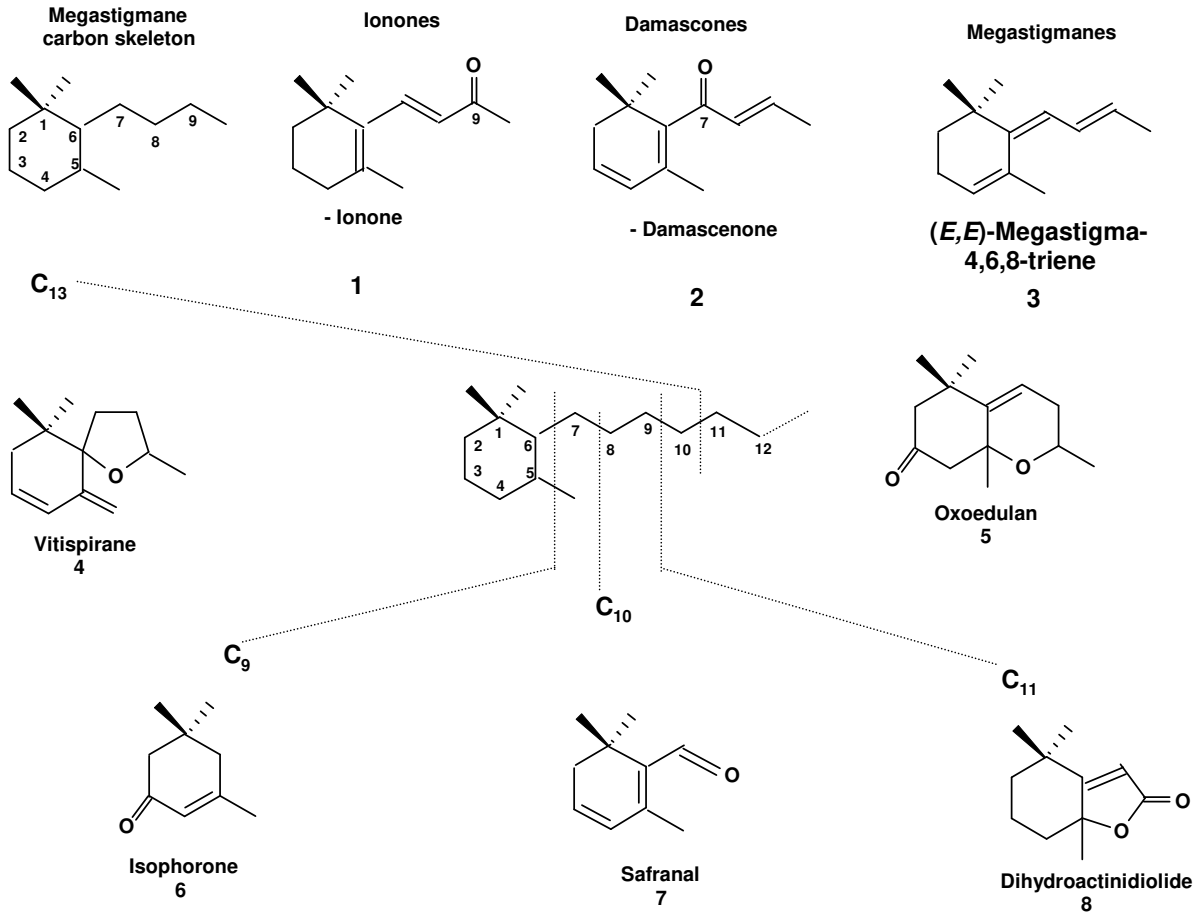


Figure 3. Different classes of degraded carotenoids and examples for potent odorants from classes of C_9-, C_{10}-, C_{11}-, and C_{13} –norisoprenoids. (Rodríguez-Bustamante & Sánchez, 2007).

3.5.1.1. α- and β-ionone

The C_{13} ketones α- and β-ionone are cyclic terpenoid derivatives that occur in many essential oils. α-Ionone has appearance of colorless to pale-yellow liquid and characteristic violet-like odor. Moreover, β-ionone has appearance of colorless to pale-yellow liquid, but has characteristic violet-like odor, more fruity and woody than α-ionone. They naturally occur in the distillate of the flowers of *Boronia megatisma* Nees, and in a few other essences. Reported uses include non-alcoholic beverages, ice cream, candy, baked goods, gelatins and puddings, chewing gum, maraschino cherries (Burdock & Fenaroli, 2010).

The biotransformation of some carotenoids to produce β-ionone has been reported. The use of the enzymes lipoxygenase and xanthise oxidase, using β-carotene as substrates, has been described by Gonçalves et al. (2006), Wu et al. (1999) and Waché et al. (2006). Moreover, the use of cells were evaluated by Zorn et al., 2003, using filamentous fungi to cleave β-carotene into flavor compounds. The production of β-ionone, β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone were observed.

3.5.1.2. Damascones and damascenones

The damascones are commercially important, due to their low odour threshold values and attractive sensory properties. α-Damascone occurs in the natural flavor of tea and tobacco, and has a very powerful floral complex fruity note reminiscent of plum, rose and blackcurrant. On the other hand, β-damascenone is found as a volatile constituent of many natural materials such as rose oils and extracts. This ketone is a constituent of Bulgarian rose oil, where it is an important contributor to the characteristic aroma, although present in a concentration of just 500ppm (Winterhalter & Rouseff, 2001).

Recently, the microbial bioconversion of damascones was studied. Bioconversion of α-damascone was studied with four strains of *Botrytis cinerea* in grape must (pH 3.2). As biotransformation products of compound 1, 3-oxo-α-damascone, *cis*- and *trans*-3-hydroxy-α-damascone, γ-damascenone, 3-oxo-8, 9-dihydro-α-damascone, and *cis*- and *trans*-3-hydroxy-8,9-dihydro-α-damascone were identified (Schoch et al., 1991).

3.6. Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde), one of the most widely used flavor compound in the world, possesses a sweet, floral and spice-like odor, and is quite bitter above 30 to 40 ppm. It is the principal flavor component of vanilla extract obtained from the cured pods (beans) of the orchid *Vanilla planifolia* Andrews, at a level of about 2% by weight (Schwab et al., 2008, Burdock & Fenaroli, 2010). Its use includes non-alcoholic beverages, ice cream, candy, gelatins and puddings, chewing gum, syrups, toppings, margarine (Burdock & Fenaroli, 2010). Vanilla extracts are used extensively in chocolate and baked products, and especially in ice cream (Bauer, 2001).

Vanilla extract is valued as a natural flavor. However, due to its high cost and limited availability less than 1% of the annual world demand for vanillin is isolated from its botanical source (Walton et al., 2003). Most of the vanillin used by the flavor industry originates from chemical methods that use guaiacol, eugenol or lignin as starting materials (Rao & Ravishankar, 2000; Schwab et al., 2008). However, the difference in price of the natural compound and its chemically synthesized counterpart can be considerable. For example: the price for synthetic vanillin is about US$12 kg^{-1} with a market of 12000 tons/year, while vanillin extracted from vanilla pods costs about US$4000 kg^{-1}, with a market of 50 tons/year (Feron & Waché, 2006).). Consequently, extensive studies have been conducted to obtain vanillin by means of microbial processes.

Neither *de novo* routes in plant cell cultures of Vanilla nor those in bacteria or fungi afford anything like acceptable yields. The precursor approach holds more promise. Several starting materials appear to be suitable including lignin, eugenol, ferulic acid, curcumin and benzoe siam resin (Benz & Muheim, 1996). Turnover rates of less than 30% and production levels below 1 $g.L^{-1}$ have been reported. Again, the toxicity of both the precursor and the product, as well as product degradation in the course of fermentation, prevented a better yield (Zorn et al., 2003). Although the highest yields of biotechnological vanillin are related to patented biotransformation/bioconversion processes, with precursors such as ferulic acid and eugenol (Dausgh & Pastore, 2005), some processes obtained this compound by *de novo* means, mainly from glucose by recombinant *Escherichia coli* (Li & Frost, 1998) and *Schizosaccharomyces pombe* (Hansen et al., 2009).

A large number of patents and papers describe different bioprocesses to obtain vanillin by biotechnological means. The highest yields reported for biotechnologically-

produced natural vanillin refer to patent processes: the bioconversion of ferulic acid into vanillin by strains of *Amycolatopsis* sp. or *Streptomyces setonii* in a 10 L bioreactor. The final yields were 11.5 $g.L^{-1}$ (Rabenhorst & Hopp, 2000) and 13.9 $g.L^{-1}$ (Muheim et al., 2001) respectively. In fact, the microbial transformation of ferulic acid is recognized as the most attractive and promising alternative source of natural vanillin (Bicas et al., 2010c).

For more details about the bio-production of vanillin, see Walton et al. (2003), Walton et al. (2000) and Daugsch & Pastore (2005).

4. **Concluding remarks**

Most food flavoring ingredients are produced by means of chemical processes. However, the tendency is growing for the production of aroma compounds using biotechnological tools, which have the following advantages: (i) the process occurs under mild conditions, (ii) it presents a lower environmental impact and (iii) the compounds may be labeled as "natural". This represents an important market opportunity because of the preference of the consumer for "natural"," products. However, low transformation rates and high production costs are still obstructing their widescale adoption. In this sense, recent advances in genetic, bioprocess engineering and the use of by-products as alternative substrates stimulates research into biotechnological processes to produce aroma compounds.

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Capítulo 2

Fusarium oxysporum 152B: A potent biocatalyst for the biotransformation of terpenes

ABSTRACT

This article complements a series of studies on the strain *Fusarium oxysporum* 152B describing its versatility to the biotransformation of terpenes. The potential of this fungal strain has been well elucidated for extracellular alkaline lipase production and limonene biotransformation into α-terpineol, yielding 4 $g.L^{-1}$ of this monoterpene alcohol. In this study, *Fusarium oxysporum* 152B was able to grow on several terpenes as a sole carbon and energy source and also assimilated the terpenes *S*-(–)-limonene, limonene-1,2-epoxide, α-pinene, linalool, γ-terpinene, while some others mono-, sesqui- and tetraterpenes tested were not utilized as a carbon and energy source nor metabolized into new products. The reported concentration of 1.2 $g.L^{-1}$ of limonene-1,2-diol from the biotransformation of *S*-(–)-limonene in non-optimized conditions is one of the highest already published and encourage the studies of the production of this natural flavor obtained by *Fusarium oxysporum* 152B. Thus, this might be a promising strategy for obtaining this compound in natural forms. Considering the results of this extensive evaluation, the microorganism *Fusarium oxysporum* 152B proved to be a potent biocatalyst with great potential for the development of new flavor compounds from the biotransformation of terpenes.

Keywords: Biotransformation, Bioconversion, *S*-(–)-Limonene, Limonene-1,2-epoxide, α-Pinene, Linalool, γ-Terpinene.

1. Introduction

Terpenes are widely distributed in nature. They are secondary metabolites of plants that are produced, in part, as a defense against microorganisms and insects in addition to their pollinator-attractive properties (Gershenzon and Dudareva, 2007). These compounds also exhibit a diversity of roles in mammals because they act in stabilization of cell membranes, regulate enzymatic reactions and participate in metabolic pathways (De Carvalho and Da Fonseca, 2006).

The commercial attention around terpenes are increasing since it was observed their role in prevention and therapy of several diseases, such cancer; their activity as natural insecticide and antimicrobial agents; as building blocks for the synthesis of many highly value compounds (De Carvalho and Da Fonseca, 2006) and they are also a good starting material for the synthesis of many fine chemicals due to their similar carbon skeleton (Bicas et al., 2009).

Monoterpenes are naturally occurring branched chain C-10 hydrocarbons formed from two isoprene units (Van der Werf et al., 1997) widely distributed in nature (more than 400 structures), constituting suitable precursor substrates (Krings and Berger, 1998) which are ideal starting materials for the biotechnological production of natural aroma chemicals (NACs). Terpenoids, oxygenated derivatives of terpenes, are inexpensive, readily available and renewable natural precursors. More than 22.000 individual terpenoids structures are known at present, constituting the largest group of natural products (Demyttenaere and De Kimpe, 2001). This terpenoids have, in general, more characteristic flavor properties than their terpene hydrocarbon counterparts. Therefore, the hydroxylation of cheap and readily available monoterpene hydrocarbons such as (-)-α-pinene and (+)-limonene have been one of the target reactions for the biotransformation studies (Van der Werf et al., 1997).

The biotransformation of terpenes through microorganisms is recognized as being of great economic potential since these processes can allow the production of enantiomerically pure flavors and fragrances under mild conditions and also the products may be considered as "natural", increasing the interest in this area (De Carvalho and Da Fonseca, 2006). But, in the other hand, this complex process is technically difficult because of monoterpenes chemical instability, low solubility, toxicity and volatily and also because can occur multiple metabolic pathways leading to a mixture of products; low product concentrations and yields or the absence of product accumulation (Van der Werf et al., 1997).

The bioconversion of *R*-(+)-limonene, for example, one of the most abundant monoterpene, can lead to an interesting end product called *R*-(+)-α-terpineol. This product is a stable alcohol widely distributed in nature typically applied in household products, cosmetics, pesticide, flavor preparations and is one of the most commonly used perfume chemicals (Bauer et al., 2001; Demyttenaere et al., 2001). This monoterpenoid is commonly produced by acid-catalyzed chemical synthesis from α-pinene or turpentine oil and represents an important commercial product (Bicas et al., 2008a). On the other hand, the production of this monoterpene alcohol has been described using a wide range of microorganisms as catalysts (Tan and Day, 1998ab; Tan et al., 1998; Adams et al., 2003; Bicas et al., 2008ab).

The present study was conducted with a fungal strain identified as *Fusarium oxysporum* 152B, previously isolated as a potential extracellular alkaline lipase producer (Prazeres et al., 2006). Simultaneously, the strain was tested by Maróstica Jr. and Pastore (2007a) for the bioconversion of (R)-(+)-limonene using the agroindustrial residues cassava wastewater and orange peel oil, as substitutes for the fungal cultivation medium and substrate, respectively. The authors could observe that approximately 450 $mg.L^{-1}$ of *R*-(+)-α-terpineol accumulated in the medium indicating that the microorganism was capable of biotransforming *R*-(+)-limonene (Marostica-Jr and Pastore, 2007a).

Following the studies carried out with this biocatalysts, in an extensive study, Bicas et al. (2008a) described the optimization of the ten main process variables involved in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *Fusarium oxysporum* 152B through a Plackett-Burman matrix with 16 assays, including the effect of the medium composition, substrate concentration, cultivation conditions and inoculum size, followed by central composite design methodology. Thus, the authors improved significantly the production of *R*-(+)-α-terpineol, from 450 $mg.L^{-1}$ previously described to 2.44 $g.L^{-1}$ of this monoterpene alcohol.

Another study conducted by the same group proposed an integrated process for the co-production of lipase and *R*-(+)-α-terpineol, where the concentration of this monoterpene alcohol reached approximately 4 $g.L^{-1}$ (Bicas et al., 2010a).

Thus, this article complements a series of studies on the strain *Fusarium oxysporum* 152b aiming to evaluate the potential of this biocatalyst in the biotransformation of several other mono and sesquiterpenes to obtain new flavor compounds of industrial interest.

2. Material and methods

2.1. Microorganism and chemicals

The microbial strain employed in this study was isolated from the northeast Brazilian fruits, and was identified as *Fusarium oxysporum* 152B (Prazeres et al., 2006). The chemical standards used in this study were *S*-(–)-limonene (96% purity, Sigma-Aldrich), (–)-α-pinene (97% of purity, Sigma-Aldrich), (–)-β-pinene (97% of purity, Sigma-Aldrich), γ-terpinene (97% of purity, Sigma-Aldrich), citronellol (95% of purity, Sigma-Aldrich), (-)-carveol (97% of purity, Sigma-Aldrich), (–)-limonene-1,2-oxide (99% of purity, Sigma-Aldrich), linalool (97% of purity, Fluka), geraniol (96% of purity, Fluka), α-terpineol (97% purity, Fluka), α-farnesene (mixture of isomers, SAFC), α-bisabolol (97% of purity, Fluka), (+)-valencene (70% of purity, Sigma-Aldrich), neoxanthin and β-carotene (Sigma-Aldrich) were kept under refrigeration (4 ^{o}C). Ethyl acetate and n-decane used as solvent and internal standard, respectively, were of best commercial grade.

2.2. Inoculum for the biotransformation trials

A piece of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 50 mL of yeast and malt (YM) medium (in $g.L^{-1}$: glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3, pH ~ 6.7). The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After incubation at 30 ^{o}C and 150 rpm for 72 h, the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman n^{o}1 (Bicas et al., 2008a).

2.3. Utilization of terpene substrates for fungal growth

The biomass obtained in item 2.2 (~ 3 g) was distributed amongst 250 mL conical flask filled with 50 mL of YM medium. However, the sole carbon source consisted of 0.5% (v/v) of one of the terpenes tested (*S*-(-)-limonene, α- and β-pinenes, γ-terpinene, citronellol, linalool, geraniol, (-)-carveol, limonene-1,2-oxide, α-terpineol, α-farnesene, α-bisabolol, valencene, neoxanthin and β-carotene) to assess the ability of the fungal strain to adapt and use these substrates for growth. The flasks were incubated in rotatory shaker at 30 ^{o}C and 150 rpm for 48 h. Biomass was recovered, dried at 105

^{o}C for 24 h and weighed to evaluate the growth of biomass in alternative media. Experiments were conducted in triplicate.

2.4. Biotransformation procedure

The biomass obtained in item 2.2 (~ 3 g) was distributed amongst 250 mL conical flask filled with 50 mL of mineral (MM) medium (in g.L^{-1}: $MgSO_4.7H_2O$ = 0,5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0,5 e Fe_2SO_4 = 0,001)(Brunerie et al., 1987). Subsequently, 0.5% (v/v) of the monoterpenes and sesquiterpenes cited above were added to the medium. When the tetraterpenes were used as substrate, biomass (~ 1 g) was added to 100 mL screw-top sterile flasks, containing 20 mL of mineral medium and added with 1 mL of neoxanthin and β-carotene in each experiment (protected from exposure to light) (Dionísio, 2010). The flasks were incubated at 30 ^{o}C and 150 rpm. Periodically, 1 mL samples were taken in order to monitor the consumption of substrate and volatile compounds produced for 96 h (Bicas et al., 2008a). In the same way, chemical blanks of the biotransformation experiments were performed without mycelium, to ensure the absence of chemical transformation reactions (Maróstica Jr. and Pastore, 2007a).

2.5. Determination and quantification of the volatile compounds by GC-FID

For the monoterpenes, each sample was extracted (40 s in Vortex) with the same volume of ethyl acetate. After phase separation, 1 µL of the organic fraction was dried over sodium sulphate and injected in split mode (split ratio of 1:10) 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 lenght x 0.25 mm i.d. x 0.25 µm of film thickness). Helium was used as carrier gas (1.0 mL.min^{-1}) and the oven temperature was kept at 80 ^{o}C for 3 min, raised at 20 ^{o}C min^{-1} until 200 ^{o}C and held for 4 min. Temperatures of the injector and detector were kept at 250 ^{o}C. Substrates and products were quantified by a calibration curve using *n*-decane as internal standard. All experiments were performed in triplicate (adapted from Bicas et al. (2008b)).

For the tetraterpenes, a SPME device (Supelco, Bellefonte, PA, USA) containing a fused-silica fiber (10 mm in length) coated with a 65 µm layer of PDMS-DVB was used. Before analysis the fiber was preconditioned in the injection port of the chromatograph according to the manufacturer's instructions. The aroma compounds

were extracted from the screw-top sterile flasks, containing 20 mL of mineral medium and substrate. The system was left for 20 min at 30 ^{o}C with agitation to allow the equilibrium of the volatiles in the headspace. The fiber was exposed to the headspace for 30 min at 30 ^{o}C. Desorption proceeded in the injection port of the gas chromatograph at 250 ^{o}C with the purge valve off (splitless mode) and the fiber remained a further 10 min in the injector port to eliminate the possibility of any carry-over of analyte between samples. Analyses were performed as previously described.

2.6. Analysis of the samples by gas chromatography/mass spectrometry (GC–MS)

The analysis and identification of volatile compounds 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). A fused silica capillary column HP- 5MS (J&W Scientific, Folsom, California, USA) with 30 m lenght x 0.25 mm i.d. x 0.25 µm of film thickness was used to separate the volatile components. Helium was used as carrier gas at constant flow rate of 1.0 mL.min^{-1}. The programming of the gas chromatograph oven temperature was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 °C, impact energy of 70 +eV and a mass range 35-500 m/z. The identification of the compounds was made by comparing the spectra with NIST 2008 library over 90% similarity, and comparison with commercial standard.

3. Results and discussion

3.1. Biotransformation trials

The microorganism tested in this study was *Fusarium oxysporum* 152B, previously isolated as a potential extracellular alkaline lipase producer (Prazeres et al., 2006). In a series of tests, this strain demonstrated a great potential for the biotransformation of limonene into α-terpineol and stood out as the main fungal biocatalyst for this biotransformation process, as well as one of the biggest productions ever reported for the production of this monoterpene alcohol. In fact, as reported by Bicas et al. (2010a), the production of α-terpineol reached 4 g.L^{-1} after an extensive optimization process of culture conditions in a previous study (Bicas et al., 2008a). Several other reports dealt with this biotransformation route yielding α-terpineol in concentrations ranging from approximately 1 g.L^{-1} by *Penicillium* sp. (Pescheck et al.,

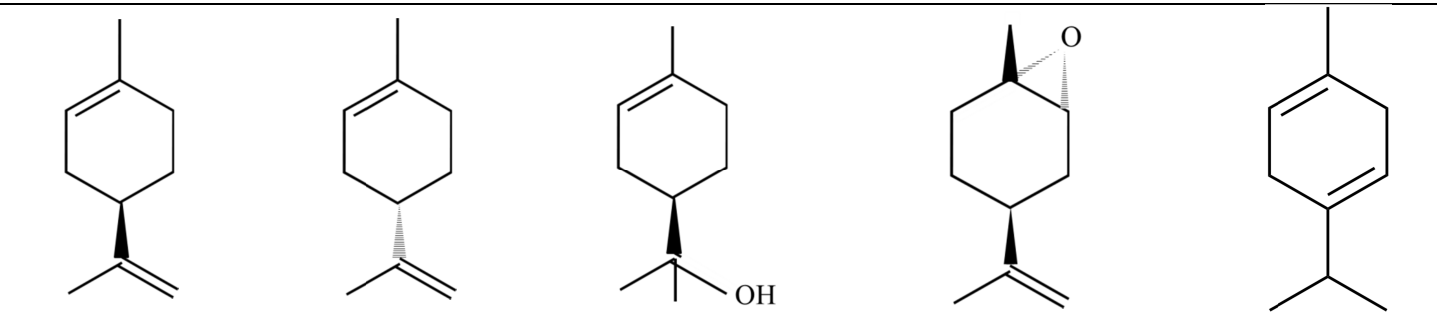
2009) to 130 $g.L^{-1}$, the highest already described, by *Sphingobium* sp. (Bicas et al., 2010b).

In this sense, aiming to extend the studies on the potential of this microorganism in biotechnological routes, this work initiated a series of trials to assess its versatility. Thus, the fungal strain was first tested for its ability to biotransform several other terpenes, including monoterpenes which structures are presented in Figure 1 (*S*-(-)-limonene, α- and β-pinenes, γ-terpinene, citronellol, linalool, geraniol, (–)-carveol, limonene-1,2-oxide, α-terpineol), sesquiterpenes (α-farnesene, α-bisabolol, valencene) and tetraterpenes (neoxanthin and β-carotene) displayed in Figure 2, in order to identify possible metabolic pathways active in the corresponding fungal strain and the production of new flavor compounds with industrial interest. To define these metabolic pathways through which the terpenes substrates were degraded and to find activities that could be exploited at a preparative scale, bioconversion experiments were carried out using fresh cells of *Fusarium oxysporum* 152B.

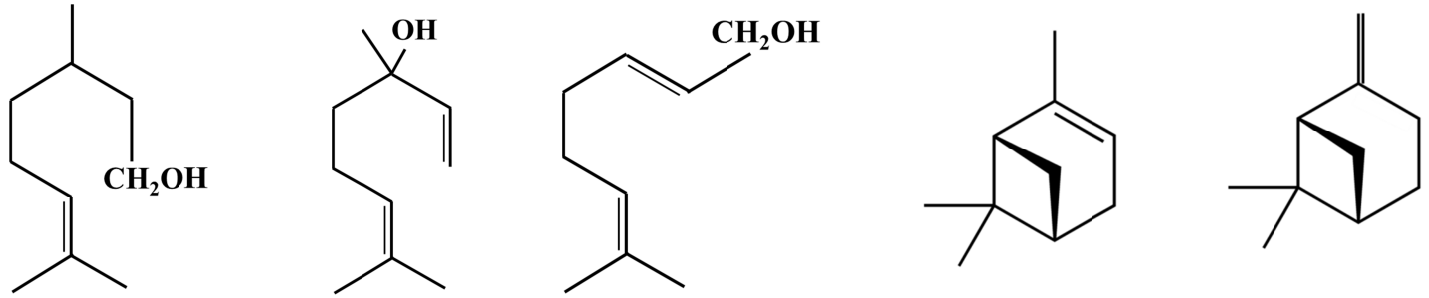
Table 1 summarizes the results observed for fungal growth using terpenic substrates as sole carbon and energy source (see Methods section 2.3), and also the results obtained through GC-FID and GC-MS analyses, describing products identified and quantified following the methodology previously described after biotransformation process. *Fusarium oxysporum* 152B was able to use several terpenes as carbon and energy source, mainly *S*-(–)-limonene and linalool producing 8.5 and 8.1 $g.L^{-1}$ of biomass after 48 h of incubation with these terpenes.

Furthermore, most of terpenes were not used as substrates accumulating new metabolites, such as β-pinene, citronellol, geraniol, (–)-carveol, α-terpineol, α-farnesene, α-bisabolol, valencene and neoxanthin and β-carotene. These terpenes have been selected by the sensory quality of the flavors derived from their biotransformation, such as the production of rose oxide from citronellol, one of the most interesting monoterpene ethers for the flavor and fragrance industries, performed by *Aspergillus niger* (Demyttenaere et al., 2004) and *Penicillium* sp. (Maróstica Jr. and Pastore, 2006), but the reported yields were low for this aroma compound.

Monoterpenes



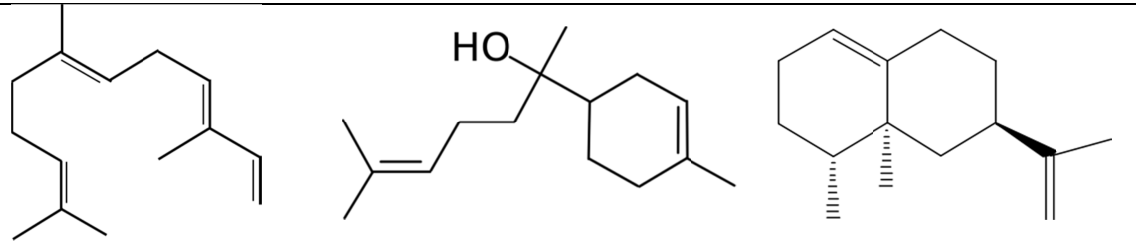
R-(+)-limonene | *S*-(–)-limonene | α-Terpineol | Limonene-1,2-oxide | γ-Terpinene



Citronellol | Linalool | Geraniol | α-Pinene | β-Pinene

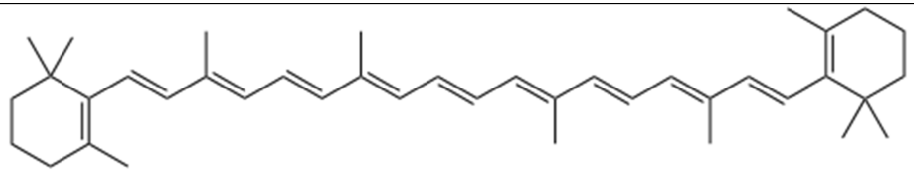
Figure 1 – Monoterpenes used as substrate for bioconversion process by *Fusarium oxysporum* 152B.

Sesquiterpenes

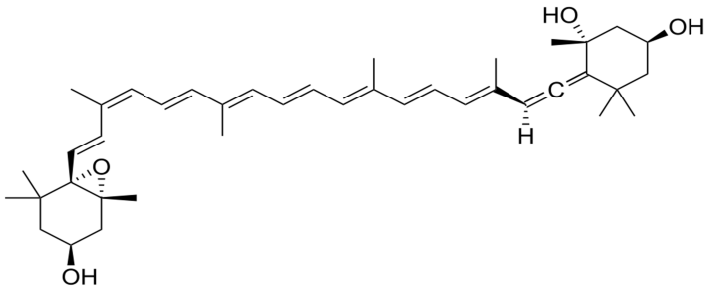


Farnesene Bisabolol Valencene

Tetraterpenes



β-Carotene



Neoxanthin

Figure 2 – Sesqui- and tetraterpenes used as substrate for bioconversion process by *Fusarium oxysporum* 152B.

Another important compound targeted in the beginning of the experiments was carvone that, due to its spearmint-like odor, has several applications as a fragrance and flavor compound (De Carvalho and Da Fonseca, 2006). The biotransformation of limonene into carvone has already been described for *Rhodococcus opacus* (De Carvalho and Da Fonseca, 2003). However, considering that previous studies with *Fusarium oxysporum* 152B reported the biotransformation of limonene into α-terpineol, this study aimed to change the substrate used focusing on the same metabolic pathway. Thus, carveol was tested as substrate but unfortunately it was not metabolized by the microorganism. Carveol has also been used as a substrate using the bacteria *Rhodococcus opacus* PWD4 (Duetz et al., 2001) or *R. erythropolis* (De Carvalho et al., 2005) as biocatalysts and concentrations up to 150 $g.L^{-1}$ of carvone have been achieved in small scale column reactors.

Considering the high production of α-terpineol from the biotransformation of limonene, as previously discussed and reported by the authors Maróstica and Pastore (2007a) and Bicas et al. (2008; 2010), this study aimed to provide α-terpineol as the substrate in the biotransformation process, to evaluate in more detail the ability of the microorganism to metabolize α-terpineol in another derivative compound, as never reported before in the literature. The investigation was not positive, given that the α-terpineol was not metabolized to the accumulation of new derivatives.

On the other hand, surprisingly, this strain showed a positive result for various substrates, which proves a large enzymatic and metabolic potential, revealing this strain as a potent biocatalyst for the production of natural flavors as described below.

3.1.1. Biotransformation of S-(-)-limonene

Much work has been done on the biotransformation of the inexpensive hydrocarbon limonene (Demyttenaere et al., 2001), which is beside α-pinene the most widely distributed terpene in nature. While *R*-(+)-limonene is present in citrus peel oils at a concentration over 90%, the *S*-(–)-isomer is found in oils from the *Mentha* species and conifers at low concentrations (Bauer et al., 2001). The microbial conversion of limonene is well documented and the first data date back to the sixties mainly using *Pseudomonas* strains (Molina et al. 2013a) and, since then, many advances have been achieved in this area, particularly using the *R*-(+)-isomer as substrate, due to its abundance and relatively low cost (Bicas et al., 2009).

Maróstica-Jr and Pastore (2007b) and Bicas et al. (2008b) have reviewed the main metabolic routes for limonene in six different degradation pathways, which are: (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 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.

Considering the recent advances with *Fusarium oxysporum* 152B for the biotransformation of *R*-(+)-limonene that was well elucidated for the production of α-terpineol, as already discussed, thus the objective was to compare the use of the *S*-(–)-isomer as substrate. Previous reports dealt with the biotransformation of *R*-(+)-limonene and *S*-(-)-limonene to *R*-(+)- and *S*-(–)-α-terpineol, respectively, by *Penicillium digitatum* (Demyttenaere et al. 2001; Adams et al. 2003). Authors observed that *R*-(+)-limonene was converted much better into the product with a high enantioselectivity, obtaining *R*-(+)-α-terpineol (ee > 99%) (Adams et al., 2003). Similarly, assays conducted by Bicas et al. (2010b) with the isomers *R*-(+)- and *S*-(–)-limonene showed that both enantiomers were converted into *R*-(+)- and *S*-(–)-α- terpineol, respectively. However, authors found out that the activity obtained for the *R*-(+)-isomer was more than 10 times higher, similar to that observed by Braddock and Cadwallader (1995) for the bacterium *Pseudomonas gladioli*.

R. erythropolis DCL14 was also able to degrade *R*-(+)-limonene initiated by a double bond epoxidation, forming (1S,2S,4R)-limonene-1,2-diol, (1S,4R)-1-hydroxy-2-oxolimonene and (3R)-3-isopropenyl-6-oxoheptanoate. The opposite enantiomers ((1R,2R,4S)-limonene-1,2-diol, (1R,4S)-limonene-1-ol-2-one, and (3S)-3-isopropenyl-6-oxoheptanoate) accumulated when *S*-(–)-limonene was employed as substrate, showing that the enzymes from this pathway are not stereoselective (Van der Werf et al., 1999).

Interestingly, the results for the biotransformation of *S*-(–)-limonene have not converged for the production of α-terpineol as expected, but resulted into (1R,2R,4S)-limonene-1,2-diol as major product accumulated after 30 h (Figure 3). The biotransformation of *S*-(–)-limonene was carried out over a period of 7 days. The first feeding of substrate was performed after 96 h, with *S*-(-)-limonene reaching concentrations of approximately 4 $g.L^{-1}$ after re-injection, and the maximum yield of limonene-1,2-diol observed was 56%, similar to *Corynespora casiicola* that was also

able to produce (1R,2R,4S)-limonene-1,2-diol with a final yield ranging from 46 to 60.6% (Demyttenaere et al., 2001).

The optimal concentration of limonene-1,2-diol found was 1.2 $g.L^{-1}$ obtained after 96 h of process, with 150 rpm and 30 ^{o}C. Figure 3 illustrates the bioconversion process, representing consumption of substrate and product formation analyzed by GC-FID and GC-MS. Also, the bioconversion of *R*-(+)- and *S*-(–)-limonene by *Fusarium oxysporum* 152B is given schematically in Figure 4. Considering the route showed in this figure, it is important to mention that the intermediate limonene-1,2-epoxide was not detected in biotransformation GC-FID and GC-MS analyses, indicating that the reaction proceeds mainly for the diol production. The observations published by Van der Werf et al. (1999) corroborates these findings, considering that in their experiment with *Rhodococcus erythropolis* DCL14 no accumulation of limonene-1,2-epoxide was observed, and attempts were made to obtain this epoxide by separating the limonene-1,2-epoxide hydrolase activity from the limonene 1,2-mono-oxygenase activity by gel filtration or anion-exchange chromatography were unsuccessful due to the instability of limonene 1,2-monooxygenase and the fact that the limonene-1,2-epoxide hydrolase activity in cell extracts was 50-fold greater than the limonene 1,2-monooxygenase activity (Van der Werf et al., 1999).

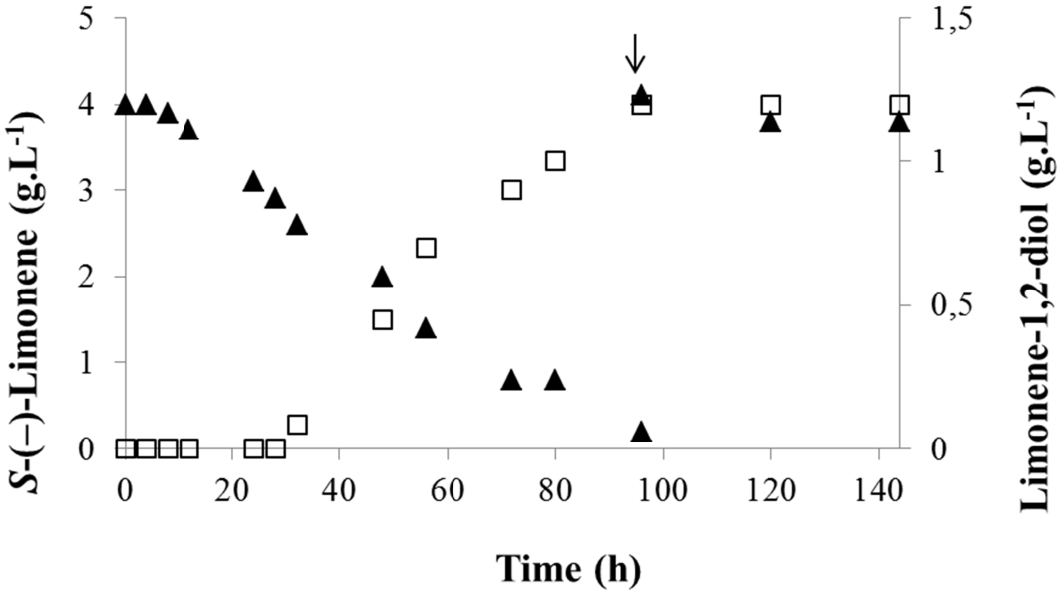


Figure 3 – Biotransformation of S-(–)-limonene (▲) into limonene-1,2-diol (□) in shaker (30 ^{o}C/150 rpm) by fresh biomass of *F. oxysporum* 152B. The arrow indicates the addition of limonene during the biotransformation process.

The production of the diol occurs with the attack of the double bond of limonene in 1 and 2 position and it is suggested that the *trans*-limonene-1,2-diol is formed by hydrolysis of the initially formed epoxide (Figure 4). In fact, some authors observed that diols are common intermediates in the monoterpene metabolism of fungi (Bicas et al. 2009; Mukherjee et al., 1973). This pathway was first found by *Cladosporium* sp. T7 yielding 1.5 $g.L^{-1}$ of *trans*-limonene-1,2-diol and small amounts of the cis-diol (0.2 g/L) in a 4-day fermentation (Mukherjee et al. 1973). Another study reported an extensive screening of 800 strains that yielded three other fungi, *Diplodia gossypin* ATCC 10936, and two *Corynespora cassiicola* strains (DSM 62474 and DSM 62475), with the same capability (Kieslich et al., 1986). Another important screening of more than 60 fungal strains reported by Demyttenaere et al. (2001) also yielded the formation of diols from limonene by *Corynespora cassiicola* strains as one of the major results. In addition, *Aspergillus sp., i.e. A. cellulosae*, was capable of converting both enantiomers of limonene into limonene-1,2-diol as the main product (Noma et al., 1992). The first distinguished process dealing with a larger-scale production of the diol has been reported by Abraham et al. (1985). Authors achieved 900 g of the (1S,2S,4R)-limonene-1,2-diol from *R*-(+)-limonene in a 100 L bioreactor filled with 70 L of culture medium with continuous substrate feeding (1,300 g) within 96 h of process.

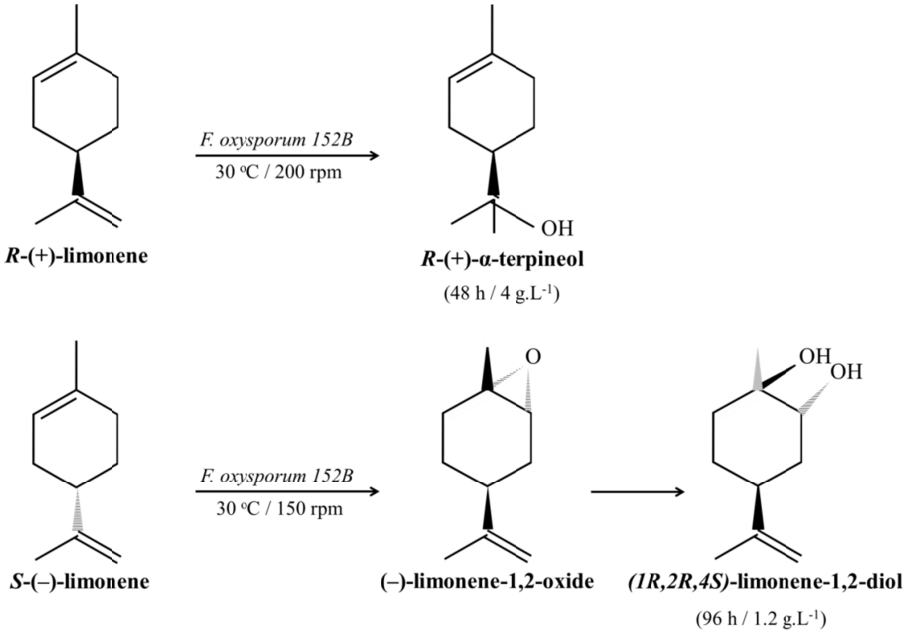


Figure 4 – Scheme of the bioconversion products of *R*-(+)- and *S*-(–)-limonene by *Fusarium oxysporum* 152B.

There are not many descriptions of the industrial potential of this compound, such as its unknown aroma threshold value. However, this product has a cool minty aroma and it is consumed mainly in flavors used in mint preparation, alcoholic and nonalcoholic beverages, chewing gun, gelatins/puddings and other food products (Burdock and Fenaroli, 2010).

Finally, the data presented show great promise for obtaining limonene-1,2-diol through biotechnological means, compound that merits further attention and studies to elucidate its potential and the variables involved in its production, as well as the optimization of these parameters. The reported concentration of 1.2 $g.L^{-1}$ in non-optimized conditions is one of the highest already published and encourage the studies of the production of this natural flavor obtained by *Fusarium oxysporum* 152B. Thus, this might be a promising strategy for obtaining this compound in natural forms.

3.1.2. Biotransformation of limonene-1,2-oxide

Van der Werf et al. (1999) confirmed the route of degradation of limonene to its corresponding diol using biochemical trials with *Rhodococcus erythropolis* strain subjected to limonene as the sole carbon source. It was observed that the strain begins its attack by ring 1,2 double bon epoxidation (through limonene-1,2-monooxygenase), and the hydrolysis of limonene-1,2-epoxide into limonene-1,2-diol is catalyzed by a limonene-1,2-epoxide hydrolase very active and inducible.

Considering the metabolic pathway for obtaining limonene-1,2-diol shown in Figure 4 has limonene-1,2-epoxide as intermediate, it was evaluated the capability of the fungal strain to consume this compound as substrate and allowing to confirm the metabolic pathway observed. The results were positive, and in a few hours of biotransformation, the epoxide substrate was converted into the corresponding diol, confirming the expectations. Bicas et al. (2008b) observed similar results with *P. fluorescens*, where the first enzyme of this metabolic route, limonene-1,2-epoxide hydrolase was cofactor independent.

The accumulation of limonene-1,2-diol during the limonene-1,2-oxide bioconversion led to suggest that *Fusarium oxysporum* 152B might have another pathway for limonene, similar to that described by Van der Werf et al. (1999).

3.1.3. Biotransformation of α-pinene

In the past few years much work has been done on the biotransformation of α-pinene, one of the most widely distributed terpene in nature (Bicas et al. 2009). This compound has a characteristic odor of pine and turpentine-like flavor, with aroma threshold of 2.5 to 62 ppb and mainly used in chewing gum, hard candy, baked good and others (Burdock and Fenaroli, 2010).

Considering its potential, the biotransformation of α-pinene was investigated and this monoterpene was converted into myrtenol by *Fusarium oxysporum* 152B, reaching 45 $mg.L^{-1}$. This product has a camphoraceous, minty, medicinal, woody odor and a good market value to nonalcoholic beverages, frozen dairy, gelatins, puddings and others, which encourages its biotechnological production. Further studies should be done with this biocatalyst and the optimization of the biotransformation of α-pinene to myrtenol is under way. Figure 5 exemplifies the compound obtained in this study.

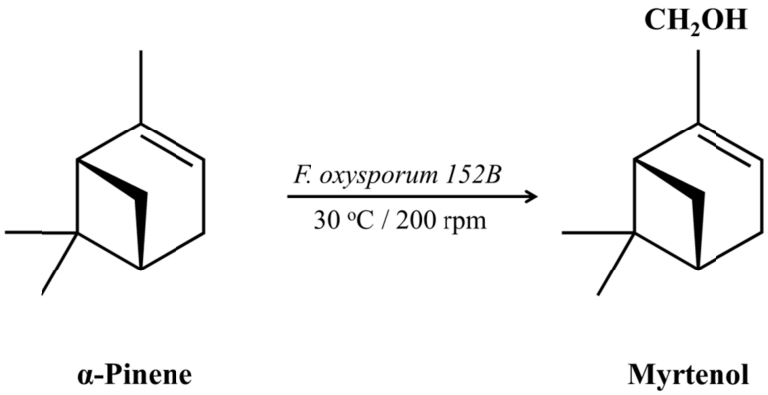


Figure 5 - Scheme of the bioconversion products of α-pinene into myrtenol by *Fusarium oxysporum* 152B.

The production of myrtenol from the biotransformation of α-pinene was previously described in the literature, although many studies found as a minor compound or mixture of metabolites generated. Lindmark-Henriksson (2003) performed the biotransformation of this substrate by culturing cells of *Picea abies*. While the main products were *trans*-verbenol and verbenone, myrtenol was only found as minority product, along with pinocarveol and α-terpineol. Thus, the biotransformation of α-pinene by the cell suspension of *P. abies* was characterized mainly by oxidation in the allylic position. Other reactions were observed oxidative cleavage of the cyclobutane

ring and oxidation of the terminal carbons producing monoterpenic monocyclic alcohols (Lindmark-Henriksson 2003).

Busmann and Berger (1994) reported the biotransformation of β-pinene in basidiomycetes. The products obtained were verbenol, verbenone, myrtenol and *trans*-pinocarveol. Additionaly, Savithry et al. (1998) conducted some trials with *Bacillus pallidus* BR425 and observed that this strain was capable of degrade α- and β-pinene, as well as limonene. In the first case, significant amounts of pinocarveol, pinocarvone, carveol, carvone, and lesser amounts of myrtenol and myrtenal were recovered.

3.1.4. Biotransformation of linalool

The optically active forms of linalool (*d*- and *l*-) are present in more than 200 oils from herbs, leaves, flowers and wood, while the *l*-form is present in the largest amounts (80 to 85%) in the distillates from leaves of *Cinnamomum camphora* var. *orientalis* and others. The presence of this monoterpene is also reported found in over 280 products including apple, citrus peel oils and juices, berries, grapes, guava, celery, peas, potato, tomato, cinnamon, passion fruit, olive, mang and so many others. Linalool has a typical pleasant floral odor, free from camphoraceous and terpenic notes, its annual consumption is approximately 21,500 lb with aroma threshold values of 4 to 10 ppb (Surburg and Panten, 2006).

When this compound was tested as substrate in biotransformation trials, it was observed that *Fusarium oxysporum* 152B was able to use this compound as the sole carbon source (Table 1) and further accumulate the product known as linalool oxide during its metabolism, in small concentrations. This product was quantified based on the substrate concentration curve by GC-FID, reaching 18 $mg.L^{-1}$ (relative to linalool).

Linalool oxide has natural occurrence in several fruits and has a powerful, sweet, woody, penetrating odor with floral, woody-earthy undertones found in many fruits, coffee, hop, lavender and other natural resources (Surburg and Panten, 2006). It is reported that the natural compound is suggested for use in tropical, vegetable, rum and wine flavor applications, with a use level of 20 to 50 ppm, an aroma threshold values of 320 ppb and taste characteristics at 50 ppm with green, floral, fatty, woody, fermented, herbal, fruity and berry (Burdock and Fenaroli, 2010). Considering the versatility of applications, it is important to increase the studies of bioconversion of linalool into this metabolite.

Table 1 – Terpenic substrates used for growth of *Fusarium oxysporum* 152B, biotransformation products and concentrations obtained.

Terpene substrate	Growth[a]	Biomass[b] ($g.L^{-1}$)	Biotransformation metabolite[c]	Product concentration[d] ($g.L^{-1}$)
Monoterpenes				
S-(–)-Limonene	+	8.5	Limonene-1,2-diol	1.2
α-Pinene	+	6.9	Myrtenol	0.045
β-Pinene	+	7.3	-	-
γ-Terpinene	+	5.8	*trans*-2-caren-4-ol, unidentified compound	0.145 0.137
Citronellol	-	-	-	-
Linalool	+	8.1	Linalool oxide	0.018
Geraniol	-	-	-	-
α-Terpineol	-	-	-	-
Carveol	-	-	-	-
Limonene-1,2-oxide	-	-	Limonene-1,2-diol	0.58
Sesquiterpenes				
α-Bisabolol	+	4.4	-	-
α-Farnesene	+	6.1	-	-
Valencene	-	-	-	-
Tetraterpenes				
β-carotene	-	-	-	-
Neoxanthin	-	-	-	-

[a] growth after 48 h under practical conditions described in Methods, section 2.3

[b] biomass produced during the process was separated from the culture medium and dried at 105°C for 24 h

[c,d] identification and quantification of products were performed using a GC-FID and GC-MS system (Method section 2.5 and 2.6)

The highest productivity of linalool oxide was reported for *Corynespora cassiicola* (Mirata et al., 2008). In this study, authors investigated the biotransformation of (±)-linalool by screening 19 fungi. Linalool oxides and 8-hydroxylinalool were the major products of fungal (±)-linalool biotransformations identified using SPME-GC-MS headspace analysis, by *Aspergillus niger* DSM 821, *Botrytis cinerea* 5901/02, and *B. cinerea* 02/FBII/2.1. With a conversion yield close to 100% and a productivity of 120 mg/L.day linalool oxides, *Corynespora cassiicola* DSM 62485 was identified as a novel highly stereoselective linalool transforming biocatalyst.

Other reports show the conversion of linalool to furanoid and pyranoid linalool oxides mediated by *Aspergillus niger* (Demyttenaere and Willemen, 1998) and into linalool oxide, ocimenol, geraniol, and also α-terpineol by using several strains during a fungal screening (Molina et al., 2013b).

In fact, linalool was the substrate in extended screenings that identified fungi producing linalool oxides, 8-hydroxylinalool and lilac aldehyde plus alcohol (Mirata et al., 2008), mainly because mono-alcohols, such as linalool, are less of a problem because of their lower solubility in the membrane of the cells and in some experiments resting cells were often used to overcome the cytotoxicity of the hydrocarbon substrates. Some pathways identified were inducible, but a general mechanistic understanding of these detoxification reactions is missing (Berger, 2009) and need some efforts to a greater understanding of the routes of metabolism of the monoterpene and others.

3.1.5. Biotransformation of γ-terpinene

γ-Terpinene has an herbaceous and citrus-like flavor; woody, terpene, tropical lemon odor with an annual consumption of 6550.00 lb. This compound is reported in papaya fruit, tea tree oil, tangerine, citrus, mandarins, papaya fruit, citrus peel oils, and others, and although its aroma threshold value is not recognized, its taste thresgold characteristics at 40 ppm, and can be applied mainly in chewing gum, candies, beverages and meat products (Burdock and Fenaroli, 2010).

Despite its great potential as a flavor compound, the routes of metabolism of this compound when used as a substrate is not thorough, and few works have studied its microbial bioconversion. Krings et al. (2005) used a wild strain of *Stemphylium botryosum* growing in the presence of γ-terpinene. The biotransformation showed the accumulation of a novel highly odour active terpene alcohol, whose structure was established by spectroscopic means as *p*-mentha-1,4-dien-9-ol (ee < 74%). This compound was further oxidized to the corresponding aromatic alcohol *p*-cymene-9-ol (ee < 70%).

Apart from the microbial transformation, the use of unconventional biocatalysts was also reported, suggesting that the larvae of the cutworm *Spodoptera litura* possesses a high level of enzymatic activity against terpenoids, some authors have tested their biotransformation potential for α-terpinene (Miyazawa et al., 1996). Additionaly, photooxidations via singlet oxygen employing dyes as photosensitizers have increasingly attracted the interest of organic chemists for industrial scale

production of flavor compounds. This green chemical approach is one attractive alternative to the traditional chemical synthesis, since it is a clean, traceless, and sustainable technology, although the high energy demand of most artificial sources is one challenge that must be overcome.It has been employed in the production of ascaridole from α-terpinene (Wootton et al., 2002).

When γ-terpinene was used in submerged culture with *Fusarium oxysporum* 152B it was metabolized into *trans*-2-caren-4-ol. Despite this possibility, the results of similarity with the NIST library were low (73 %) and the lack of description of possible metabolic pathway derived from oxidation of this substrate made it difficult to confirm the results. It was attempted to also identify this compound according by comparison of Kovats Index with the literature. In addition to the possible biotechnological production of *trans*-2-caren-4-ol, another compound remained unidentified, as shown in Figure 6. No description of its potential has been found in the bibliography used, although its presence is reported in some essential oils (Marzoug et al., 2011; Elhassan et al., 2010; Milos et al., 1998).

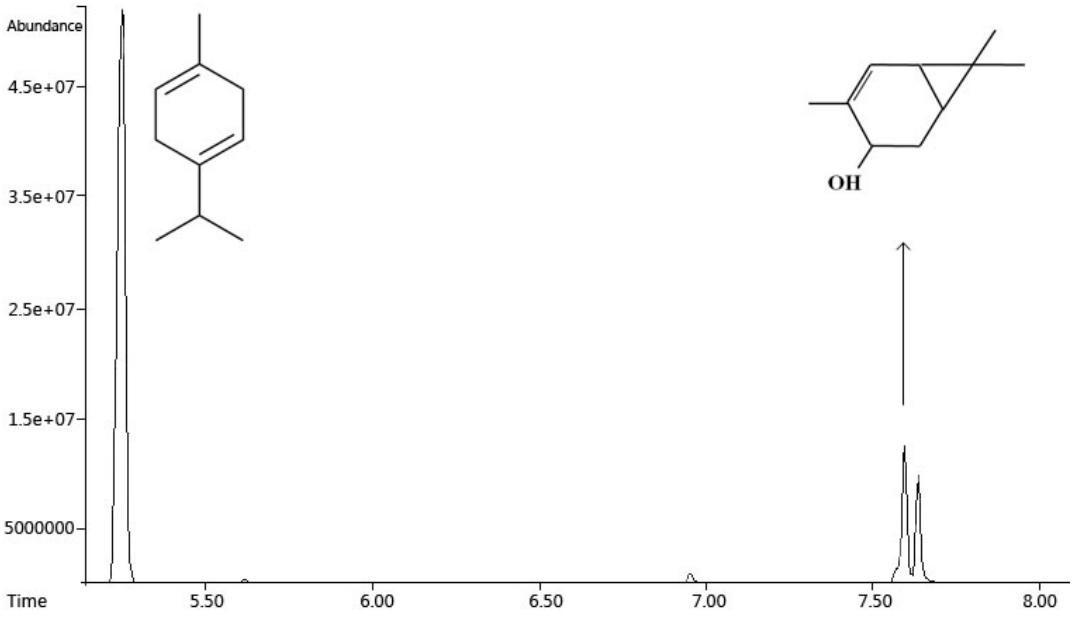


Figure 6 – Chromatogram of the *trans*-2-caren-4-ol and an unidentified compound from the biotransformation of γ-terpinene by *Fusarium oxysporum* 152B analyzed by GC-MS.

Therefore, further studies should be performed to identify this, as well as using more advanced analytical techniques and also for nuclear magnetic resonance confirmation of these derivatives. Results obtained are presented in Table 1, with the main products accumulated after 72 h of bioconversion and their respective approximate yields.

Terpinene family exhibit further potential, such as α-terpinene, that exhibited antifungal activity similar to that of commonly used antifungal drugs (Parveen et al., 2004). In general, antifungal therapy utilizing terpenes and their derivatives is very promising but may be difficult to apply because high doses of terpenes or terpenoids have to be used. For that reason, such therapy might result in serious side effects. These substances may nevertheless serve as supplementary agents that could improve standard, conventional antifungal therapies (Paduch et al., 2007).

Also, considering this potential to their flavor technology, the product obtained in this study should be evaluated to test its biological effect being a field to be explored due to the high potential of the substrates α- and γ-terpinenes.

4. Conclusion

Results achieved in this study demonstrated that *Fusarium oxysporum* 152B could be considered as a specialist for the degradation of several terpenes, especially the biotransformation of *R*-(+)-limonene well elucidated yielding α-terpineol and the innovative finding of this work that showed the production of limonene-1,2-diol from *S*-(-)-limonene. Besides that, this strain also metabolized limonene-1,2-oxide, α-pinene, linalool and γ-terpinene, producing metabolites of interest. Thus, this strain might be considered a high versatile microorganism and further studies are need to elucidate new metabolic pathways and also the optimization of bioconversion process.

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Capítulo 3

Characterization of *S*-(–)-limonene bioconversion into limonene-1,2-diol by *Fusarium oxysporum* 152B

ABSTRACT

In the past few years much work has been done on the biotransformation of limonene, an inexpensive hydrocarbon monoterpene, which is one of the most widely distributed terpene in nature. Recently, the strain *Fusarium oxysporum* 152B has been selected based on its high production of extracellular alkaline lipase and other publications have also described the biotransformation of *R*-(+)-limonene into *R*-(+)-α-terpineol catalyzed by the same strain. The main characteristics of this conversion were described in recent papers but no information deals with the bioconversion of its isomer, *S*-(–)-limonene. Thus, this paper focused on the biotransformation of *S*-(–)-limonene by *F. oxysporum* 152B with an extensive characterization of the biotransformation process for the production of limonene-1,2-diol, including the influence of inoculum age, biocatalyst induction, presence of co-solvent in biotransformation media and sequential feeding of substrate. These results encourage further studies with this biocatalyst and the characterization of its enzyme system involved in the biotransformation of *S*-(–)-limonene and other terpenes is of great importance, and could be source of novel aroma compounds with industrial interest.

Keywords: *S*-(–)-limonene, limonene-1,2-diol, *Fusarium oxysporum*, monoterpenes, terpenoids, aroma compounds, flavor compounds.

1. Introduction

Extensive research has been devoted to the biotechnological production of flavours and fragrances (Berger, 1995). Via biotransformation or bioconversion ‘‘natural’’ flavors can be produced, applying to the consumers’ demand for natural products (Adams et al., 2003). Terpenes and especially their oxygenated derivatives are extensively used in the flavour industry. Via biotransformation, monoterpene precursors are converted into their more valuable oxygenated derivatives (Van der Werf et al., 1997). The biotransformation of terpenes through microorganisms is recognized as being of great economic potential because can allow the production of enantiomerically pure flavors and fragrances under mild conditions and the products may be considered as “natural”, increasing the interest in this area (De Carvalho and Da Fonseca, 2006). But, in the other hand, this complex process is technically difficult because of monoterpenes chemical instability, low solubility, toxicity and volatily and also because can occur multiple metabolic pathways leading to a mixture of products; low product concentrations and yields or the absence of product accumulation (Van der Werf et al., 1997).

In the past few years much work has been done on the biotransformation of limonene, an inexpensive hydrocarbon monoterpene, which is one of the most widely distributed terpene in nature (Duetz et al., 2003). As its chemical structure is similar to that of many oxygenated monoterpenoids presenting a pleasant fragrance, e.g. perillyl alcohol, carveol, carvone, menthol and α-terpineol, it may be used as a precursor in the synthesis of these flavor compounds (Bicas et al., 2009).

Many papers describe enzyme production by species of the genus *Fusarium*, e.g. *F. graminearum, F. solani* and *F. oxysporum* (Bicas et al., 2010). Recently, the strain *F. oxysporum* 152B has been selected based on its high production of extracellular alkaline lipase (Prazeres et al., 2006). Simultaneously, other publications have also described the biotransformation of *R*-(+)-limonene into *R*-(+)-α-terpineol catalyzed by the same strain (Bicas et al., 2008a; Maróstica Jr and Pastore, 2007). The main characteristics of this conversion were described in recent papers (Bicas et al. 2008a) but no information deals with the bioconversion of its isomer, *S*-(–)-limonene.

In this paper, the bioconversion of *S*-(–)-limonene into limonene-1,2-diol by *F. oxysporum* 152B was partially characterized using different techniques, in order to enhance the production of this product.

2. Material and methods

2.1. Microorganism and chemicals

The microbial strain employed in this study was isolated from the northeast Brazilian fruits, and it was identified as *Fusarium oxysporum* 152B (Prazeres et al., 2006). The chemical standard used as substrate in this study was *S*-(–)-limonene (96% purity, Sigma-Aldrich), and the reagents ethanol, methanol, hydrogen peroxide, glycerol and Tween 80, used as co-solvents, ethyl acetate and *n*-decane, used as solvent and internal standard, respectively, were of best commercial grade and kept under refrigeration (4 ^{o}C) until use.

2.2. Inoculum for the biotransformation trials

A piece of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 50 mL of yeast and malt (YM) medium (in $g.L^{-1}$: glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3, pH ~ 6.7). The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After incubation at 30 ^{o}C and 150 rpm for 72 h, the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman n^{o}1 (Bicas et al., 2008a).

2.3. Biotransformation procedure

The biomass obtained in item 2.2 (~ 3 g) was distributed amongst 250 mL conical flask filled with 50 mL of mineral (MM) medium (in $g.L^{-1}$: $MgSO_4.7H_2O$ = 0,5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0,5 e Fe_2SO_4 = 0,001) (Brunerie et al., 1987). Subsequently, 4 $g.L^{-1}$ of *S*-(-)-limonene was added to the medium. The flasks were incubated at 30 ^{o}C and 150 rpm and this experiment was used as control for all experiment described in the sequence. Periodically, 1 mL samples were taken in order to monitor the consumption of substrate and volatile compounds produced for 300 h (adapted from Bicas et al., 2008a). In the same way, chemical blanks of the biotransformation experiments were performed without mycelium, to ensure the absence of chemical transformation reactions (Maróstica Jr. and Pastore, 2007).

The biotransformation process of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B was characterized following the methodologies described below. All the experiments were performed at least in triplicate.

2.4. Influence of inoculum age experiments

The influence of inoculum age on the production of limonene-1,2-diol was evaluated by *Fusarium oxysporum* 152B. Initially, it was plotted the fungal growth curve for a greater knowledge of the stages and behavior of the biocatalyst during its biomass development in synthetic medium using glucose as carbon source. For that, a piece of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 50 mL of YM medium. The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. The flasks were incubated at 30 ^oC and 150 rpm, and samples were taken every 4 h to plot the growth kinetic of *Fusarium oxysporum* 152B. Biomass produced during the process was filtered by vacuum filtration and dried at 105^oC for 24 h.

Subsequently, five different inoculum ages (24, 48, 72, 96 and 120 h) were selected to evaluate their influence on biotransformation process. The same protocol above was repeated (triplicate for each time interval) and after the disruption of the solid matter, the flasks were incubated at the same conditions described above. After each time interval (24, 48, 72, 96 and 120 h), the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman n^{o}1 (Bicas et al., 2008a) and re-suspended in mineral medium initiating the biotransformation process with the addition of 4 $g.L^{-1}$ of *S*-(–)-limonene.

2.5. Influence of biocatalyst induction experiments

To investigate the effect of induction of *Fusarium oxysporum* 152B cells in its growth and production of bioconversion compounds, this work developed a three phase system consisting of: i) biomass production in YM; ii) adjusting biomass to the substrate and induction of fungal strain and iii) biotransformation process. For the first phase, the fungal strain was developed in YM liquid medium as described in item 2.2. After 48 hours, the second phase was initiated with the decreasing concentration of glucose and addition of 1 $g.L^{-1}$ of *S*-(–)-limonene was added as a carbon and energy source to induce the fungal strain. After 24 hours incubated at 30 ^oC and 150 rpm, biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman ^{o}1 (Bicas et al., 2008a) and re-suspended in mineral medium initiating the biotransformation process with the addition of *S*-(–)-limonene (4 $g.L^{-1}$). Glucose was

quantified by constructing a calibration curve using the method described by Miller (1959).

2.6. Influence of co-solvent on the bioconversion process

The influence of the presence of several co-solvents on the bioconversion process of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B was evaluated. Initially, a pre-test was proposed to evaluate the effect of the co-solvent tests during *Fusarium oxysporum* growth. For that, inoculum production was performed as described in Section 2.2, with the addition of the co-solvents at the same concentrations tested in the co-solvent experiments. Influence of co-solvent on the bioconversion process was performed using the same protocol described above and five different co-solvents were added to the biotransformation media: i) ethanol ii) methanol iii) hydrogen peroxide iv) glycerol and v) Tween 80. Three different solvent concentrations were tested: i) low level (1%, $v.v^{-1}$), medium level (3%, $v.v^{-1}$) and high level (5%, $v.v^{-1}$), while for Tween 80 only 0.1 and 0.5 % ($v.v^{-1}$). For each culture, the same amount of substrate was added and two additions of 250 mL of a 20% ($v.v^{-1}$) limonene/solvent solution took place (adapted from Adams et al., 2003). The flasks were incubated at 30 ^{o}C/150 rpm and samples were extracted for GC-FID analysis at 0, 24, 48, 72 and 96 h, and results were compared with the control experiment as previously described.

2.7. Influence of sequential addition of substrate

The influence of a sequential addition of substrate was tested, adding the same amount of substrate as presented elsewhere (Item 2.3) but dividing it in two and three additions to compare the results observed when substrate was added at once (Item 2.3). In the two-fold addition, a total amount of 3 g of *S*-(–)-limonene was added after inoculation and 1 g after 30 hours of process. In case of a three-fold addition, first addition contained 2 $g.L^{-1}$ and after 30 h followed by twice of 1 $g.L^{-1}$ was administered to the cultures with a time interval of 4 h (adapted from Adams et al., 2003). Samples were taken after 24 h after the final substrate addition until 96 h for GC-FID analysis, and results were compared with the control experiment as previously described.

2.8. Determination and quantification of the volatile compounds

Samples were extracted (40 s in Vortex) with the same ethyl acetate volume. After phase separation, 1 µL of the organic fraction was dried over sodium sulphate and

injected in split mode (split ratio of 1:10) 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 lenght x 0.25 mm i.d. x 0.25 µm of film thickness). Helium was used as carrier gas (1.0 $mL.min^{-1}$) and the oven temperature was kept at 80 ^{o}C for 3 min, raised at 20 $^{o}C\ min^{-1}$ until 200 ^{o}C and held for 4 min. Temperatures of the injector and detector were kept at 250 ^{o}C. Substrates and products were quantified by a calibration curve using *n*-decane as internal standard. All experiments were performed in triplicate (adapted from Bicas et al. (2008b)).

2.9. Analysis of the samples by gas chromatography/mass spectrometry (GC–MS)

The analysis and identification of volatile compounds 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). A fused silica capillary column HP- 5MS (J&W Scientific, Folsom, California, USA) with 30 m lenght x 0.25 mm i.d. x 0.25 µm of film thickness was used to separate the volatile components. Helium was used as carrier gas at constant flow rate of 1.0 $mL.min^{-1}$. The programming of the gas chromatograph oven temperature was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 °C, impact energy of 70 +eV and a mass range 35-500 m/z. The identification of the compounds was made by comparing the spectra with NIST 2008 library over 90% similarity, and comparison with commercial standard.

2.10. Statistical analysis

An analysis of variance (ANOVA) and a Tukey test of multiple comparisons with a significance level of 5% were run using the Statistic 7.0 software for comparison of the differences between means and the results observed during bioconversion experiments using inoculum with different ages, presence of co-solvents and sequential substrate feeding. Finally, it must be said that experiments were run with control flasks.

3. Results and discussion

3.1. Kinetics of the biotransformation of *S*-(–)-limonene by *Fusarium oxysporum* 152B

Biotransformation of *S*-(–)-limonene kinetics was presented in Chapter 2, where limonene-1,2-diol concentration reached 1.2 $g.L^{-1}$ after 96 h of process and remained

stable until 144 h. In this experiment, the biotransformation process was monitored for a longer period of time, aiming to better understand the kinetics of the process and the metabolic pathway used by *Fusarium oxysporum* 152B for transformation of this substrate, and also to verify the possibility of maximizing this production.

Figure 1 presents the biotransformation of *S*-(–)-limonene into limonene-1,2-diol which showed a similar profile and behavior to that described above. Small differences are justifiable since biological systems may be complex since there is a great range of process, both from the biological and an engineering viewpoint (Montague et al., 1989).

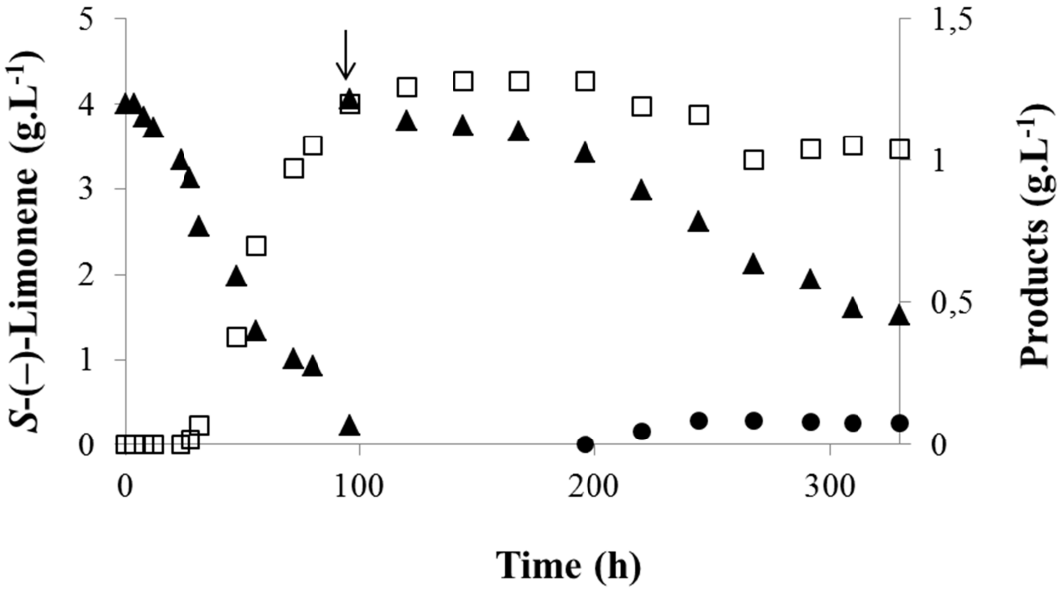

Figure 1 - Biotransformation of *S*-(–)-limonene (▲) into limonene-1,2-diol (□) and 1-hydroxy-2-oxolimonene (●) in shaker (30 ^{o}C/150 rpm) by fresh biomass of *F. oxysporum* 152B. The arrow indicates the addition of limonene during the biotransformation.

Furthermore, limonene-1,2-diol concentration remained constant not only up to 144 hours, but close to 200 hours. Following the fermentation process and evolution of time, a new product was detected, identified by GC-MS as (*1R,4S*)-1-hydroxy-2-oxolimonene (85% of similarity), which did not reach concentrations higher than 90 mg.L^{-1}. The pathway followed in this process may be seen in Figure 2, with the structural representation of the compounds obtained in this study.

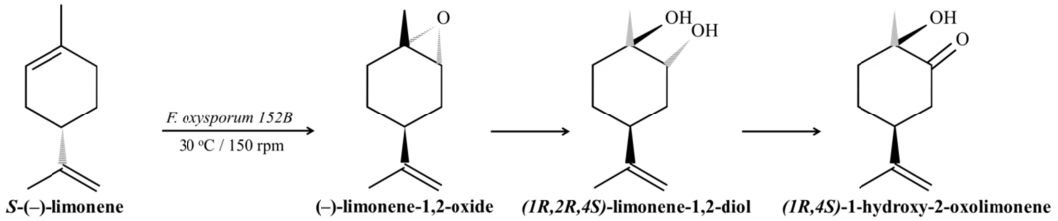

Figure 2 – Pathway for the bioconversion of *S*-(–)-limonene into limonene-1-2-diol, followed by 1-hydroxy-2-oxolimonene by *Fusarium oxysporum* 152B.

Additionally, no description about its potential as aroma compound was found in literature. This indicates that for further investigations in this process may be more important to consider only obtaining limonene-1,2-diol and not a long process to obtain the other compound 1-hydroxy-2-oxolimonene. Nevertheless, future studies should be devoted to the description of its aroma potential, description and threshold, which can make it as one more derivative product obtained from the biotransformation of limonene important for future biotechnological applications.

Finally, based on analysis of the results, the sequence observed in the metabolic pathway, where *S*-(–)-limonene was converted into limonene-1,2-diol (enzyme involved is a limonene-1,2-epoxide hydrolase) and this compound was further metabolized into 1-hydroxy-2-oxolimonene (enzyme involved is a limonene-1,2-diol dehydrogenase), led to suggest that *Fusarium oxysporum* 152B might have a recent discovered pathway for limonene, similar to that described by van der Werf et al. (1999) for *Rhodococcus erythropolis* DCL14 and Bicas et al. (2008b) for *Pseudomonas fluorescens*. These results show that this strain also metabolizes both enantiomers of limonene via epoxidation at the 1,2 double bond forming limonene-1,2-epoxide and further conversion to limonene-1,2-diol followed by 1-hydroxy-2-oxolimonene (van der Werf et al., 1999).

3.2. Influence of inoculum age experiments

The inoculum age and density markedly influence the productivity and economics of bioprocesses (Sen and Swaminathan, 2004). In this sense, this study aimed to evaluate the influence of inoculum age ranging between 24, 48, 72, 96 and 120 h during the biotransformation of *S*-(–)-limonene into limonene-1,2-diol, whereas the biomass concentration remained fixed at the value initially set.

Figure 3 presents the kinetic growth of *Fusarium oxysporum* 152B in YM medium and glucose (used as carbon and energy source) consumption. This figure was important to check at which phase of microbial growth the fungal strain stood during the selected times (24, 48, 72, 96 and 120 h) for inoculum age study. During batch culture, a typical bacterial growth curve shows five distinct phases of growth: i) lag phase; ii) exponential phase; iii) stationary phase, iv) death phase v) long-term stationary phase (which can extend for years) (Finkel, 2006; Rolfe et al., 2012), while some authors consider the normal microorganism growth curve only having four stages without the last cited one (Al-Qadiri et al., 2008).

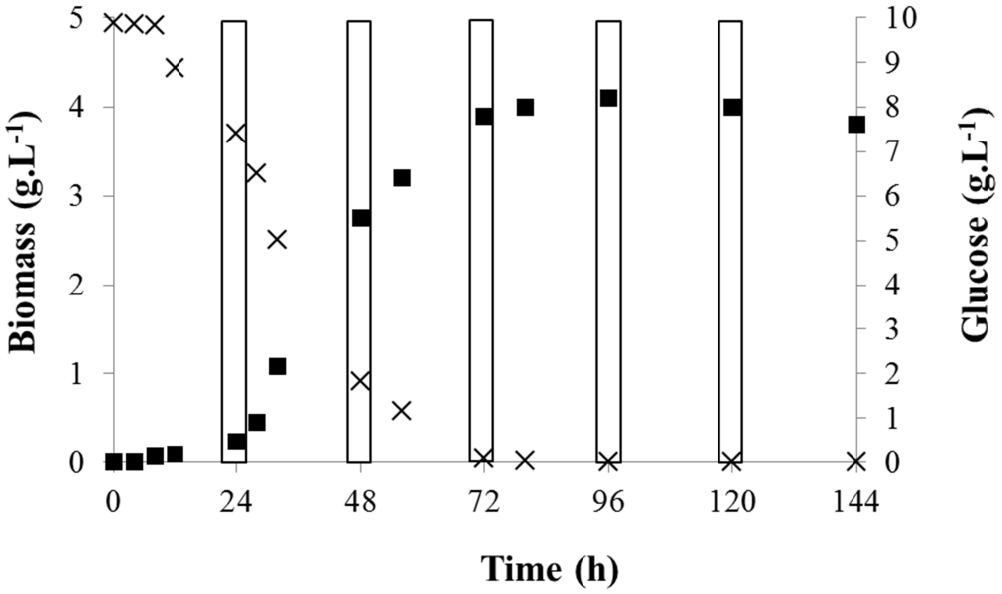

Figure 3 – Biomass growth (■) in YM medium and glucose consumption (x) in shaker (30 ^{o}C/150 rpm) by fresh biomass of *F. oxysporum* 152B. The rectangles indicate the inoculum age used in biotransformation experiments (24, 48, 72, 96 and 120 h).

The results collected after each inoculum with different ages tested in bioconversion process showed inferior results than expected in the lower and upper time values (24 and 120 h). In accordance with the highlighted rectangles in Figure 3, the inoculum used with 24 h of growth consisted mainly of cells in the end of lag phase, characterized as the delay before the start of exponential growth where is still happening the synthesis of cellular components necessary for growth (Monod, 1949). Despite the knowledge held on physiological data about this phase simply show that lag-phase microorganism are metabolically active (Rolfe et al., 2012) in this experiment the

concentration of cells was low and probably not well developed as described just above, due to the results presented in Table 1.

Table 1 – Biotransformation of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B using inoculum with different ages.

Biomass growth parameters				Bioconversion parameters		
Inoculum age (h)	**Biomass ($g.L^{-1}$)**	**$Y_{x/s}$ [1] ($g.g^{-1}$)**	**Pr_x [2] ($g.L^{-1}.h^{-1}$)**	**IPT [3] (h)**	**MPT [4] (h)**	**Lim-diol ($g.L^{-1}$) [5]**
24	0.24 ± 0.02	0.10	0.010	-	-	-
48	2.55 ± 0.03	0.54	0.053	32	96	1.15 [a]
72	3.80 ± 0.04	0.41	0.053	30	96	1.18 [a]
96	4.10 ± 0.05	0.41	0.043	38	100	0.82 [b]
120	4.00 ± 0.05	0.00	0.033	43	98	0.27 [c]

[1] $Y_{x/s}$, Yield of biomass per substrate consumed;

[2] Pr_x, Productivity of biomass per process time;

[3] *IPT*, Initial production time of limonene-1,2-diol;

[4] *MPT*, Maximum production time of limonene-1,2-diol;

[5] Lim-diol, Concentration of limonene-1,2-diol quantified by GC-FID;

[a,b,c] Different letter superscripts in the same column indicate a statistically significant difference ($p < 0.05$).

Meanwhile, inoculum ages used after 48 and 72 h of growth in YM medium can be characterized in the log phase, whereas the second case is at the limit of this phase. In this period, exponential growth can occur with a doubling time as short as 20 min as observed for *Salmonella enterica* serovar Typhimurium and requires a number of factors to be present in excess in the growth medium, including sources of carbon, nitrogen, phosphate, and certain trace elements, such as iron. The physiology of exponential bacterial growth and replication involves multiple rounds of DNA synthesis, coupled with transcription and translation, to synthesize necessary macromolecules (Faith et al., 2007).

As may be seen in Table 1, concentration of biomass obtained after 48 and 72 h was 2.5 and 3.8 $g.L^{-1}$, respectively. When these biomass were applied in bioconversion process the yield of limonene-1,2-diol was similar, reaching 1.15 and 1.18, respectively. In both times, it was also observed the best values for yield and productivity of biomass. From the economic point of view, this result indicates that the use of an inoculum of 48 h is possible, saving overall process time and maximizing the production of biomass for

the biotransformation process, considering the proximity of the obtained results although with different values of biomass.

Finally, inoculum with 96 and 120 h of growth in synthetic medium provided cells in stationary phase for the bioconversion process, characterized by the inhibition of microorganism growth since the essential nutrients required for bacterial growth are exhausted and metabolic inhibitory byproducts accumulate (Garbutt, 1997). Exponential and stationary phases have been extensively studied, representing the processes of cell division and the cessation of division, respectively (Rolfe et al., 2012). Sen and Swaminathan (2004) conducted an optimization of the age and size of the inoculum in batch reactor studies for the production of surfactin. Authors found the optimal values of inoculum age and size as 56 h and 5.5% (v/v), respectively. On the other hand, Bicas et al. (2008a) evaluated only the influence of the inoculum size on α-terpineol production, and results showed that this variable had no statistically significant influence ($p < 0.1$) on the process using inoculum with 48 h.

Finally, it is important to highlight that this study has proven the importance of using an inoculum with an adequate time of growth, which can impact on the reduction of time and cost of the processes. In future studies, optimization of biomass concentration will be also evaluated, but the results collected so far collaborate to the knowledge of the important ranges to this process.

3.3. Influence of biocatalyst induction experiments

This study developed a three-phase system aiming to investigate the influence of induction of *Fusarium oxysporum* 152B cells on the production of limonene-1,2-diol from the bioconversion of *S*-(–)-limonene. According to Figure 4, it is possible to observe: i) biomass production in YM medium with glucose consumption as carbon source (Fig. 4A); ii) adjusting cells to the substrate and induction of fungal strain with *S*-(–)-limonene concentration kept constant for 24 h at 1 $g.L^{-1}$ (Fig. 4B) and iii) bioconversion process of *S*-(–)-limonene into limonene-1,2-diol with induced cells.

Comparison of biomass productivity in the period comprised between 48 and 72 h presented in Figure 3 and Figure 4B showed a decrease from 3.69 to 3.01 $g.L^{-1}.h^{-1}$. This clearly indicates that, even in the presence of glucose, when limonene is added to the medium becomes necessary a cellular adaptation, since limonene is toxic to cells (Onken and Berger, 1999).

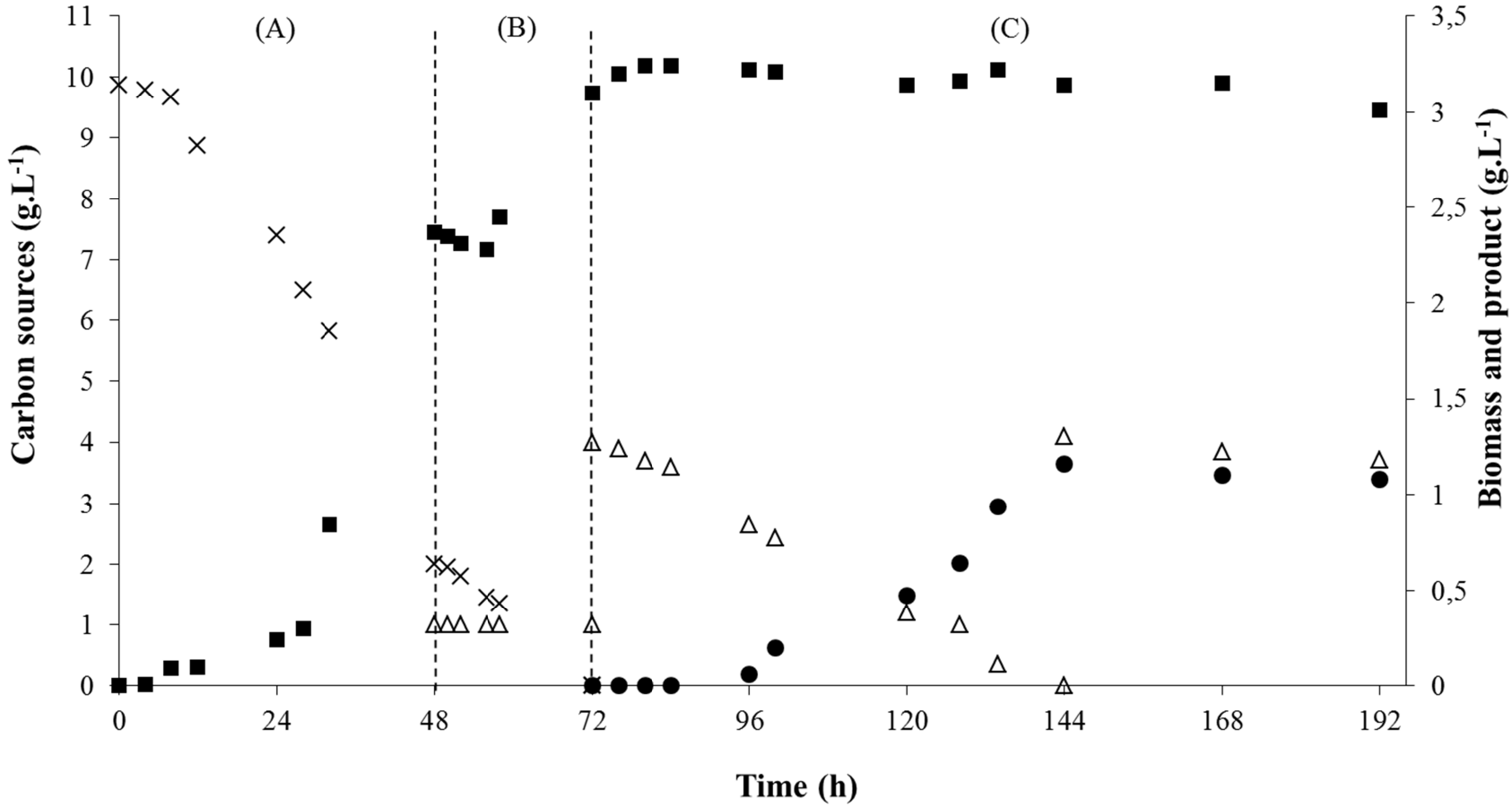

Figure 4 – Three phase system adoption: a) Biomass growth (■) in YM medium and glucose consumption (x) in shaker (30 °C/150 rpm) by fresh biomass of *F. oxysporum* 152B; b) Induction of biomass with the addition of *S*-(–)-limonene as carbon source (Δ); c) Biotransformation of *S*-(–)-limonene into limonene-1,2-diol (●) by induced biomass of *F. oxysporum* 152B.

Furthermore, it is important to analyze a considerable reduction of limonene-1,2-diol generation phase, which represents a faster adaptation of cells to the presence of limonene. The comparison of Figure 1 and Figure 4C show this difference, where the production of limonene-1,2-diol started after 30 and 24 h, without induction and with induces cell, respectively. Consequently, it was observed that optimal production was relocated for approximately 72 h, a significant reduction when compared to 96 h observed in previous experiments. Despite this apparent reduction, the final concentration of limonene-1,2-diol was slightly lower than usual process, reaching 1.15 $g.L^{-1}$. This can be explained by the possible presence of limonene remaining cells during the induction, possibly causing the increase of intracellular concentration of the substrate and affecting overall production (Molina et al., 2013).

Contrary to that observed by Onken and Berger (1999) using *Pleurotus sapidus*, in this case the adaptation proposed caused the general improvement of resistance to the toxic substrate as shown by the dry matter after substrate addition that remained constant (Figure 4), while transformation activity of the fungus remained almost the same during bioconversion. Therefore, a general adaptation mechanism of the fungus seemed to play a major role while the induction of the terpene transforming enzymes might be unaffected.

Substrate induction of cytochrome P-450 monooxygenases has been reported for several fungi transforming polycyclic aromatic hydrocarbons or steroids (Masaphy et al., 1995), for the limonene transforming fungus *Penicillium digitatum* (Tan et al., 1998a.b) and also for the basidiomycete *Pleurotus sapidus* (Onken and Berder, 1999), where authors obtained more than 100 $mg.L^{-1}$ of *cis*/*trans*-carveol and carvone when limonene was added continuously via the gas phase.

To conclude, it is important to highlight that the results obtained in this work have shown that this reaction was catalyzed by a limonene-1,2-epoxide hydrolase non inducible, as observed for the same fungal strain by Maróstica Jr and Pastore (2007), considering that α-terpineol or limonene-1,2-diol were still produced by a biocatalyst grown on glucose. However, the three-phase system adoption proved the importance for the cells adaptation prior the biotransformation process, being responsible for the reduction of process time and maximum production of the compound of interest.

3.4. Influence of co-solvent on the bioconversion process

The influence of co-solvents addition on the bioconversion process of *S*-(–)-limonene into limonene-1,2-diol was evaluated. This technique aims to bring adjustments to the process as most flavor substrates, such as limonene, are insoluble in aqueous media and/or are highly cytotoxic (Laane et al., 1987; Salter and Kell, 1995). In these cases, organic co-solvents can be applied to bioconversion systems in three different ways, in a single-phase, two-phase, or reversed micelles system (Tan and Day, 1998a,b).

In this perspective, several authors discusses that the selection of this organic solvent plays an important role on the process increasing the concentration of poorly water-soluble substrates; reducing product and/or substrate inhibition; reducing mass-transfer limitations and altering the partitioning of the substrate/product (Adams et al., 2003; Salter and Kell, 1995; Tan and Day, 1998a,b).

Moreover, this process must consider the fact that most organic solvents are also highly cytotoxic and/or inhibitory and their choice could be the greatest difficulty in using organic solvents with viable cells (Tan and Day, 1998b). Those that are non-toxic usually have restricted solvating power and consequently are of limited use. Thus, selection the solvent becomes the key determining factor for biotransformations in organic solvent systems (Salter and Kell, 1995).

Thus, the biggest concern in this general selection of the suitable solvents can be based on the log P rule (Laane et al., 1987) that indicates that biocatalyst stability decreases as log P increases (Tan and Day, 1998b). The parameter log P, or octanol-water partition coefficient, represents the level of toxicity of organic solvents to microorganisms. Substances with log P between 1 and 5 are usually considered toxic to microbial cells. In the case of monoterpenes, overall, the log P value is approximately 2.0-5.0 (e.g. limonene is 4.83) (Heipieper et al., 1994).

As proposed by Laane et al. (1987), the biocatalysis in organic solvents is low in polar solvents having a log P < 2, is moderate in solvents having a log P between 2 and 4, and is high in a polar solvent having a log P > 4. Furthermore, it was found that this correlation between polarity and activity parallels the ability of organic solvents to distort the essential water layer that stabilizes the biocatalysts.

Stability reaches a minimum for log P values between 0 to 2 for enzymes, and between 2 and 4 for microorganisms. Above these ranges increasing log P of the solvent (or for that matter substrate) results in increased biocatalyst stability; i.e. biocatalysts are

more stable in less polar solvents. The transition point from cytotoxicity to non-toxicity for solvents typically occurs between a log P of 3-5 (Vermue et al., 1993).

The substrate used in this study, *S*-(–)-limonene, is a lipophilic compound with a low solubility in aqueous media. Thus, in this study 5 different organic solvents in three different concentrations were tested for their ability to enhance the bioconversion of *S*-(–)-limonene into limonene-1,2-diol. The following strategies were applied to increase bioconversion rates: i) addition of single-phase water-organic co-solvent systems (ethanol, methanol, glycerol and hydrogen peroxide) and (2) use of reversed micelle systems using non-ionic surfactants (Tween 80) (adapted from Tan and Day, 1998b).

The results are shown in Table 2, and it is possible to observe that three co-solvents enhanced the bioconversion using *Fusarium oxysporum* 152B free cells. Ethanol (1.0 %, $v.v^{-1}$), methanol (1.0 %, v/v) and Tween 80 (0.1 %, $v.v^{-1}$) increased the bioconversion yields by 3, 3 and 8%, respectively. Despite these observations, the solvents selected for this study and concentrations tested had little significant effect on the production of limonene-1,2-diol and even positive results were not statistically different from control.

When ethanol was evaluated as co-substrate, the results were close to control yields, showing a small variation in the higher concentration (with 85% of relative activity). Despite the variation of 3% when compared with control test, this co-solvent was previously reported as a key co-solvent to increase the bioconversion yields of limonene (Adams et al., 2003).

Using higher concentration of methanol, yields declined and relative activity were 91 and 81%, with 3 and 5% of this solvent, respectively. In contrast, methanol at 0.5% had impacting effect on α-terpineol production and increased the yields 50% for bioconversion with free cells of *Penicillium digitatum* (Tan and Day, 1998b). Also, this solvent can be also used to improve the reaction rate in steroid bioconversions (Sode et al., 1989).

The addition of the non-ionic surfactant Tween 80 had an effect on production of limonene-1-2-diol. When it was added 0,1 % ($v.v^{-1}$) of this compound in bioconversion system, the relative activity observed was 108 % and yield of product per grams of biomass was 0.40 $g.g^{-1}$. Tan and Day (1998b) also observed positive results using Tween 80 during the bioconversion of limonene into α-terpineol using immobilized cells of *Penicillium digitatum*. Without Tween 80, the reaction took more than 120 h to reach the maximum yield and in its presence only 72 h. Authors also found out that

organic solvents are less cytotoxic to immobilized cells than to free cells (Tan and Day, 2008a). Additionally, Tween 80 has been used to accelerate cholesterol degradation rates by *Mycobacterium strain* DP (Smith et al., 1993).

Table 2 – Biotransformation of *S*-(–)-limonene into limonene-1,2-diol by fresh cells of *Fusarium oxysporum* 152B in the presence of co-solvents after 72 h of process.

Solvent	Log P [1]	Concentration (% v.v^{-1})	Growth [2]	Limonene-1,2-diol (g.g^{-1} dry cells)	% Bioconversion
Control	–	–	3.75 ± 0.4	0.37 ± 0.01^{a}	100
Ethanol	- 0.31	1.0	++	0.38 ± 0.01^{a}	**103**
		3.0	++	0.34 ± 0.03^{a}	92
		5.0	++	0.32 ± 0.02^{b}	85
Methanol	- 0.77	1.0	++	0.38 ± 0.01^{a}	**103**
		3.0	++	0.35 ± 0.01^{a}	95
		5.0	+	0.30 ± 0.02^{b}	81
Glycerol	- 3.01	1.0	++	0.32 ± 0.01^{b}	85
		3.0	++	0.28 ± 0.04^{b}	76
		5.0	++	0.30 ± 0.03^{b}	81
Hydrogen peroxide	- 0.45	1.0	+	0.27 ± 0.02^{c}	73
		3.0	-	0.25 ± 0.03^{c}	68
		5.0	-	0.21 ± 0.01^{c}	60
Tween 80	–	0.1	++	0,40 ± 0.01^{a}	**108**
		0.5	++	0.30 ± 0.05^{b}	81

[1] Values following Laane et al. (1987), Tan and Day (1998) and Adlercreutz (2008)

[2] ++ Lower values than control with a maximum of 10% variation (3.37 to 3.75 g.L^{-1}),
+ Lower values than control with a maximum of 25% variation (2.81 to to 3.75 g.L^{-1})
- Values with more than 25% of variation (< 2.81 g.L^{-1})

[a,b,c] Different letter superscripts in the same column indicate a statistically significant difference ($p < 0.05$).

Bicas et al. (2008a) evaluated the effect of surfactin, another surfactant, produced by a *Bacillus subtilis*, along with various other process variables in the production of α-terpineol with the same microorganism employed in this study using a Placket-Burmam screening. Results showed no statistical effect (at $p < 0.1$) on the response, for either 72 or 96 h of biotransformation of *R*-(+)-limonene.

At 0.5%, Tween 80 hindered the extraction process and concentration of limonene-1,2-diol obtained was lower than expected. Thus, from the product recovery and downstream process viewpoint, the utilization of this co-solvent is recommended at low concentrations.

De Carvalho and Da Fonseca (2004) conducted an extensive experiment to evaluate the effects of certain solvents, present in biphasic reaction systems, on the cells of two gram-positive bacterial strains (*Rhodococcus erythropolis* DCL14 and *Arthrobacter simplex*) and of two gram-positive or variable strains (*Xanthobacter* Py2 and *Mycobacterium* sp. NRRL B-3805). Authors evaluated the presence of co-solvent in morphological factors of non-viable cells, adaptation time, sampling time, morphological factors and others.

Overall, the observed negative results may be related to the fact that many organic solvents partition preferentially to the microbial cell membrane, increasing its fluidity (Heipieper et al., 1994) and impacting on its viability and ability to carry out a biotransformation process since the permeation of compounds through the membrane is altered (De Carvalho and Da Fonseca, 2004). Since cofactor regeneration is required for most of the biotransformations of interest, cells should be able to stay viable under the reaction conditions (De Carvalho and Da Fonseca, 2004). Due to these changes and adverse conditions, cells may change their morphology and in a more advanced proportion could lead to turgor and plasmolysis of cells (Morbach and Kramer, 2002).

3.5. Influence of sequential addition of substrate

The production of new aroma compounds from the biotransformation of terpenes still has many challenges to be overcome, some of the main problems commonly encountered is the toxicity of these compounds for microorganisms in high concentrations and also their higher volatilization causing lost due to evaporation (Duetz et al., 2003). In order to provide the fungal cells with appropriate amounts of substrate at each time, the effect of a sequential addition of substrate was tested, according the protocol presented in Section 2.7.

Table 3 comprises the results obtained with this bioconversion strategy. No improvements were observed in the production of limonene-1,2-diol, despite the initial production phase has begun before for test with lower initial averaged concentration of substrate, indicating easier adaptation of the microorganism to the medium conditions. Values observed for limonene and final biomass were lower and higher, respectively, for the strategy consisting of three-fold feedings, while the relative biotransformation (%) for limonene-1,2-diol for the two-fold strategy was almost the same as the control, or one-fold feeding, reaching 98% and 1.20 $g.L^{-1}$.

Table 3 – Production of limonene-1,2-diol by fresh cells of *Fusarium oxysporum* 152B after 96 h of process with sequential feeding of *S*-(–)-limonene.

Feeding Strategy	SPF [1] ($g.L^{-1}$)	IPT [2] (h)	FB [2] ($g.L^{-1}$)	LC [3] ($g.L^{-1}$)	Lim-diol [4] ($g.L^{-1}$)	Conversion (%)
One fold	4	32	3.90 ± 0.5	0.19	1.23 [a]	100
Two fold	3-1	29	3.99 ± 0.4	0.27	1.20 [a]	98
Three fold	2-1-1	29	4.14 ± 1.0	0.10	1.12 [b]	91

[1] Substrate profile feeding

[2] Final biomass

[3] Limonene concentration remaining in the medium quantified by GC-FID

[4] Limonene-1,2-diol was quantified by GC-FID

[a,b] Different letter superscripts in the same column indicate a statistically significant difference ($p < 0.05$).

Pescheck et al. (2009) studied the sequential feeding of limonene into non-toxic portions for a *Penicillium digitatum* DSM 62840, capable to regioselectively convert limonene into α-terpineol in bioreactor. This technique was adopted since shake flasks experiments revealed a pronounced growth inhibition when initial concentrations of the substrate exceeded 1.9 mM.

In contrast to what was observed in this experiment, Adams et al. (2003) reported the beneficial effect of a sequential addition of limonene, when yields were higher when a sequential addition of substrate was applied. However, it is important to note that the strategy of sequential addition can be positive for this experiment adapting different strategies, considering changes in times of addition and substrate concentrations, which can be considered in greater details in a future research.

4. Conclusion

The fungal strain *Fusarium oxyusporum* 152B was subjected to a series of tests in order to increase the production of limonene-1,2-diol from the bioconversion of *S*-(–)-limonene. Bioconversion trials showed additional information on its limonene metabolic pathway, where *S*-(–)-limonene was converted into limonene-1,2-diol and this compound was further metabolized into 1-hydroxy-2-oxolimonene, which led to suggest that *Fusarium oxysporum* 152B might have a recent discovered pathway for the bio-degradation of this substrate. It was adopted a three-phase bioconversion process, proving the importance of the contact of the cells with substrate prior the

biotransformation process, being responsible for the reduction of process time and maximum production of the compound of interest. Additionally, although expected, the results collected for the presence of co-solvent in the bioconversion system and the sequential feeding of substrate showed no major impact on response, considering the ranges tested. Finally, to date, the estimates indicate this process as one of the highest yields using fungal strain as biocatalyst for the production of biotechnological limonene-1,2-diol. Furthermore, based on the observed results, it is important to emphasize that this biocatalyst has shown pronounced substrate and product tolerance, and several studies should be directed at achieving higher concentrations of this product based on a better knowledge of the bioconversion process.

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Capítulo 4

Optimization of biomass development using agro-industrial by-products and biotransformation of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B

ABSTRACT

This study comprised a multiple stage process optimizations, which initially aimed to produce biomass using agro-industrial wastes to reduce costs related to raw materials and then apply this biocatalyst in the optimization of process conditions involved in the bioconversion of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B. For biomass development, the most appropriate alternative medium of those tested was cassava wastewater (or manipueira) and the influence of process conditions such as the dilution of this agro-industrial residue (%), process temperature (^{o}C) and agitation speed (rpm) has been evaluated by means of a central composite design. Furthermore, the main variables involved in the bioconversion of *S*-(–)-limonene into limonene-1,2-diol were assessed by a sequential optimization strategy, firstly involving the screening of variables followed by a central composite design, analyzing pH medium, limonene concentration (g/L), process temperature (^{o}C) and agitation (rpm), to optimize these variables and assess their impact on the production of this natural aroma compound. The results have been statistically analyzed by the response surface methodology and the optimal process conditions for limonene-1,2-diol bio-production have been determined. The process variables showed a significant impact the production of limonene-1,2-diol and the optimal process conditions, which are pH 6.5, 5 g.L^{-1} of *S*-(–)-limonene, 28 ^{o}C and 250 rpm of temperature and agitation, respectively, led to approximately 3.7 g.L^{-1} of this bioflavor. It is important to note that this is the first description of a fungal biocatalyst grown on agro-industrial residues and capable of producing limonene-1,2-diol, as well as one of the highest concentrations ever reported for this biotechnological aroma compound.

Keywords: Terpenes, monoterpenes, terpenoids, natural flavor, aroma compound.

1. Introduction

The production of natural products by direct recovery from nature has been applied for decades, although some serious drawbacks are encountered, such as the low concentrations of the compound of interest, the variability in the composition and yield of the final products which is dependent on geographical, seasonal and climatic features, and the possible ecological problems that are associated with the extraction of the product (Bicas et al., 2010a), increasing interest in products obtained by biotechnological methods, such as biotransformation of bioconversion of specific substrates (Van der Werf et al., 1997).

Biotransformation can be defined as the use of biological systems to produce chemical changes on compounds that are not their natural substrates (Borges et al., 2007). A certain molecule is modified through the transformation of its functional groups with or without the degradation of its carbon skeleton. Such modifications result in the formation of novel and useful bioactive compounds that are not easily prepared through chemical methods (Borges et al. 2009). Among these products, many have wide-ranging applications, including flavorings, agrochemicals, antibiotics, antioxidants and anticancer agents (Molina et al., 2013a).

In modern biotechnology an area of great interest is the production of natural flavors through the biotransformation of terpenes as substrates. These compounds are secondary metabolites of plants that are produced, in part, as a defense against microorganisms and insects, in addition to their pollinator-attractive properties. Terpenoids, particularly mono and sesquiterpenoids, are the main flavor and fragrance impact molecules in the essential oils of higher plants (Gershenzon and Dudareva, 2007).

Limonene is the most abundant naturally occurring monoterpene and the *R*-(+)-isomer represents up to 90% of orange peel oil, while the *S*-(–)-isomer is found in oils from the *Mentha* species and conifers at low concentrations, representing an interesting and inexpensive citrus byproduct (Bauer et al., 2001). Thus, limonene has become one of the most studied precursors in bioconversion experiments for the production of high-value derivatives, which may prove to be a good strategy for the enrichment of the commercial value of agro-industrial residues, such as orange peel oil (Maróstica Jr and Pastore, 2007a). In the scientific literature, the biotransformation of limonene by microorganisms has been well documented (Duetz et al., 2003; Krings and Berger, 1998).

Maróstica and Pastore (2007b) and Bicas et al. (2008a) have recently reviewed the main metabolic routes for limonene in six different degradation pathways, which are: (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 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.

In this perspective, the use of bioprocessing is a promising alternative in the recovery of terpene-derivate natural flavor compounds, but a commercial process with high productivity and low manufacturing costs must be developed (Berger et al., 1999). In this context, biotechnological improvements to enhance the production rate, yields, recovery efficiency (Welsh, 1994) also the reduction of process costs such as for obtaining raw materials as substrates of synthetic medium for biocatalyst growth (Van der Werf et al., 1997) are indispensable.

Thus, considering these challenges, this study describes the development of biomass using agro-industrial wastes to reduce costs and utilization of this biocatalyst in the optimization of process conditions involved in the biotransformation of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B, both strategies proposed were evaluated using an experimental optimization by means of a central composite design methodology.

2. Material and methods

2.1. Chemicals

The chemical standard used as substrate in this study was *S*-(–)-limonene (96% purity, Sigma-Aldrich), ethyl acetate and *n*-decane were used as solvent and internal standard, respectively. All solvents were of the best available commercial grade and kept under refrigeration (4 ^{o}C) until use.

2.2. Microorganism cultivation

The microbial strain employed in this study was isolated from the northeast Brazilian fruits, and it was identified as *Fusarium oxysporum* 152B (Prazeres et al., 2006). The fungal strain was maintained on Yeast Malt (YM) Agar (in g.L^{-1}: agar = 20; glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3, pH ~ 6.7) and stored at

4°C.

2.3. Biomass development using alternative culture medium

2.3.1 Agro-industrial residues

The agro-industrial residues used in this study were: i) Cassava wastewater (CWW); ii) Corn steep liquor (CSL); iii) Whey (WH); iv) Glycerol (GLY) and v) Banana pulp wastewater (BPW). The materials were obtained from specific industrial plants situated in the state of São Paulo - Brazil, kept under refrigeration until directly used or treated to be applied in experiments as described in the following.

2.3.2. Alternative medium preparation

Cassava water effluent obtained from the manufacture of cassava flour was collected and stored at -18 ^{o}C until needed. The medium was prepared by heating the waste to boiling to remove solids. After cooling, the substrate was centrifuged at 10.000 x *g* for 20 min. The supernatant was sterilized (121 ^{o}C for 20 min) (Maróstica Jr. and Pastore, 2007a). The resulting pH prior to autoclaving was 5.6 and it was not adjusted.

Corn steep liquor was obtained from the manufacturer of corn syrup and centrifuged at 10.000 g for 20 min. The liquor was adjusted with water to 15-20 % solids (adapted from Liggett and Koffler, 1948). The supernatant was sterilized (121 ^{o}C for 20 min). The resulting pH prior to autoclaving was 4.8 and it was not adjusted.

Whey, or sweet milk serum, was obtained from a small cheese production at Campinas, state of São Paulo, Brazil. This material was sterilized at 121 ^{o}C for 20 min and directly used in biomass development trials.

Crude glycerol was obtained from biodiesel synthesis by transesterification of soybean oil. Conventional procedures were used for glycerol separation, such as neutralization with sulfuric acid, filtration, decantation and evaporation of residual ethanol (Santos et al., 2012). The raw glycerol contained approximately 85% (w/w) of glycerol. This substrate was diluted with the same volume in water and sterilized at 121 ^{o}C for 20 min prior utilization.

Banana pulp liquid residue was obtained from a small processing starch production plant. Residual water was obtained through successive washings of pulp fruit for the isolation of starch and subsequently autoclaved (121 ^{o}C for 20 min) to be directly used.

2.3.3. Analysis of the composition of cassava and corn wastes media

The composition of two residues used, cassava wastewater and corn steep liquor, was evaluated. In the first case, the material was characterized as proposed by Maróstica-Jr and Pastore (2007a), and levels obtained in each test were compared with the same literature.

2.3.3.1. Total carbohydrates analyses

Total carbohydrates were determined by a colorimetric method based on Phenol reaction with glucose (Daniels et al., 1994).

2.3.3.2. Determination of reducing sugars contents

Total reducing sugars were quantified according to Somogy (1945).

2.3.3.3. Determination of nitrogen content

Protein content was calculated from nitrogen determination by the Kjeldahl procedure using 6.25 as conversion factor (AOAC, 1995).

2.3.3.4. Determination of minerals contents in cassava medium

One milliliter of cassava medium was incinerated for 3 h at 500 ^{o}C and after this period 5.0 mL of HCl 6M was added and evaporated. The solid was transferred to a volumetric balloon of 50 mL and filtered. The minerals were determined by an Atomic Emission Spectrophotometer (Jobin Yvon, model JY 50P). The spectral lines for each element were (in nm): P, 178.2; K, 766.5, Ca, 317.9; Mg, 279.9; Mn, 257.6; Fe, 259.9; Cu, 324.7; Zn, 213.8; and S, 180.6.

2.3.3.5. Analysis of chemical oxygen demand (COD) and total solids

Chemical oxygen demand (COD) and total solids analysis of CWW and CSL were done according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

2.4. Inoculum for biomass development trials and optimization experiments

A piece of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 50 mL each alternative culture medium in test. The material was homogenized under sterile

conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. An aliquot of this liquid was removed for the quantification of microorganism biomass, used as control. After incubation at 30 °C and 150 rpm for 48 h, the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman n°1 (Bicas et al., 2008b), dried at 105 °C for 24 h and weighed to evaluate the growth of biomass in alternative media. Experiments were conducted in triplicate. Inoculum for the optimization of biomass development experiments (Section 2.5) was prepared similarly to that described above.

2.5. Optimization of biomass production using cassava wasterwater medium

To determine the influence of the process conditions, such as the cassava wastewater dilution, temperature and agitation on the development of biomass (dependent variable) using this agro-industrial residue as substrate, a central composite design 2^3 was performed, with three replicates at the central point and six axial points, totaling 17 experiments (Rodrigues and Iemma, 2005). Experimental design and the coded and real values of the independent variables, as well as results for regression coefficients and analysis of variance (ANOVA) for the biomass development in cassava wastewater medium, are given in Table 1. The study ranges had been defined in preliminary tests.

2.6. Optimization of limonene-1,2-diol production

2.6.1. Strategy for optimization process

The strategy for the optimization of limonene-1,2-diol production was initially based on a 2^{5-1} fractional design, carried out in order to evaluate the effects of the process variables on the response. The variables selected were: i) initial pH (5.3 to 8.7); ii) inoculum concentration produced in cassava wastewater (1.0 to 5.0 g/L); iii) substrate concentration (4.0 to 12.0 g/L); iv) temperature (20 to 40 °C) and v) agitation (50 to 250 rpm). Table 2 presents the coded values and real values used in this screening of impacting variables on limonene-1,2-diol production.

To determine the influence of the most influential variables, a central composite design 2^4 was performed, with six replicates at the central point and eight axial points, totaling 30 experiments (Rodrigues and Iemma, 2005). The dependent variable (response) was limonene-1,2-diol concentration after 72 h of bioconversion process. The experimental design and the coded and real values of the independent variables are

given in Table 3. The study ranges had been defined in preliminary tests and all the experiments were carried out in a randomized way. In the best conditions, the cultivations were performed in triplicate in order to validate the mathematical model for limonene-1,2-diol concentration at 72 h of process.

Table 1 - Central composite design 2^3 matrix with coded values and real values (in parenthesis), results for regression coefficients and analysis of variance (ANOVA) for the biomass development into cassava wastewater medium.

Optimization experiments[a]					**Statistical analysis**		
	X_1	**X_2**	**X_3**	**Biomass (g/L)**	**Parameter**		**Biomass (g/L)**
1	-1 (60)	-1 (27)	-1 (60)	4.92	Mean	β_0	16,52
2	+1 (90)	-1 (27)	-1 (60)	4.55	*Linear*		
3	-1 (60)	+1 (33)	-1 (60)	9.58	X_1	β_1	1.95
4	+1 (90)	+1 (33)	-1 (60)	7.12	X_2	β_2	–
5	-1 (60)	-1 (27)	+1 (240)	8.24	X_3	β_3	3.35
6	+1 (90)	-1 (27)	+1 (240)	23.42	*Quadratic*		
7	-1 (60)	+1 (33)	+1 (240)	10.43	X_1	β_1^2	–
8	+1 (90)	+1 (33)	+1 (240)	12.05	X_2	β_2^2	-3.43
9	-1.68 (50)	0 (30)	0 (150)	14.36	X_3	β_3^2	-1.78
10	+1.68 (100)	0 (30)	0 (150)	21.87	*Interaction*		
11	0 (75)	-1.68 (25)	0 (150)	10.85	X_1X_2	β_{12}	-1.96
12	0 (75)	+1.68 (35)	0 (150)	6.44	X_1X_3	β_{13}	2.45
13	0 (75)	0 (30)	-1.68 (0)	8.00	X_2X_3	β_{23}	-2.05
14	0 (75)	0 (30)	+1.68 (300)	18.58	*ANOVA*		
15	0 (75)	0 (30)	0 (150)	17.03	$F_{calculated}$		10.18
16	0 (75)	0 (30)	0 (150)	15.68	F_{listed} [b]		3.29
17	0 (75)	0 (30)	0 (150)	16.67	$F_{calculated}$:F_{listed}		3.1
					p-value		< 0.0001
					R^2 [c]		0.88

[a] X_1 = Substrate dilution (%), X_2 = Temperature (°C), X_3 = Agitation (rpm)

[b] Values of F_{listed} at $p < 0.05$

[c] R^2 = coefficient of determination

2.6.2. Pre-culture preparation

Four pieces of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 200 mL of CWW medium. The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After incubation at 30 °C and 150 rpm for 48 h, these pre-culture was added aseptically in the bioreactor.

2.6.3. Biocatalyst production

The biocatalyst was produced in a bioreactor with a working volume of 4 L (Bioflo 310, Bioflo & Celligen, New Brunswick, USA), using 200 mL of the pre-culture as inoculum and 3.8 L of sterile CWW medium. Temperature, agitation and aeration were kept at 30 ºC, 500 rpm, and 1.0 slpm, respectively, while the pH was set at 6.5. After 48 h, *S*-(–)-limonene was added to the system reaching 1 g/L. This concentration was kept constant by re-addition of substrate and it was monitored by GC-FID (Section 2.7). After 24 h of contact with *S*-(–)-limonene, the biomass was recovered from the bioreactor by vacuum filtration using a Buchner funnel and paper filter Whatman n°1. The biomass was used for optimization of limonene-1,2-diol production experiments, following the concentration values set for each test, as presented in Table 2 (for the variables screening shown below (Section 2.6.1)).

2.6.4. Bioconversion experiments

The biomass obtained in item 2.6.3 was distributed amongst 250 mL conical flask filled with 50 mL of mineral (MM) medium (in $g.L^{-1}$: $MgSO_4.7H_2O$ = 0,5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0,5 e Fe_2SO_4 = 0,001) (Brunerie et al., 1987). Subsequently, *S*-(−)-limonene was added to the medium following the values set for the optimization experiments. The flasks were incubated under proposed temperature and agitation conditions (as may be seen in Table 2). After 72 and 96 h of process, 1 mL samples were taken in order to monitor the consumption of substrate and volatile compounds produced (Adapted from Bicas et al., 2008b).

2.7. Analytical conditions

Samples from culture medium were extracted (40 s in Vortex) with the same volume of ethyl acetate. After phase separation, 1 µL of the organic fraction was dried over sodium sulphate and injected in split mode (ratio of 1:10) 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 lenght x 0.25 mm i.d. x 0.25 µm of film thickness). Helium was used as carrier gas (1.0 $mL.min^{-1}$) and the oven temperature was kept at 80 ^oC for 3 min, raised at 20 $^oC\ min^{-1}$ until 200 ^oC and held for 4 min. Temperatures of the injector and detector were kept at 250 ^oC. Substrate and product were quantified by a calibration curve using *n*-decane as internal standard (adapted from

Bicas et al. (2008a). The concentrations were expressed as mass of product per liter of medium.

2.8. Data analysis

Statistical analysis of the experimental data, screening of variables and the response surface methodology were performed using the Statistica 7.0 software (StatSoft Inc, Oklahoma, USA).

Table 2 - Coded values, real values (in parentheses) and experimental data obtained from the assays in the 2^{5-1} fractional design for limonene-1,2-diol production.

	Independent variables[a]					Results[b]	
	X_1	X_2 (g/L)	X_3 (g/L)	X_4 (^oC)	X_5 (rpm)	LD-72h (g/L)	LD-96h (g/L)
1	-1 (5.3)	-1 (1.0)	-1 (4.0)	-1 (20)	1 (250)	2.90	2.70
2	1 (8.7)	-1 (1.0)	-1 (4.0)	-1 (20)	-1 (50)	0.12	0.16
3	-1 (5.3)	1 (5.0)	-1 (4.0)	-1 (20)	-1 (50)	0.68	0.68
4	1 (8.7)	1 (5.0)	-1 (4.0)	-1 (20)	1 (250)	2.30	2.36
5	-1 (5.3)	-1 (1.0)	1 (12)	-1 (20)	-1 (50)	0.20	0.27
6	1 (8.7)	-1 (1.0)	1 (12)	-1 (20)	1 (250)	1.10	1.11
7	-1 (5.3)	1 (5.0)	1 (12)	-1 (20)	1 (250)	1.49	1.46
8	1 (8.7)	1 (5.0)	1 (12)	-1 (20)	-1 (50)	0.08	0.09
9	-1 (5.3)	-1 (1.0)	-1 (4.0)	1 (40)	-1 (50)	0.11	0.11
10	1 (8.7)	-1 (1.0)	-1 (4.0)	1 (40)	1 (250)	0.87	0.85
11	-1 (5.3)	1 (5.0)	-1 (4.0)	1 (40)	1 (250)	1.84	1.80
12	1 (8.7)	1 (5.0)	-1 (4.0)	1 (40)	-1 (50)	0.06	0.09
13	-1 (5.3)	-1 (1.0)	1 (12)	1 (40)	1 (250)	0.95	0.97
14	1 (8.7)	-1 (1.0)	1 (12)	1 (40)	-1 (50)	0.02	0.03
15	-1 (5.3)	1 (5.0)	1 (12)	1 (40)	-1 (50)	0.05	0.04
16	1 (8.7)	1 (5.0)	1 (12)	1 (40)	1 (250)	0.47	0.49
17	0 (7.0)	0 (3.0)	0 (8.0)	0 (30)	0 (150)	1.10	1.07
18	0 (7.0)	0 (3.0)	0 (8.0)	0 (30)	0 (150)	1.11	1.09
19	0 (7.0)	0 (3.0)	0 (8.0)	0 (30)	0 (150)	1.09	1.12
20	0 (7.0)	0 (3.0)	0 (8.0)	0 (30)	0 (150)	1.13	1.12

[a] X_1 = pH; X_2 = Inoculum concentration (g/L); X_3 = Substrate concentration (g/L); X_4 = Temperature (^oC) and X_5 = Agitation (rpm)

[b] LD-72h = Limonene-1,2-diol production after 72 h; LD-96h = Limonene-1,2-diol production after 96 h

Table 3 - Central composite design 2^4 matrix with coded values and real values (in parenthesis), results for regression coefficients and analysis of variance (ANOVA) for the production of limonene-1,2-diol from the biotransformation of *S*-(–)-limonene.

Optimization experiments[a]						Statistical analysis		
	X_1	X_2 (g/L)	X_3 (^oC)	X_4 (rpm)	LD-72h (g/L)	Parameter		Biomass (g/L)
1	-1 (5.5)	-1 (4.0)	-1 (20)	-1 (125)	1.24	Mean	β_0	3.12
2	1 (7.5)	-1 (4.0)	-1 (20)	-1 (125)	0.87	*Linear*		
3	-1 (5.5)	1 (8.0)	-1 (20)	-1 (125)	0.45	X_1	β_1	–
4	1 (7.5)	1 (8.0)	-1 (20)	-1 (125)	0.31	X_2	β_2	-0.36
5	-1 (5.5)	-1 (4.0)	1 (30)	-1 (125)	1.09	X_3	β_3	–
6	1 (7.5)	-1 (4.0)	1 (30)	-1 (125)	0.55	X_4	β_4	0.84
7	-1 (5.5)	1 (8.0)	1 (30)	-1 (125)	0.62	*Quadratic*		
8	1 (7.5)	1 (8.0)	1 (30)	-1 (125)	0.47	X_1	β_1^2	-0.63
9	-1 (5.5)	-1 (4.0)	-1 (20)	1 (275)	3.31	X_2	β_2^2	-0.39
10	1 (7.5)	-1 (4.0)	-1 (20)	1 (275)	2.34	X_3	β_3^2	-0.43
11	-1 (5.5)	1 (8.0)	-1 (20)	1 (275)	1.85	X_4	β_4^2	-0.21
12	1 (7.5)	1 (8.0)	-1 (20)	1 (275)	1.97	*Interaction*		
13	-1 (5.5)	-1 (4.0)	1 (30)	1 (275)	3.51	X_1X_2	β_{12}	–
14	1 (7.5)	-1 (4.0)	1 (30)	1 (275)	3.20	X_1X_3	β_{13}	–
15	-1 (5.5)	1 (8.0)	1 (30)	1 (275)	1.94	X_1X_4	B_{14}	–
16	1 (7.5)	1 (8.0)	1 (30)	1 (275)	1.20	X_2X_3	β_{23}	–
17	-2 (4.5)	0 (6.0)	0 (25)	0 (200)	0.50	X_2X_4	β_{24}	-0.22
18	2 (8.5)	0 (6.0)	0 (25)	0 (200)	0.25	X_3X_4	β_{34}	–
19	0 (6.5)	-2 (2.0)	0 (25)	0 (200)	1.69			
20	0 (6.5)	2 (10)	0 (25)	0 (200)	0.97			
21	0 (6.5)	0 (6.0)	-2 (15)	0 (200)	0.26			
22	0 (6.5)	0 (6.0)	2 (35)	0 (200)	2.08			
23	0 (6.5)	0 (6.0)	0 (25)	-2 (50)	0.46			
24	0 (6.5)	0 (6.0)	0 (25)	2 (350)	3.70			
25	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.13	*ANOVA*		
26	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.10	$F_{calculated}$		29.50
27	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.09	F_{listed}[b]		2.46
28	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.07	$F_{calculated}$:F_{listed}		12.00
29	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.15	*p*-value		< 0.0001
30	0 (6.5)	0 (6.0)	0 (25)	0 (200)	3.16	R^2 [c]		0.89

[a] X_1 = pH; X_2 = Inoculum concentration (g/L); X_3 = Substrate concentration (g/L); X_4 = Temperature (^oC) and X_5 = Agitation (rpm); LD-72h = Limonene-1,2-diol production after 72 h

[b] Values of F_{listed} at $p < 0.05$

[c] R^2 = coeficient of determination

3. Results and discussion

3.1. Biomass production in alternative culture medium: Economical viewpoint

The field of production of natural aroma compounds is still in development and now requires highly innovative processes to increase the diversity of compounds produced and to decrease the costs of production (Aguedo et al., 2004).

This sentence indicates the importance of searching for new techniques to reduce overall process costs. In general, the biotransformation of terpenes process can be divided into two main stages. At first, the inoculum is produced in conventional synthetic media, with the aim of developing the required concentration of biocatalysts that will be later applied in the process of interest (Bicas et al., 2008a,b; Maróstica Jr. and Pastore 2007a). In this phase, the culture medium plays an important role and should be rich in essential elements for cellular growth such as carbon, nitrogen, and, to a lesser extent, phosphorus and sulfur. Microorganisms can use a variety of carbon sources, from complex ones, such as starch and wood hydrolyzates, to refined sources, such as glucose syrup or sucrose. Trace metal requirements must be met with salt solutions. Complex nitrogen and carbon sources are required in some fermentation where the slow release of nutrients may be important in regulating these metabolisms (Blanch and Clark, 1997). Also, this phase may consider biocatalyst induction techniques to improve enzymatic performance (Onken and Berger, 1999; Tan et al., 1998; Molina et al., 2013b). Economic aspects are very important for an industrial process once the raw material represents 30% of the total costs of biotechnological processes (Cameotra and Makkar, 1998).

Meanwhile, the second step of the process is the biotransformation or bioconversion of precursors, which can be developed in complex media (Krings et al., 2006) or using mineral medium (Maróstica Jr and Pastore, 2007a), employing the specific substrates for each process. As reported above, several monoterpenes have a relatively low cost, such as limonene, which is interesting from the economic viewpoint, while the cost is related to the requirements of nutrients in the medium (Bauer et al., 2001).

Despite that, up to now, most studies dealing with biotransformation of terpenes described are not suitable for industrial application, due to problems encountered during the process. Van der Werf et al. (1997) listed the main limiting features which are: (i) chemical instability of substrates; (ii) low solubility of substrate; (iii) high volatility of substrates and products; (iv) toxicity of substrate and products; (v) absence of product

accumulation and product degradation; (vi) multiple metabolic pathways resulting in the formation of a mixture of products; (vii) low product concentrations/yields; (viii) long incubation times; (ix) short biocatalyst lifetimes and (x) overall process costs (Van der Werf et al., 1997).

In this sense, some attempts have been made in order to avoid or reduce these problems and achieve a feasible yield and product concentration, including the use of a biphasic system (Bicas et al., 2008a; Bicas et al. 2010b), co-solvents addition (Tan and Day, 1998), use of statistical tools (Bicas et al., 2008b) and also the use of agro-industrial residues as substrate has also been considered for the production of aroma compounds using biotransformations. This last strategy seems to be a growing trend to overcome the high costs involved in microbial transformations (Bicas et al., 2010a).

In the same perspective, the choice of a substrate will depend on the raw material contribution to the production cost and on its influence in downstream processing operations, as well as on final product specifications. For high-value products, the raw materials may not comprise a significant part of the production cost, but selection of raw material may nevertheless be important in maintaining consistent product quality. For low-value products, the cost of raw materials determines the economic success of a process such as single-cell protein production, for example (Blanch and Clark, 1997).

Considering the above, the biotransformation of *R*-(+)-limonene by *Fusarium oxysporum* 152B was very well described and established in previous works allowing several process improvements with this fungal biocatalyst. For example, Maróstica Jr. and Pastore (2007a) showed a distinguishable approach using cassava wastewater to support fungal growth; in the sequence, such biomass was applied in biotransformation processes using by-products as source of terpene substrate, *e.g.* orange oil as source of *R*-(+)-limonene reaching 450 $mg.L^{-1}$ of the monoterpene alcohol α-terpineol (Maróstica Jr. and Pastore 2007a). Following, Bicas et al. (2008b) described the optimization of the ten main process variables involved in the biotransformation of *R*-(+)-limonene to R-(+)-α-terpineol by *Fusarium oxysporum* 152B through a Plackett-Burman matrix with 16 assays, including the effect of the medium composition, substrate concentration, cultivation conditions and inoculum size, followed by central composite design methodology. Thus, the authors improved significantly the production of *R*-(+)-α-terpineol, from 450 $mg.L^{-1}$ previously described to 2,44 $g.L^{-1}$ of this monoterpene alcohol. Another study conducted by the same group has proposed an integrated process

for the co-production of lipase and *R*-(+)-α-terpineol, where the concentration of this monoterpene alcohol reached approximately 4 $g.L^{-1}$ (Bicas et al., 2010c).

Meanwhile, this doctoral thesis has achieved many advances in studies on the biotransformation of *S*-(–)-limonene into limonene-1,2-diol, which reached 1.2 $g.L^{-1}$ after 72 h of process, using conventional synthetic medium for the growth of biomass and under non optimized process conditions. In this sense, this chapter aimed to optimize these two processes to further optimization of both biomass production in agro-industrial residues as the production of limonene-1,2-diol from the statistical analysis applied to the influence of the main process variable.

To reduce process costs related to the raw materials used for the production of biomass/biocatalyst, some agro-industrial residues were evaluated as alternative culture media, as the following examples: i) Cassava wastewater (CWW); ii) Corn steep liquor (CSL); iii) Whey (WH); iv) Glycerol (GLY) and v) Banana pulp wastewater (BPW). Figure 1 shows an aliquot of each liquid residue used as an alternative substrate.

Figure 1 – Agro-industrial residues treated and used as substrates for *Fusarium oxysporum* 152B biomass development. From left to right: i) Cassava wastewater, ii) Corn steep liquor, iii) Whey, iv) Industrial glycerol and v) Banana pulp wasterwater.

The first substrate, CWW, has a high organic content and is an important economic product of traditional and rural low technology agro-industry in many parts of the world (Kaewkannetra et al., 2011). This residue was already used as substrate for the production of biosurfactants (Nitschke and Pastore, 2006; Barros et al., 2008), the bioflavor α-terpineol (Maróstica-Jr and Pastore, 2007a), biohydrogen (Sreethawonga et al., 2010), as a resource for electricity generation by microbial fuel cells (Kaewkannetra et al., 2011) and others.

Manipueira is a residue generated in great amounts during the manufacture of cassava flour, a very common ingredient in Brazilian cookery. The major nutrients present in cassava waste are sugars (sucrose, glucose, fructose and maltose), nitrogen and mineral salts. Although disposal of this residue is a problem due to its high organic load, it is also a very attractive substrate for biotechnological processes (Maróstica Jr and Pastore, 2007a). On the other hand, this residue also contains high contents of substances that could limit the growth or inhibit the capacity of biotransformation, such as cyanide (Maróstica Jr and Pastore, 2007a), becoming important its primary treatment. The composition of the CWW used in this study is shown in Table 4, showing a comparison with other compositions described in the specific literature.

When applied as medium for biomass development following the methodology described in Section 2.4, CWW was shown to be an excellent medium for the growth of this fungal strain, reaching 23 $g.L^{-1}$ of biomass after 48 h of process with an approximately 68% of carbohydrates converted into biomass (Figure 2). Thus, this medium becomes a promising alternative for replacing the medium used in the prior studies, known as Yeast and Malt medium (composition in $g.L^{-1}$: glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3, pH ~ 6.7). The nutrients used traditionally in most of the fermentative media, particularly yeast extract and peptone, are very expensive. Because of this, the search for alternative, financially competitive nutrients sources is particularly interesting. Generally, fermentation production with agricultural byproducts as nutrient sources is more attractive than defined medium at the point of cost (Wei et al., 2007).

Table 4 – Composition of cassava wastewater and corn steep liquor, compared with the specific literature.

	Unit	CWW[1]	Literature[2]	CSL[3]	Literature[4]
Total solids	g/L	64.0	61.0 ± 1.0	71.0	-
COD[a]	g/L	56.0	54.6 ± 1.2	61.0	-
Total sugars	g/L	40.5	40.4 ± 0.9	16.0	-
Non-red. sugars	g/L	20.5	21.7 ± 1.6	8.5	-
Reducing sugars	g/L	20.0	18.8 ± 0.5	7.5	-
Total nitrogen	g/L	2.3	1.9 ± 0.2	3.2	3.5 ± 0.1
Phosphorous	mg/L	252.0	307.0 ± 62.0	1.7	1.5 ± 0.1
Potassium	mg/L	2290.0	3556.0 ± 84.0	2780.0	2753.9 ± 69.7
Calcium	mg/L	240.0	264.5 ± 28.5	641.0	239.8 ± 2.9
Magnesium	mg/L	291.0	478.5 ± 40.5	585.0	723.8 ± 1.9
Sulphur	mg/L	0.1	107.7 ± 46.3	0.0	0.34[*]
Iron	mg/L	282.5	5.2 ± 2.5	465.0	15.6 ± 8.1
Zinc	mg/L	127.5	2.9 ± 0.1	160.0	10.7 ± 0.1
Manganese	mg/L	10.0	2.5 ± 0.8	42.5	55.3 ± 1.0
Copper	mg/L	1.3	1.0 ± 0.1	2.5	1.1 ± 0.3
pH	-	5.6	5.3 - 5.8	4.8	3.7 - 4.1[*]

[a]COD, Chemical Oxygen Demand

[1] CWW, Cassava wastewater

[2] Data obtained from the literature Maróstica Jr. and Pastore (2007); Nitschke and Pastore (2003) related to CWW

[3] CSL, Corn Steep Liquor

[4] Data obtained from the literature Valduga et al. (2007) related to CSL

[*] Additional reference Liggett and Koffler (1948)

The use of cassava wastewater has also been considered for directly as medium for bioflavor production to reduce the manufacturing costs (Bicas et al., 2010a). As presented in Table 4, this agro-industrial residue is rich in sugars (~40 $g.L^{-1}$), nitrogen (~2 $g.L^{-1}$) and a variety of minerals (K, Mg, P, Ca, S, Fe, Zn, Mn, Cu) and therefore it has been considered very attractive for the growth of many microorganisms (Maróstica Jr and Pastore, 2007a). The first description of bioflavor production through biotransformation processes using cassava wastewater for biomass production was reported for a *Penicillium* sp strain. When transferred to a fresh mineral medium with citronellol as sole carbon source, this microorganism produced a mixture of *cis*- and *trans*-rose oxides in a total concentration of ~100 $mg.L^{-1}$ (Maróstica-Jr and Pastore, 2006). Using a similar technique, *Fusarium oxysporum, Aspergillus sp. or Penicillium*

sp. grown in cassava wastewater could biotransform *R*-(+)-limonene to produce *R*-(+)-α-terpineol (Maróstica-Jr and Pastore, 2007c). In both cases, cassava wastewater was suitable for biomass production, but the use of mineral media during the biotransformation was recommended to increase yields (Maróstica-Jr and Pastore, 2007a,c).

Another residue studied as substrate is derived from the industrial production of corn syrup, known as CSL. Corn is one of the largest agricultural products in the world and there is a large supply of this residue which demands new uses for the valorization of this product (Wei et al., 2007, Valduga et al., 2007). The composition of this residue is presented in Table 4, and it is possible to observe the high content of nitrogen and the presence of several salts.

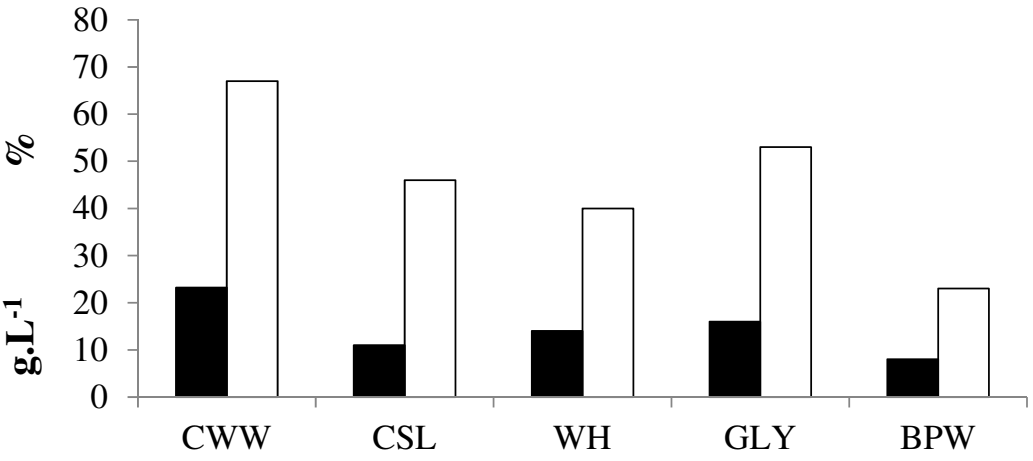

Figure 2 – Biomass concentration (■) and conversion percentage (□) using agro-industrial residues as substrates for *Fusarium oxysporum* 152B biomass development. Conversion percentage = grams of cells produced/grams of substrate consumed x 100. CWW (Cassava wastewater), CSL (Corn steep liquor, WH (Whey), GLY (Industrial glycerol) and BPW (Banana pulp wasterwater).

Figure 2 displays that the biomass concentration obtained using CSL, that reached 11 $g.L^{-1}$. The low values found for both biomass concentration and for the conversion rate can be justified by the fact that this material contained a low sugar content and carbon source for fungal metabolism. In fact, several sources report corn steep liquor has been used as a rich source of nutrients and has successfully been

employed in replacement of yeast extract and peptone (Kona et al., 2001; Lee, 2005), while ethanol production from glucose increased to 5.0% (w/v) when CSL was added in the fermentation medium (Sreenath and Jeffries, 1996). Additionally, the low pH value (~ 4.8) can also be a limiting factor for many biocatalysts growth. As the aim of this work is to study future practical applications, it was decided to not continue the study with this residue which, despite having a lot of potential, requires a series of preliminary treatments for their preparation, which can be a time consuming in industrial scales.

Whey is a by-product remaining after milk fat and casein have been separated from whole milk and one of the major disposal problems of the dairy industry, and demands simple and economical solutions (Panesar and Kennedy, 2012). In this analysis, pH of the residue was 5.2 (characterized as sweet whey), total nitrogen reached 1.3 g/L, while calcium, phophorous and potassium 0.37, 0.38 and 1.57, respectively, in accordance with published results by Moulin and Gauzy (1984). Table 4 present the results collected, reaching 14 $g.L^{-1}$ of biomass with 40% of conversion. Apart from the possibility of producing biomass as alternative culture medium, such as presented in this study with *Fusarium oxysporum* 152B, whey can also be used directly as a substrate for the growth of different microorganisms to obtain various products such as ethanol, single-cell protein, enzymes, lactic acid, citric acid, biogas and so on (Panesar and Kennedy, 2012).

Meanwhile, during the biodiesel production approximately 10% (w/w) of glycerol is generated as the main byproduct, representing a great opportunity for new applications and culminating in intensive investigations that focus on the utilization of crude glycerol directly as substrate (Yang et al., 2012). Some applications include the production of 1, 3-propanediol Mu et al. (2006), citric acid (Papanikolaou and Aggelis, 2003), several fuels (Ito et al., 2005) and many others. The use of this waste as substrate generated about 16 $g.L^{-1}$ of biomass with an estimate conversion of 53%. It is important to note that many improvements can still be made when using this substrate, such as the variation of its concentration. Moreover, the steps of purification require a careful process, because the remaining methanol can hinder the development of biocatalyst, besides being a waste with many other metabolites that may influence the development of the fungal strain.

The last alternative substrate tested was banana pulp liquid residue, obtained from a small processing starch production plant. Residual water was obtained through successive washings of pulp fruit for the isolation of starch and subsequently autoclaved

(121 ^{o}C for 20 min) for use. To the point that was researched, this seems the first description of an attempt to use this waste as substrate and application in biotechnology. Results showed that, although with a favorable pH (6.4) and also total sugars content (~ 27 g.L^{-1}), it was observed that the growth of the microorganism was not satisfactory. Biomass concentration reached only 8 g.L^{-1} of biomass and thus presented the worst results among others. After a careful analysis, the low values observed might be related to the presence of metabisulfite in the medium (from the treatment followed for the extraction of starch as shown in Pelissari et al. (2012)). This compound, a preservative commonly added to intravenously administered pharmaceuticals as an antioxidant, has inhibitor effect on various microorganisms (Franka and Patel, 2007), which may have negatively affected the development of *Fusarium oxysporum* 152B. Thus, if there is an initial step of removing the metabisulfite, this may become a residue of biotechnological interest in the near future, featuring a further innovation proposed in this study.

Finally, the objective was attempted to develop a cost-effective media for the fungal development, based on several agro-industrial residues treated to be used as stating material. The selected media (CWW, CSL, WH, GLY and BPW) were treated as minimum as possible, to characterize a cheap and practical process to use this residues as substrates, making it feasible to apply in biotechnological processes. The results showed a great potential for the production of biomass using these by-products, making it possible to adapt this stage to replace the commonly used synthetic media. It can be seen that CWW medium was the most promising medium for the production of biomass. In this sense, this study conducted an optimization process for the production of biocatalysts in CWW medium, aiming to evaluate the best dilution of CWW and also the process conditions such as temperature and agitation.

3.2. Optimization of biocatalyst production in CWW medium

The influence of the process conditions, such as the appropriate cassava wastewater dilution (%), temperature (^{o}C) and agitation (%) suitable for the development of biomass, a central composite design 2^3 was performed, with three replicates at the central point and six axial points, totaling 17 experiments (Rodrigues and Iemma, 2005).

Table 1 presents the experimental design, containing the coded and real values used for the independent variables, as well as the results for regression coefficients and

analysis of variance (ANOVA) for the biomass development into cassava wastewater medium.

The experiments performed at the central points showed a good reproducibility of the data, averaging 16.46 ± 0.52 $g.L^{-1}$ of biomass. These results performed under the same conditions as presented previously (30 ^{o}C of temperature and 150 rpm of agitation speed), only by changing the medium dilution (in this case, 75 % of CWW in water), already indicate the harmful effects of this dilution with a decreasing in biomass concentration with a decreasing concentration of biomass previously observed (23 $g.L^{-1}$, Section 3.1). This makes the application of this residue as substrate even more interesting by the use of concentrated CWW medium, as it was obtained after the initial treatment process (Section 2.3.2). Meanwhile, Table 1 also reveals that the best results were obtained under practical conditions of concentrated CWW medium (experiment 6) and agitation (experiment 10), reaching 21.87 and 23.42, respectively.

These data were submitted to statistical analyses and followed by an analysis of variance (ANOVA) at 95% confidence level. Only the statistically significant parameters have been used for analysis of the behavior of the fitted mathematical model for biomass production, presented below:

$$Biomass = 16.52 + 1.95\,X_1 + 3.35\,X_3 - 3.43\,X_2^2 - 1.78\,X_3^2 - 1.96\,X_1X_2 + 2.45\,X_1X_3 - 2.05\,X_2X_3 \quad (Eq.1)$$

where X are the coded independent variables (X_1 = CWW dilution (%), X_2 = Temperature (^{o}C) and X_3 = Agitation (rpm).

Table 1 also summarizes the results of the ANOVA, including the regression coefficients for the coded second order polynomial equation, the coefficients of determination (R^2), and the F and p values. Finally, to determine whether the fitted equations are predictive, they must satisfy a certain criterion based on the F_{test} values ($F_{calculated}$ and F_{listed}), which in turn depends on the calculated F ratio value for the regressions related to the residuals ($F_{calculated}/F_{listed}$). The criterion is that the value of this ratio must be higher than that of F_{listed} (Khuri and Cornell, 1996), is this case was 3 times higher, thereby enabling plotting of the response surfaces. These results suggest that the model fitted for the biomass production and lead to significant regression, low residual values, no lack of fit, and satisfactory coefficient of determination (R^2 = 0.88).

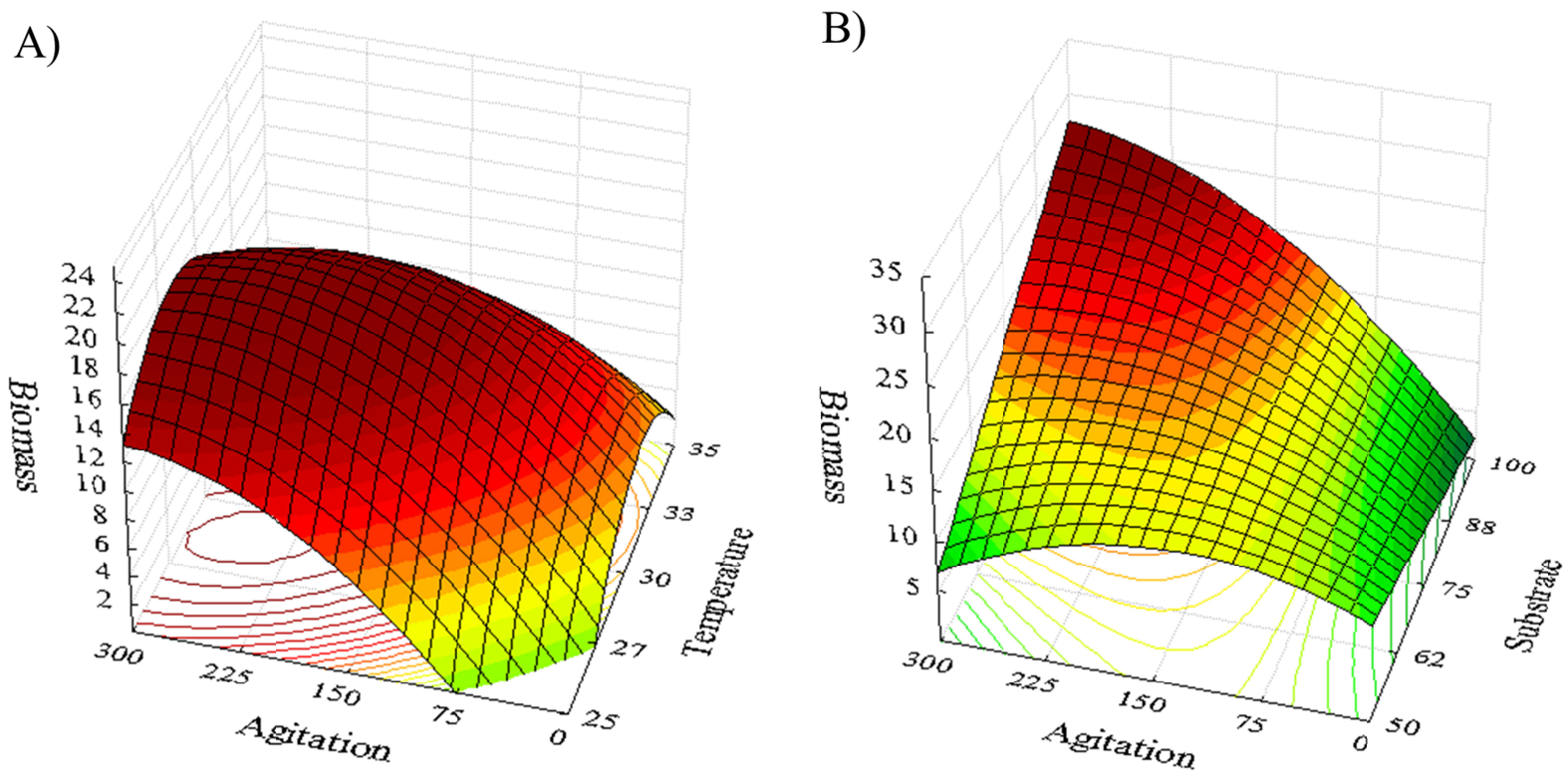

Figure 3 – Response surfaces of biomass production using CWW medium as a function of (a) agitation (rpm) and temperature (^{o}C), (b) agitation (rpm) and substrate dilution (%).

Analyzing the response surfaces presented in Figure 3 for biomass production using CWW medium as a function of agitation (rpm) and temperature (^{o}C) (Fig. 3a), the best results can be obtained by varying the agitation between 200 and 300 rpm and keeping the temperature near the central condition (30 ^{o}C). Meanwhile, the relation between agitation (rpm) and substrate dilution (%) (Fig. 3b) shows that the increasing of stirring and higher concentration of CWW medium can lead to higher biomass production, *i.e*., confirming the previously described assumption that it should be used concentrated CWW medium.

The optimal medium and process condition for biomass of *Fusarium oxysporum* 152B development contained 100% of CWW medium at 30 ^{o}C and 240 rpm. In these conditions, the biomass obtained was approximately 29.22 ± 0.85 g.L^{-1}. These studies shown the CWW medium was similar in performance to a nutrient-rich medium, but the cost of production was smaller than the use of synthetic medium containing yeast and malt extract. In this sense, it was presented an economical process for the production of *Fusarium oxysporum* 152B cells as biocatalyst for the further bioconversion of *S*-(–)-limonene into limonene-1,2-diol. This also seems to be the first description of an optimization process using CWW medium for fungal biomass support.

3.3. Screening of variables for limonene-1,2-diol production

The selection strategy to evaluate the impact of process variables in the bioconversion was started by a 2^{5-1} fractional design. The variables selected were: i) initial pH (5.3 to 8.7); ii) inoculum concentration produced in cassava wastewater (1.0 to 5.0 g/L); iii) substrate concentration (4.0 to 12.0 g/L); iv) temperature (20 to 40 ^{o}C) and v) agitation (50 to 250 rpm).

As may be seen in Table 2, the center points for the screening design were chosen based on the conditions usually applied for this biotransformation. All variables related to the process were in the same range as described above and as performed in initial experiments (Chapter 2 and 3), except for the usual concentration of limonene (4 g.L^{-1}) that has been applied as level -1, to allow extrapolation of the experimental data and analysis of the effect of this variable on the response of interest in higher concentrations.

Table 2 also displays the coded values and real values (in parenthesis) used in this experiment, as well as the experimental data obtained from the assays in the 2^{5-1} fractional design for limonene-1,2-diol production. The triplicate of the center points

showed that the production reached 1.10 ± 0.01 and 1.10 ± 0.02 $g.L^{-1}$ of limonene-1,2-diol, similar average values for 72 and 96 h. In this sense, the concentration of product observed for both times might not be big enough to justify an extra 24 h of process. For this reason, both times were statistically analyzed but only the first period was considered in the statistical analysis for the central composite design.

Figure 4 presents the statistical evaluation of the results considering the estimates of the effects of the limonene-1,2-diol production from the bioconversion of *S*-(–)-limonene after 72 and 96 h of process. In this case, a *p* value of 0.1 was used, and it is currently recommended since it is more conservative and lowers the risk of false excluding statistically significant parameters (Rodrigues and Iemma, 2005).

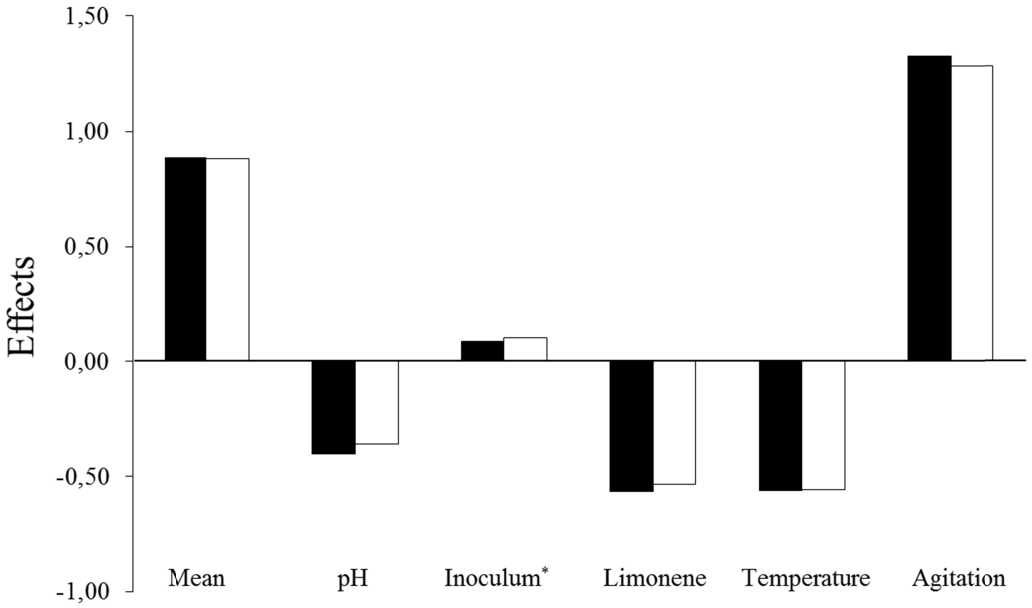

Figure 4 – Estimates of the effects of the limonene-1,2-diol production from the biotransformation of *S*-(–)-limonene after 72 h (■) and 96 h (□). All variables were statistically significant effect ($p < 0.1$) for both times analyzed, except inoculum concentration (*).

In industrial fermentation processes, it is a well-known fact that the age and density of the inoculum used directly influences the duration of the lag phase, specific growth rate, biomass yield, sporulation and quality of the final product, and hence the production costs (Sen and Swaminathan, 2004). Despite this importance, it was

demonstrated that the inoculum concentration presented no statistical effects on the responses after 72 or 96 h, considering the ranges tested, and thus this variable was of no significant interest (at $p < 0.1$) to this process. The same fact was observed in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *F. oxysporum* (Bicas et al., 2008b). Therefore, this variable was kept at 3 g as the usual process, facilitating the practical work.

Furthermore, according to Figure 4 it can be seen that the variables pH, limonene concentration and temperature showed a negative effect on the levels tested for 72 and 96 h ($p < 0.1$). In the first case, the variation of pH biotransformation can be considered an important and limiting variable for the survival and development of microorganism (Grimont, 1998). Thus, the selected value of 8.7 (level +1) may have inhibitory effect for this strain, while another *Fusarium solani* IHEM 8026 strain showed a good potential for cyanide biodegradation even under alkaline conditions, with pH ranging from 9.2 to 10.7 (Dumestre et al., 1997). However, with the *Fusarium oxyposrum* 152B, Bicas et al. (2008b) reported that there was any statistical effect of this variable on the production of α-terpineol, allowing working with pure distilled water with a pH between 5.2 and 8.2. In this process, a decrease of pH value becomes important for the next step of the process. In fact, considering the stability of the substrate, Adams et al. (2003) have shown that no acid catalyzed conversion of the substrate was observed at pH 3.5.

Regarding the negative effect of limonene concentration, as shown above, the previous process was conducted with 4 $g.L^{-1}$ of substrate, and the rise in this concentration may have an inhibitory effect on the microorganism due to the toxic activity of the substrate to the cells of *Fusarium oxysporum* in the ranges tested. In this case, smaller amounts of substrate ranging from levels -1 to 0 (4-8 $g.L^{-1}$) are indicated, considering that the production of limonene-1,2-diol was clearly affected with 12 $g.L^{-1}$ of *S*-(–)-limonene. This data is in accordance within the limonene concentration usual applied in biotransformation processes ranging from 0.2 to 1.0% (Bicas and Pastore, 2007), although some authors have suggested that substrate induction might enhance the process yield (Adams et al., 2003; Fontanille and Larroche, 2003).

Finally, the negative effect of temperature in the range tested might be related to the inhibition of microbial growth and enzyme denaturation at temperatures close to 40 ^{o}C (Grimont, 1998). In this case, mild temperatures, close to 30 ^{o}C (level 0), should be considered due to the fact that temperatures close to 40 ^{o}C (level +1) showed a

considerable reduction in the production of limonene-1,2-diol. Bicas et al. (2008b) observed that close to 40 ^{o}C there was virtually no product formation, while results obtained for *Penicillium digitatum* NRRL 1202 (Tan et al., 1998) and for *Pseudomonas putida* (Speelmans et al., 1998) showed dramatic decreases in bioconversion at temperatures above 32 and 30 ^{o}C, respectively.

3.4. Central composite design for limonene-1,2-diol production

According to the variables screened and the levels tested for the process under study, the significant factors (presented in Figure 4, $p < 0.1$), which were worth considering in the further optimization design were pH, substrate concentration, temperature and agitation speed. These variables were evaluated using a central composite design 2^4, with six replicates at the central point and eight axial points, totaling 30 experiments (Rodrigues and Iemma, 2005).

Table 3 comprises the matrix with coded and real values (in parenthesis) studied and, in addition, presents the results for regression coefficients and analysis of variance (ANOVA) for the production of limonene-1,2-diol from the biotransformation of *S*-(–)-limonene. In general, it is possible to observe a great increase in the concentration of limonene-1,2-diol, due to the new levels evaluated. Experiments performed at the central points (pH = 6.5, limonene concentration = 6.0, temperature = 25 ^{o}C and agitation = 250 rpm) reached 3.11 ± 0.05 g.L^{-1}. Meanwhile, the highest concentration was obtained in experiment 13, reaching 3.5 g.L^{-1}.

These data were treated by the software Statistica 7.0 which generated the regression coefficient followed by an analysis of variance (ANOVA) at 95% confidence level. Only the statistically significant parameters have been used for analysis of the behavior of the fitted mathematical model for limonene-1,2-diol production, presented below:

$$Lim - Diol = 3.12 - 0.36\,X_2 + 0.84\,X_4 - 0.63\,X_1^2 - 0.39\,X_2^2 - 0.43\,X_3^2 - 0.21\,X_4^2 - 0.22\,X_2X_4 \quad \text{(Eq. 2)}$$

where X are the coded independent variables (X_1 = CWW dilution (%), X_2 = Temperature (^{o}C) and X_3 = Agitation (rpm).

The same observations cited for the ANOVA reported above (Section 3.2. for biomass development in CWW medium) are valid for this model. In this particular case

for the production of the bioflavor, there was a satisfactory coefficient of determination was 0.89, indicating that the model explain 89% of the observed data variation. Bicas et al. (2008b) obtained a coefficient of determination of 0.83 for α-terpineol production using the same biocatalyst *Fusarium oxysporum* and these values are perfectly acceptable value for biological systems (Rodrigues and Iemma, 2005). In addition, the fitted equation is predictive for the process, since the F ratio value ($F_{calculated}/F_{listed}$) is 12 times higher than the corresponding F_{listed} value (Table 3), and these results suggest that the models fitted for the production of biotechnological limonene-1,2-diol.

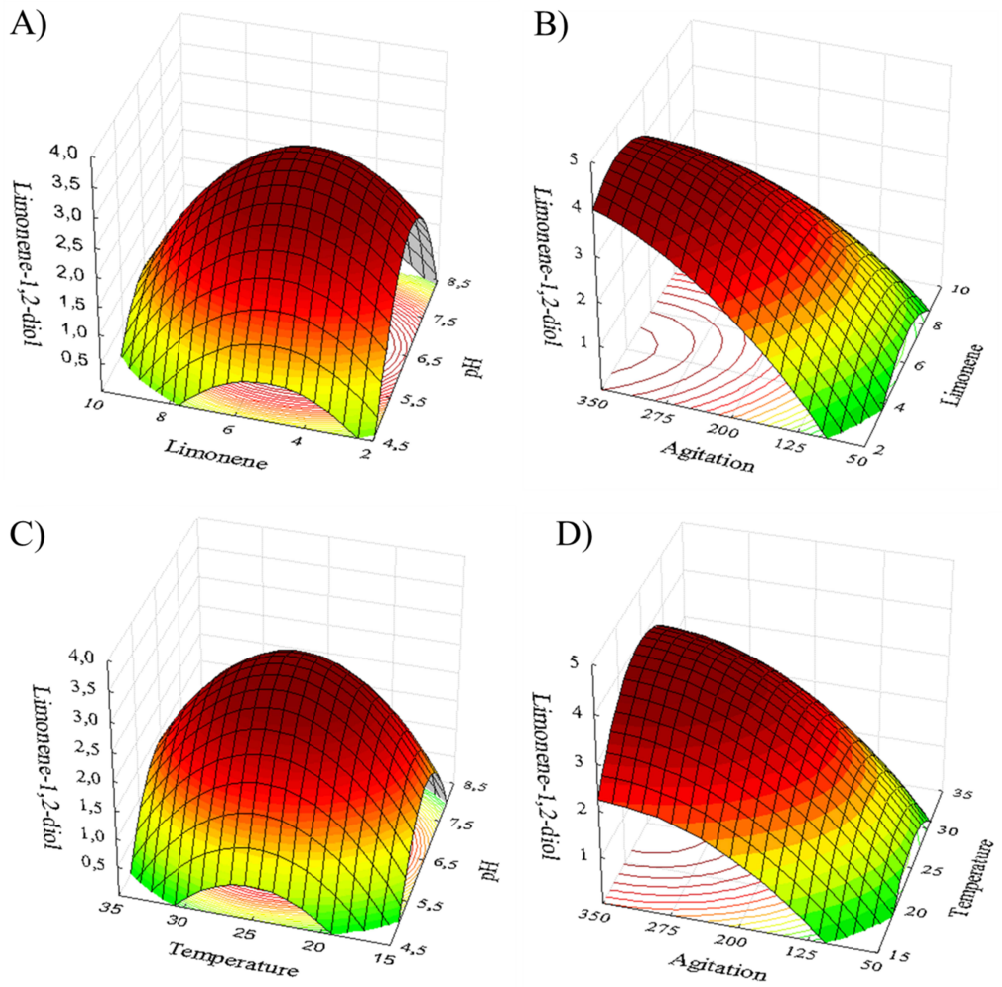

Figure 5 – Response surfaces of limonene-1,2-diol production from the biotransformation of *S*-(–)-limonene as a function of (a) limonene (g/L) and pH, (b) agitation (rpm) and limonene (g/L), (c) temperature (°C) and pH, (d) agitation (rpm) and temperature (°C).

Figure 5 depicts the response surfaces obtained for bioflavor production, as a function of (a) limonene (g/L) and pH, (b) agitation (rpm) and limonene (g/L), (c) temperature (^{o}C) and pH, (d) agitation (rpm) and temperature (^{o}C). The profile of the response surfaces obtained was ideal, since all the figures presented the predicted optimal regions comprised inside the levels studied.

Regarding Fig. 5a, it can be observed that the system allows an important flexibility for this process, which applying the central range for both variables can lead to a great production of limonene-1,2-diol. In other words, pH value could range from 5.8 to 7.2 and limonene from 3.8 to 6.2 $g.L^{-1}$, maintaining the process at optimal conditions. In a comparable process, Bicas et al. (2008b) observed that the medium pH could vary from 5.2 to 8.2 without any significant changes in α-terpineol yield. The relation between agitation and limonene (Fig. 5b) clearly demonstrated the influence of the stirring for this process, where the results are increased as this factor is also increased, with best results close to 300 rpm. This behavior is in accordance with previous report with *Fusarium oxysporum* for *R*-(+)-limonene biotransformation, where the agitation of 200 to 310 rpm allows the best results for α-terpineol production (Bicas et al., 2008b). In addition, analyzing surface response, it is possible to observe that the best results can be obtained with temperatures ranging from 23 to 30 ^{o}C for the optimal bioconversion of *S*-(–)-limonene, while for *R*-(+)-limonene from 24 to 28 ^{o}C.

The most important to observe is that a strict control of the bioconversion conditions was not necessary, which simplifies the process even more. An analysis of Eq. 1 showed that the optimal conditions could be determined using mathematical methods (equation derivation), and the values obtained were: i) pH = 0 (codified value) and 6.5 (real value); limonene concentration = +0,46 (codified value) and 6.96 g/L (real value); temperature = 0 (codified value) and 25 ^{o}C (real value); agitation = +2 (codified value) and 350 rpm (real value). In these conditions, the predicted production of limonene-1,2-diol from the bioconversion of *S*-(–)-limonene would reach 3.72 g of product per liter of medium. Due to practical and economic reasons, and also considering the surface responses analyzed above and the important ranges to this process, it was decided to use pH 6.5; 5 $g.L^{-1}$ of *S*-(–)-limonene, 28 ^{o}C and 250 rpm of temperature and agitation, respectively, keeping the production of approximately 3.62 ± 0.05 $g.L^{-1}$.

4. Conclusion

This extensive study tested various agro-industrial residues as alternative culture medium for *Fusarium oxysporum* 152B biomass production, seeking economic viability and development of a sustainable process. The most promising results were obtained using cassava wastewater as a medium, and this biomass production was assessed by means of a 2^3 central composite design. The optimal medium and process condition for biomass of *Fusarium oxysporum* 152B development contained 100% of CWW medium at 30 ^oC and 240 rpm. In these conditions, the biomass obtained was approximately 29.22 ± 0.85 $g.L^{-1}$. The biocatalyst was then applied to the biotransformation of *S*-(–)-limonene into limonene-1,2-diol, through a sequential optimization strategy, starting with the selection of impacting variables followed by a 2^4 central composite design. The production of the bioflavor reached approximately 3.7 $g.L^{-1}$ under optimized conditions, pH 6.5; 5 $g.L^{-1}$ of *S*-(–)-limonene, 28 ^oC and 250 rpm of temperature and agitation, respectively. Thus, this study is the first description of the use of agro-industrial wastes for the production of biocatalyst used in the production of limonene-1,2-diol. Moreover, as far as the authors know, this study can be considered as the first full optimization process for the production of this compound, as well as one of the highest concentrations ever reported for this biotechnological aroma.

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Capítulo 5

Comparative study of the bioconversion process using the *R*-(+)- and *S*-(–)-limonene as substrates for *Fusarium oxysporum* 152B

ABSTRACT

This study focused on the comparison of the bioconversion process of *S*-(–)-limonene into limonene-1,2-diol with the already established biotransformation of *R*-(+)-limonene into α-terpineol using the same biocatalyst in both processes, *Fusarium oxysporum* 152B. The bioconversion of the *S*-(–)-isomer was tested with cell permeabilization, in anaerobic conditions and using a biphasic system. When submitted to permeabilization trials, the biocatalyst has shown a relatively high resistance, notwithstanding no production of limonene-1,2-diol and loss of activity of the biocatalyst was observed with intense cell treatment, indicating complete loss of cell viability. Furthermore, results showed that this can be characterized as an aerobic system, catalyzed by a limonene-1,2-epoxide hydrolase, with an intracellular nature and cofactor dependent, considering that the product was not detected with an anaerobic process. Finally, this is the first report that characterizes the bioconversion of *R*-(+)- and *S*-(–)-limonene by cellular detoxification using ultra-structural analysis.

Keywords: Monoterpenes, Biotransformation, Natural flavor compounds, Anaerobic biotransformation, Cell permeabilization.

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) (Berger, 2007; Bicas et al., 2010a). The scientific literature contains many examples of reviews dealing with the chemical reactions of terpenes to produce flavors (Swift 2004) and the biotransformation of volatile terpenes for aroma production (Bicas et al., 2009; De Carvalho and 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). 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 (Van der Werf et al., 1997; Krings and 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 (Krings and Berger, 1998).

A number of reports in the literature concerned with the biotransformation of limonene leading to many oxygenated derivatives were reviewed by Maróstica and Pastore (2007a) and Duetz et al. (2003). 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.

One interesting product obtained from the biotransformation of limonene is known as α-terpineol. This product is a stable alcohol widely distributed in nature

typically applied in household products, cosmetics, pesticide, flavor preparations and is one of the most commonly used perfume chemicals (Bauer et al., 2001; Demyttenaere et al., 2001), and the production of this monoterpene alcohol has been described using a wide range of microorganisms as catalysts (Braddock and Cadwallader, 1995; Tan and Day, 1998ab; Tan et al., 1998; Adams et al., 2003; Bicas et al., 2008ab). Another product obtained from the bioconversion of the monoterpenic substrate is limonene-1,2-diol, described as possessing a cool minty aroma and consumed mainly in flavors used in mint preparation, alcoholic and nonalcoholic beverages, chewing gun, gelatins/puddings and other food products (Burdock and Fenaroli, 2010).

Few reports studied the bioconversion of both isomers of limonene, *R*-(+)- and *S*-(–)-limonene, such as performed by Demyttenaere et al. (2001), Adams et al. (2003) and Bicas et al. (2008b), aiming to comparing the results, metabolic pathways and characterization of metabolism.

Thus, the main objective of this work was to establish a characterization study of the biotransformation of *S*-(–)-limonene into limonene-1,2-diol, studying improvements to this processo with cells permeabilization, in anaerobic conditions and with the adoption of a biphasic system. In addition, these results were compared with the literature recently produced using the same microorganism with the *R*-(+)-limonene as substrate for the production of α-terpineol. Furthermore, this study also aimed to characterize and compare the bioconversion of *R*-(+)- and *S*-(–)-limonene using ultra-structural analysis.

2. Material and methods

2.1. Microorganism and chemicals

The microbial strain employed in this study was isolated from the northeast Brazilian fruits, and it was identified as *Fusarium oxysporum* 152B (Prazeres et al., 2006). The fungal strain was maintained on Yeast Malt (YM) Agar (in $g.L^{-1}$: agar = 20; glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3, pH ~ 6.7) and stored at 4°C.

The chemical standard used as substrate in this study were *R*-(+)-limonene (98% purity, Sigma-Aldrich) and *S*-(–)-limonene (96% purity, Sigma-Aldrich). The reagents *n*-hexadecane (99% purity, Sigma-Aldrich) and cyclohexanol (99%, Sigma-Aldrich) were kept under refrigeration (4 ^{o}C). Ethyl acetate and *n*-decane used as solvent and internal standard, respectively, were of best commercial grade.

2.2. Inoculum preparation

A piece of agar (approximately 1.5 cm^2) with a pre-grown culture of *F. oxysporum* 152B (72-h old) was transferred to a 250 mL conical flask filled with 50 mL of the agro-industrial residue known as cassava wastewater (or manipueira) medium. The material was homogenized under sterile conditions with an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After incubation at 30 °C and 200 rpm for 48 h, the biomass was recovered by vacuum filtration using a Buchner funnel and paper filter Whatman n°1 (Bicas et al., 2008a).

2.3. Comparative study of the bioconversion process using *R*-(+)- and *S*-(–)-limonene

The biotransformation process of *S*-(–)-limonene into limonene-1,2-diol by *Fusarium oxysporum* 152B was characterized following the methodologies described below and the results were compared with specific references to the biotransformation of *R*-(+)-limonene into α-terpineol, recently published by Bicas et al. (2008a) and Bicas et al. (2010b) using the same fungal strain *Fusarium oxysporum* 152B. All the experiments were performed at least in triplicate.

2.3.1. Biotransformation procedure under optimized conditions

The biomass was weighed and 3 g was distributed amongst 250 mL conical flask filled with 50 mL of mineral (MM) medium (in $g.L^{-1}$: $MgSO_4.7H_2O$ = 0,5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0,5 e Fe_2SO_4 = 0,001) (Brunerie et al., 1987). Subsequently, 5 $g.L^{-1}$ of *R*-(+)- and *S*-(-)-limonene were added to each experiment. The flasks were incubated under optimized conditions for each substrate, for the *R*-(+)-isomer, the conditions were 30 °C of termperature and 200 rpm of agitation (Bicas et al., 2010b), while for the *S*-(–)-limonene were pH 6.5, 28 °C and 250 rpm of temperature and agitation, respectively. Periodically, 1 mL samples were taken in order to monitor the consumption of substrate and volatile compounds produced until 96 h by gas chromatography (GC-FID).

2.3.2. Influence of cell permeabilization

The influence of cell permeabilization on the biotransformation yield was evaluated following different protocols. The biomass obtained (Section 2.2) was treated as follows: i) for 1 h at 30°C and 150 rpm with 6% ($v.v^{-1}$) diethyl ether/chloroform

mixture; ii) successive cycles of freezing (4 h at freezing temperature and 30 minutes at 30 ^{o}C, repeated three times); iii) sonication cycle (1, 5 and 10 min repeated three times, keeping the system at low temperature, approximately 5 ^{o}C) and iv) lyophilization process in a freeze-drier (Equipamentos Terroni, model LS 3000, São Paulo, Brazil) (adapted from Fontanille and Larroche (2003) and Bicas et al. (2010b)).

2.3.3. Biotransformation in anaerobic conditions

For the anaerobic biotransformation, 10.0 g of the fresh biomass (Section 2.2), 0.5%, v.v^{-1} of substrate *S*-(–)-limonene and 200 mL of mineral medium were transferred to a conical flask with two entries (one at the base and another at the neck) plugged with a rubber stopper. At the beginning of the conversion, as well as after each sampling, the medium was flushed by bubbling N_2 from the base entry for 5 min to eliminate the oxygen dissolved in both the medium and headspace. The flasks were incubated at the same conditions as described above for 96 h (adapted from Bicas et al., 2008a).

2.3.4. Biotransformation in biphasic system

For the biphasic biotransformation system, biomass produced following the protocol described in section 2.2 was distributed amongst 250 mL conical flask filled with 25 mL of aqueous phase (consisting of MM medium) and 25 mL of the organic phase in test. For this study, the organic phases selected were hexadecane and cyclohexanol. *R*-(+)- and *S*-(–)-Limonene was added to reach 5 g.L^{-1} of organic phase. The flasks were incubated at 30 ^{o}C and 200 rpm. Periodically, 1 µL of the organic phase was directly injected on GC-FID port in order to monitor the consumption of substrate and production of limonene-1,2-diol (adapted from Bicas et al., 2008b).

2.4. Determination and quantification of the volatile compounds

Samples were extracted (40 s in Vortex) with the same ethyl acetate volume. After phase separation, 1 µL of the organic fraction was dried over sodium sulphate and injected in split mode (split ratio of 1:10) 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 lenght x 0.25 mm i.d. x 0.25 µm of film thickness). Helium was used as carrier gas (1.0 mL.min^{-1}) and the oven temperature was kept at 80 ^{o}C for 3 min, raised at 20 ^{o}C min^{-1} until 200 ^{o}C and held for 4 min. Temperatures of the injector and detector were kept at 250 ^{o}C. Substrates and products were quantified by a

calibration curve using *n*-decane as internal standard. All experiments were performed in triplicate.

2.5. Ultra-structural analysis

2.5.1. Scanning electron microscopy (SEM)

The fungal strain was fixed for 24 hours in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in ascending alcohol series (70–100%), subjected to two acetone 100% baths of 15 min each and critical point dried (Balzers CPD 030). After dehydration the material was placed on aluminum supports attached with double-faced tape and sputter coated with gold (in sputtering Balzers SD 050). The fungi were examined with a Jeol P15 SEM and photographed.

2.5.2. Transmission electron microscopy (TEM)

The microorganism was fixed for 4 hours in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Post-fixation was carried out in 1% osmium tetroxide in the same buffer. En bloc staining with 2% uranyl acetate was followed by a series of ethanol dehydration steps. The microorganism was then embedded in Eppon resin and sectioned with a Leica ultramicrotome. Ultra-thin sections were stained with uranyl acetate and lead and photographed with a CM 100 Philips TEM at 80 kV.

3. Results and discussion

3.1. Comparison of bioconversion process using different isomers of limonene

In former studies, Bicas et al (2008a) described the optimization of the ten main process variables involved in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *Fusarium oxysporum* 152B through a Plackett-Burman matrix with 16 assays, including the effect of the medium composition, substrate concentration, cultivation conditions and inoculum size, followed by central composite design methodology. Thus, the authors improved significantly the production of *R*-(+)-α-terpineol, from 450 $mg.L^{-1}$ previously described by Maróstica Jr and Pastore (2007b) to 2.44 $g.L^{-1}$ of the monoterpene alcohol. Following, another study conducted by the same group has proposed an integrated process for the co-production of lipase and *R*-(+)-α-terpineol was proposed, which was the concentration of this product was re-quantified with another technique and reached approximately 4 $g.L^{-1}$, after 48 h of process which remained stable until reaching 96 h (Bicas et al., 2010b).

However, as observed in this thesis, the biotransformation of *S*-(-)-limonene did not result in the same product. The compound obtained was identified by mass spectrometry as limonene-1,2-diol (90% similarity), which was confirmed with the mass spectra and also the retention index of the commercial standard (Sigma-Aldrich). The production of limonene-1,2-diol reached 1.2 $g.L^{-1}$, under non optimized conditions (Chapter 2 and 3).

This bioconversion process is a result from the ring double bond epoxidation of limonene, followed by the corresponding diol formation. However, as proved elsewhere (Chapter 2 and 3), the production of the intermediate limonene-1,2-epoxide was not detected, indicating that the reaction proceeds mainly for the diol production. When the microorganism was evaluated by its potential to convert limonene-1,2-epoxide, the accumulation of limonene-1,2-diol led to suggest that *Fusarium oxysporum* might have another pathway for limonene, similar to that described by Van der Werf et al. (1999).

Thus, the bioconversion processes of *R*-(+)- and *S*-(–)-limonene were performed according to the optimized process conditions for both production of α-terpineol (as published recently by Bicas et al. (2008b)) and limonene-1,2-diol (as described in Chapter 4 of this PhD Thesis) by *Fusarium oxysporum* 152B. It was decided to repeat the experiment using *R*-(+)-limonene as substrate to ensure experimental control and improve the parameters for a suitable process comparison.

Figure 1 graphically represents a comparison of the profile of the two bioconversion processes. It is possible to observe that, in this process, the results obtained for the biotransformation of *R*-(+)-limonene reached approximately 3.9 $g.L^{-1}$ of α-terpineol, under the conditions presented by Bicas et al. (2010b). The difference observed when compared with the reported value (~ 4 $g.L^{-1}$) by the authors may be related to the analyser, with small variations in concentrations, rounding or calibration curve. Some slight variations in the bioconversion profile with different isomers of limonene can be observed. However, the products converge to a similar production. Table 1 summarizes all comparisons observed in bioconversion of *R*-(+)- and *S*-(–)-limonene characterizations in bioconversion process.

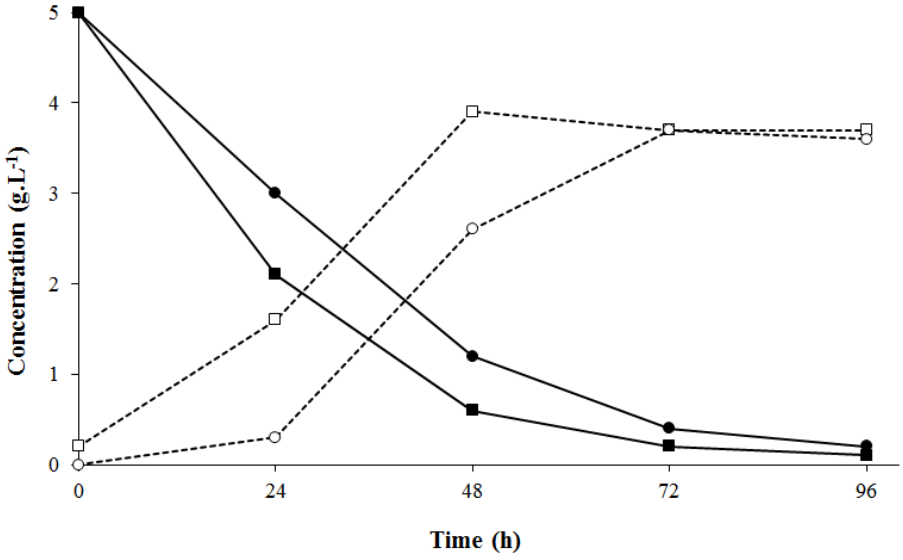

Figure 1 - Comparison of the profile between the biotransformation of *R*-(+)-limonene (■) into α-terpineol (--□--) and the bioconversion of *S*-(–)-limonene (●) into limonene-1,2-diol (--○--) by *Fusarium oxysporum* 152B.

Assays carried out with a mixture of both substrate at the same concentration, and added to the same procedure (reaching a final concentration of 5 $g.L^{-1}$ of substrate) showed that the rate of conversion was much higher for α-terpineol, while limonene-1,2-diol was found only in small concentrations, indicating that the reaction proceeds favourably for the production of α-terpineol where the activity obtained for the *R*-(+)-isomer was at least three times higher than for *S*-(–)-limonene (data not shown). Braddock and Cadwallader (1995) observed that a *Pseudomonas gladioli* was capable to convert *R*-(+)- and *S*-(–)-limonene, and the activity obtained for the *R*-(+)-isomer was more than 10 times higher. Additionally, as may be seen in Table 1, the production of α-terpineol reached maximum concentration at 48 h while limonene-1,2-diol only at 72 h by *Fusarium oxysporum* 152B.

Braddock and Cadwallader (1995) observed that the enzyme, from the bacterium *Pseudomonas gladioli*, responsible for converting limonene into α-terpineol was enantiospecific and enantioselective for the conversion of *R*-(+)-limonene into *R*-(+)-α-terpineol. On the other hand, a *Sphingobium* sp. strain showed lower selectivity as it converted both *R*-(+)- and *S*-(–)- isomers specifically into *R*-(+)- and *S*-(–)-α-terpineol, respectively, virtually with the same efficiency (Bicas, 2010c). In this case, authors

found traces of limonene-1,2-diol as a by-product of the biotransformation of *S*-(–)-limonene.

Table 1 – Comparative study of the bioconversion of *R*-(+)- and *S*-(–)-limonene performed by *Fusarium oxysporum* 152B.

Variables	Substrates	
	***R*-(+)-limonene**[a]	***S*-(-)-limonene**
Product	α-Terpineol	Limonene-1,2-diol
Concentration	4 $g.L^{-1}$	3.7 $g.L^{-1}$
Time	48 h	72 h
Extraction efficiency	32.6%	42.2%
Enzyme involved	Hydratase	Limonene-1,2-epoxide hydrolase
Inducibility	Non inducible	Non inducible
Nature	Intracellular	Intracellular
Conditions	Anaerobic	Aerobic
Metabolism	Detoxification	Energy

[a] Data obtained for the specific literature Bicas et al. (2008a) and Bicas et al. (2010b) with the same biocatalyst used in this process, *Fusarium oxysporum*, with *R*-(+)-limonene as substrate for the biotransformation process.

It is important to observe the high capacity of this fungal strain in bioconversion of limonene, since the concentrations observed for both products are very promising. Several processes studied the biotechnological production of α-terpineol, considering that concentrations reported are higher only for the distinct process developed by Bicas et al. (2008b) and Bicas et al. (2010c) using *Sphingobium* sp. as biocatalyst.

Meanwhile, the production of limonene-1,2-diol was less explored in recent years, and the yield obtained from this process is also among the highest concentrations ever described. Abraham et al (1985) described a well distinguished process of recovering good yields of *1S,2S,4R*-limonene-1,2-diol from *R*-(+)-limonene with continuous substrate feeding in a 100 L bioreactor filled with 70 L of culture medium. When 1,300 g of substrate were used, 900 g of *1S,2S,4R*-limonene-1,2-diol and small amounts of the *1R,2R,4R*-diasteroisomer were recovered after a 96 h-process, representing an economic way of preparing diols. In addition, *Aspergillus* sp., i.e. *A. cellulosae*, was capable of converting both enantiomers and the racemate of limonene into limonene-*trans*-1,2-diol as the main product (Noma et al., 1992). In fact, some authors concluded that diols are common intermediates in the monoterpene metabolism of fungi (Mukherjee et al., 1973).

3.2. Influence of biomass permeabilization treatment on biotransformation

The microbial cell membrane acts as an osmotic barrier and, most often, precursors and products involved in biotransformation processes move across this structure by passive diffusional transport. When intact microbial cells are used in biocatalysis, therefore, the cell membrane may severely hamper a bioconversion from working optimally because substrate influx and product efflux by diffusion can be slow with regard to the maximal reaction rate (Van der Werf et al., 1995, 1992).

For these reasons, cells used in commercial bioconversions are often permeabilized, in order to improve the rate of exchange between intra- and extra-cellular media. Several methods have been described for this purpose (Felix 1982). Microbial cells, for example, can also be effectively broken by physical means. Sonication is convenient and effective on the laboratory scale, while high-pressure extrusion equipment is currently the method of choice for industry (Cabral, 2001).

In this sense, the purpose of the research presented has been to determine the effect of certain factors upon the fungal biocatalyst. These factors include after successive freezings and length of storage between each successive freezing, solvent permeabilization and the effect of lyophilization in the fungal biomass. In this sense, bioconversion experiments were carried out with either concentrated fresh cells or crude enzymatic extracts. The latter could be considered as a form of the biocatalyst unable to perform cofactor-dependent reactions (Bicas et al., 2008b).

It can be noted in Figure 2 that in the case of a single freezing-thawing, *Fusarium oxysporum* manifests a percentage survival which is constant and independent of the initial cell concentration, evidencing a good resistance when treated with this initial technique. When submitted to successive cycles (see Methods), it is also possible to observe a considerable decrease in its capacity when applied in the bioconversion of *S*-(–)-limonene process, where activity was less than 50 %.

The positive effect of the freezing–thawing procedure on the bioconversion efficiency was demonstrated in a recent study, where authors showed that this treatment led to a large increase in both reaction rate and yield of isonovalal production (Fontanille et al., 2002). This process lead to a semi-permeabilization technique that causes cell ruptures releasing part of the intracellular enzymes (Fontanille and Larroche, 2003). Bicas et al. (2010c) using this technique shown that freeze–thawed cells accelerated the process for the biotransformation of *R*-(+)-limonene into *R*-(+)-α-

terpineol, indicating release of the enzyme responsible for this conversion and confirming its intracellular nature.

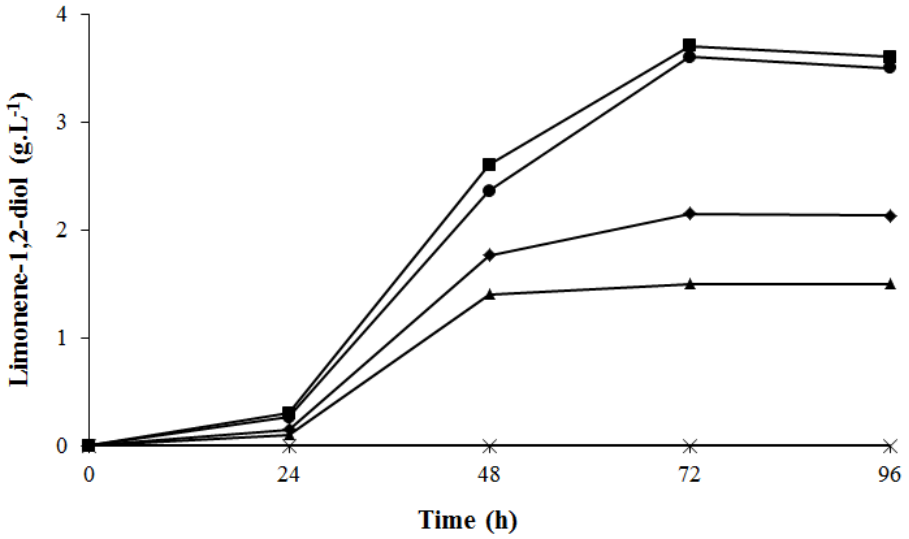

Figure 2 - Influence of cell permeabilization in the production of limonene-1,2-diol (control experiment: ■). The techniques employed were: One cycle of freezing (●), successive cycles of freezing–thawing (▲), solvent permeabilization (♦) and solvent permeabilization combined with successive cycles of freezing–thawing (x).

When solvent treatment and successive cycles of freezing–thawing were combined, it was found that there was no production of limonene-1,2-diol and loss of activity of the biocatalyst, indicating complete loss of cell viability.

Lyophilization/freeze-drying is defined as stabilizing process in which the substances are first frozen followed by a sublimation (primary drying) and desorption (secondary drying) step in order to reduce the water content to levels that will no longer support biological growth or chemical reactions (Jennings, 1999). The lyophilization process was tested due to great interest from a technical point of view, since it standardizes and simplifies the inoculation besides enabling biocatalyst storage (Bicas et al., 2010b,c). When the concentrated biomass was lyophilized and tested in bioconversion process, it was not detected the production of limonene-1,2-diol due to a possible inactivation of the biocatalyst, which showed no activity. Hence, it is supposed that, somehow, the lyophilization process damages the enzyme, reducing its activity.

Despite that, the *Fusarium oxysporum* 152B strain employed in this study has shown a relatively high resistance to cell permeabilization.

The suspension resulting from the permeabilization operation was membrane filtered (0.45 μm) to remove the remaining viable cells and tested in bioconversion process. The results obtained with the crude enzymatic extracts of *Fusarium oxysporum* for the biotransformation of *S*-(–)-limonene into limonene-1,2-diol have shown that this reaction was catalyzed by a cofactor-dependent enzyme. Conversely, the enzyme responsible for the biotransformation of *R*-(+)-limonene into α-terpineol by the same fungal strain seemed cofactor-independent (Maróstica Jr and Pastore, 2007b; Bicas et al., 2010b), such as the α-terpineol dehydratase from *P. gladioli* (Cadwallader et al., 1992).

Furthermore, membrane permeabilization did not improve the biotransformation rate processes, since it was proven that the cofactor was needed for this bioconversion process, it was not possible to use resting cells without respiratory activity.

3.3. Biotransformation in anaerobic conditions

Biotransformation in anaerobic conditions is one of the tools used to characterize the enzyme involved in the process, especially when related to required cofactors (Bicas et al., 2008b). From a technical point of view, this is also an important piece of information since it establishes the need for the system aeration (Bicas et al., 2010c).

The conduction of the bioconversion process of *S*-(–)-limonene in anaerobic conditions showed the need for oxygen for its activity. In this approach, limonene-1,2-diol production was not detected in the culture medium, characterizing an aerobic system, and thus, cofactor dependent (see Table 1). This is in agreement with the findings published by Bicas et al. (2008b) that showed that the limonene-1,2-epoxide hydrolase, first enzyme of the metabolic route, was cofactor-independent, but the following steps necessarily needed cofactors and, thus, could be performed only by aerobically operated whole cells. In addition, Bicas et al. (2010b) showed that the biotransformation of *R*-(+)-limonene into *R*-(+)-α-terpineol, although it occurred at a lower rate, was catalyzed by an oxygen-independent enzyme and, consequently, independent of cofactors. The independence of cofactors would also be related to the increase in the biotransformation rate with freeze–thawed biomass observed by these authors, because only cofactor-independent enzymes would act in the extracellular medium.

Concluding, these observations reinforce the hypothesis that *Fusarium oxysporum* 152B presents two parallel metabolisms for isomers of limonene, depending on the substrate. The bioconversion of *S*-(–)-limonene passing through limonene-1,2-oxide and limonene-1,2-diol, an aerobic process leading to energy production, while the with *R*-(+)-limonene, leading to α-terpineol, was oxygen-independent and could be viewed as a process for detoxification of the medium (Griffin et al. 1999; Bakkali et al., 2007).

3.4. Biotransformation in biphasic system

In microbial cultivation process, particularly on high-cell-density cultures, productivity is often limited by the transport of a substrate, of which oxygen is one important example (Puthli et al., 2005). In this perspective, there is a great concern about the disadvantages associated with the high aeration and agitation rates necessary to maintain high levels of oxygen solubility in the medium, due to the cell sensitivity to hydrodynamic stress, the high power consumption and increasing in operation costs (Gomes et al., 2007).

Thus, some authors discuss the possibility of addition of an organic solvent to the system, a water-immiscible phase in which oxygen has a higher solubility aiming to improve oxygen transfer rate (Gomes et al., 2007; Ju et al., 1991; Mehrnia et al., 2005). The addition of an appropriate phase can reduce the problems associated with the low solubility of substrate and product, while minimizing their toxic effects for microorganisms (Bicas et al., 2013). One practical example of this technique is the well-developed process for the production of α-terpineol through the biotransformation of limonene, where concentrations up to 120-130 $g.L^{-1}$ of product were obtained using a *Sphingobium* sp. (Bicas et al., 2010c).

Despite the advantages provided by the adoption of this kind of system for some processes, in this study it was observed that limonene-1,2-diol produced from the bioconversion of *S*-(–)-limonene partitioned preferentially to the aqueous phase. This is in agreement with the results published by De Carvalho et al. (2000), who conducted partition studies with limonene-1,2-diol where this compound was dissolved in the aqueous phase (0–20 mM), while limonene-1,2-oxide in the organic phase. The consequence of this phenomena was the production of low concentrations of limonene-1,2-diol which (less than 1 $g.L^{-1}$ of organic phase), when accumulated in the aqueous phase, clearly affected the biocatalyst.

In this sense, the organic phase was changed to cyclohexanol, looking for another phase with a greater chance of success in the experiment in order to solubilize both substrate and product at this phase. However, the results showed that, even though a higher diol concentration was attained to this organic phase, the results were not satisfactory, reaching less than 2 $g.L^{-1}$ of organic phase, with more than 75 % of product detected in the aqueous phase.

It is important to emphasize that the accumulated product in the aqueous phase can play a significant inhibitory effect to biocatalyst cells that could, in part, explain the results observed. Although the results collected with this second solvent were better, the production of limonene-1,2-diol through the biotransformation of *S*-(–)-limonene was not possible. Similarly, Bicas et al. (2010b) reported the same inability to perform the process for the production of α-terpineol from the biotransformation of *R*-(+)-limonene.

3.5. Ultra-morphological and ultra-structural analysis of *Fusarium oxysporum* 152B

The ultra-morphological analysis was performed using a scanning electron microscopy (SEM) and aimed to detect similarities and differences between the ascomycete fungus *Fusarium oxysporum* 152B grown in absence of terpenes (glucose as carbon source), and compare with the biomass in contact with the terpenes *R*-(+)- and *S*-(–)-limonene used as substrates during the bioconversion process.

The ultra-morphological comparative analysis of the samples studied showed no significance differences between them (Figures 3, 4 and 5). These results shows that although in the presence of isomers of limonene in two of the culture media analyzed in this study, these terpenes do not have toxic activity that could promote morphological changes in this biocatalyst. Ultra-morphological and ultra-structural studies performed with the *Trichophyton mentagrophytes*, a dermatophyte fungus, describes morphological changes in their hyphae, which had presented collapsed and deformed when the microorganism was challenged with different concentrations of the terpene citral, eugenol, nerolidol and α-terpineol (Park et al., 2009). In this referred study, the toxicity of the terpene tested showed clear effects in the microorganism.

In detriment of the ultra-morphological analyzes that have not shown changes, the ultra-structural analysis showed the conspicuous differences between the fungus grown in absence of terpenes (Figures 6 and 7), and the fungi grown in the presence of terpenes *R*-(+)- and *S*-(–)-limonene (Figures 8-14). In the last cases, there are the

presence of abundant smooth endoplasmic reticulum (Ser), numerous vacuoles and vesicles, in which some of these are seen as Ser product (Figure 10). Therefore, these are evidences of the intense action of this microorganism in cellular detoxification that may occur through hydroxylation, isomerization and bioconversion of xenobiotics by the cytochrome P450 enzymes present in the membranes of smooth endoplasmic reticulum (Bernhardt, 2006; Isin and Guengerich, 2007; Mansuy, 1998).

During the detoxification and bioconversion process of terpenes, the presence of vesicles and autophagic vacuoles are evident. These features show the action on recycling membranes that are being actively used by the microorganism metabolism (Figure 9 - sections 26 and 27, and Figure 13 - sections 46 and 47).

The electron micrograph of *F. oxysporum* grown without the presence of terpenes clearly shows the cytoplasm rich in ribosome along with several mitochondria (Figures 6 and 7). These features demonstrate the high metabolic activity, where the intense protein synthesis of cells can be observed. However, the electron micrographs of cultured fungi with both *R*-(+)- and *S*-(–)-limonene showed a different morphological and physiological profile. In this case, the cytoplasm is presented mostly occupied by smooth endoplasmic reticulum, vesicles and vacuoles that characterize cells in detoxification state (Figures 8-14).

Bicas et al (2008b) observed that traces of limonene-1,2-diol were present throughout the growth phase (< 38 h) of *Pseudomonas fluorescens* and that α-terpineol was accumulated at low levels between 38 h to 44 h (end of exponential phase-beginning of the stationary phase). As discussed above, these observations support the hypothesis that *Fusarium oxysporum* has two parallel metabolisms to limonene isomers: one that occurs through the primary metabolism to produce energy, and the other passing through the secondary metabolism, which is dedicated to the limonene detoxification. The latter could be confirmed by the analysis of electron micrographs, which are the first ultra-structural report that characterize the bioconversion of terpenes by cellular detoxification.

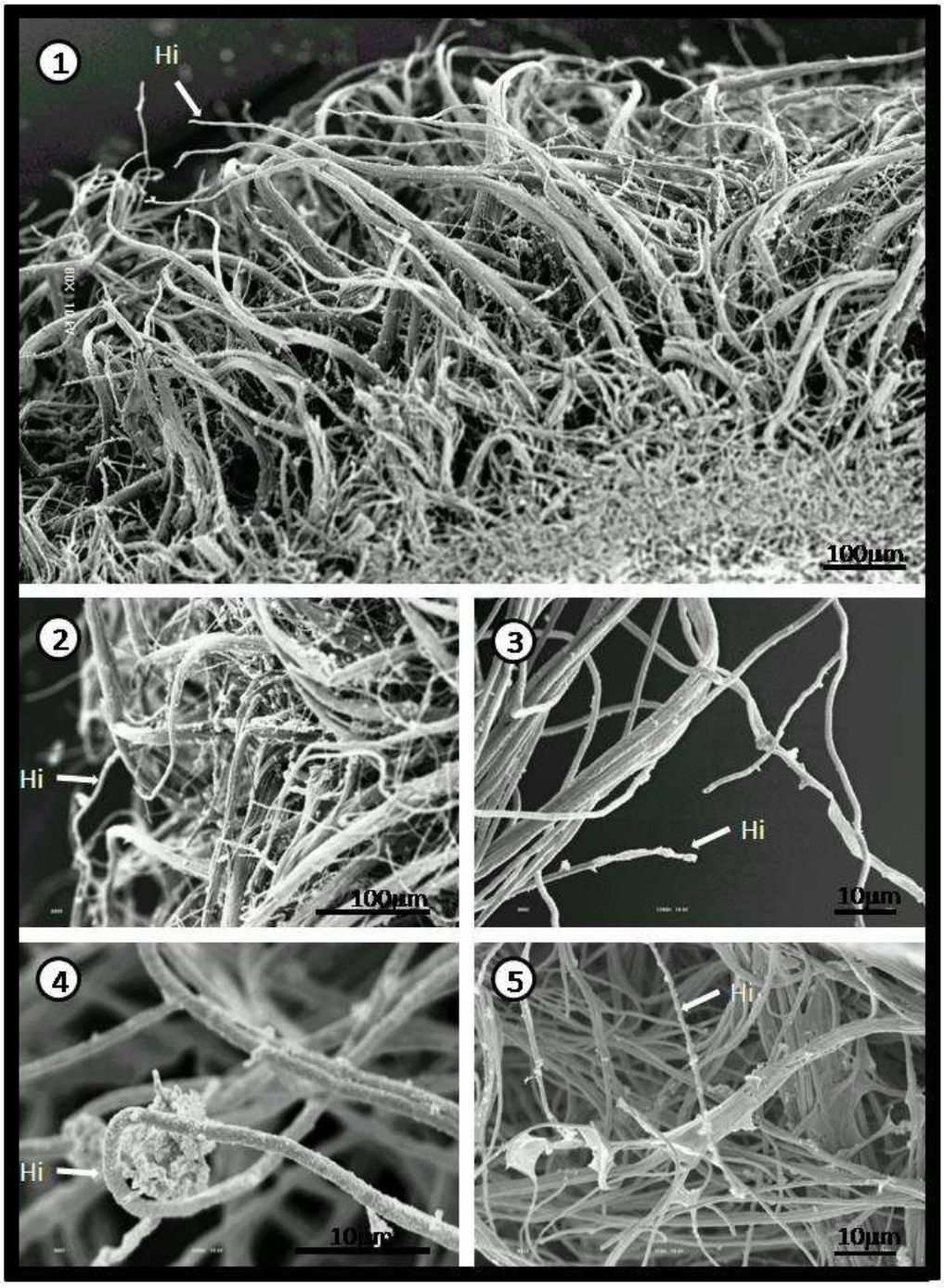

Figure 3 (Sections 1-5) - Scanning electron microscopy (SEM) showing the ultra-morphology of filamentous fungus grown in the absence of terpene using glucose as carbon source (control). Hi = hyphae.

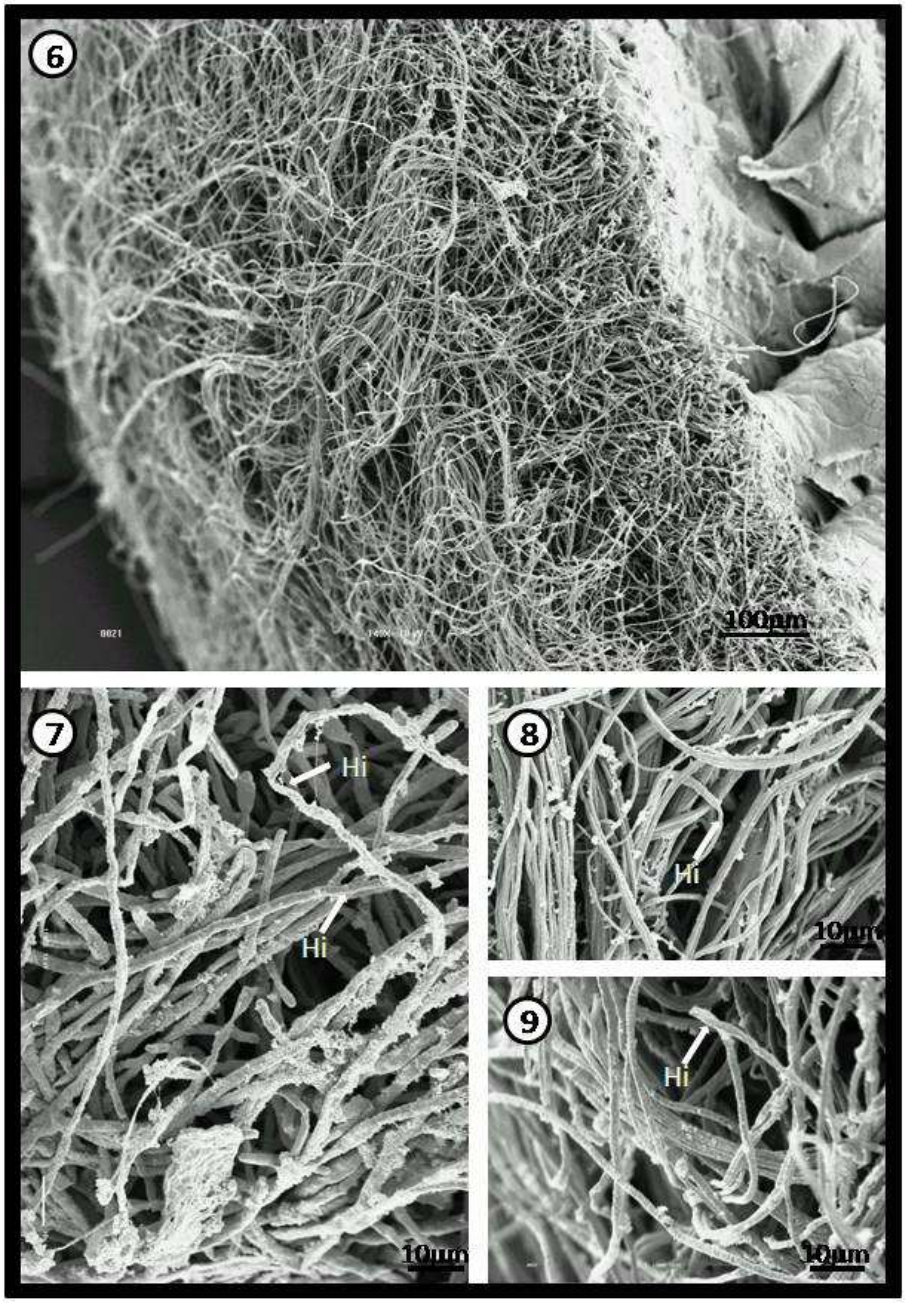

Figure 4 (Sections 6-9) - Scanning electron microscopy (SEM) showing the ultra-morphology of *Fusarium oxysporum* strain grown in the presence of *R*-(+)-limonene during biotransformation process to α-terpineol. Hi = hyphae.

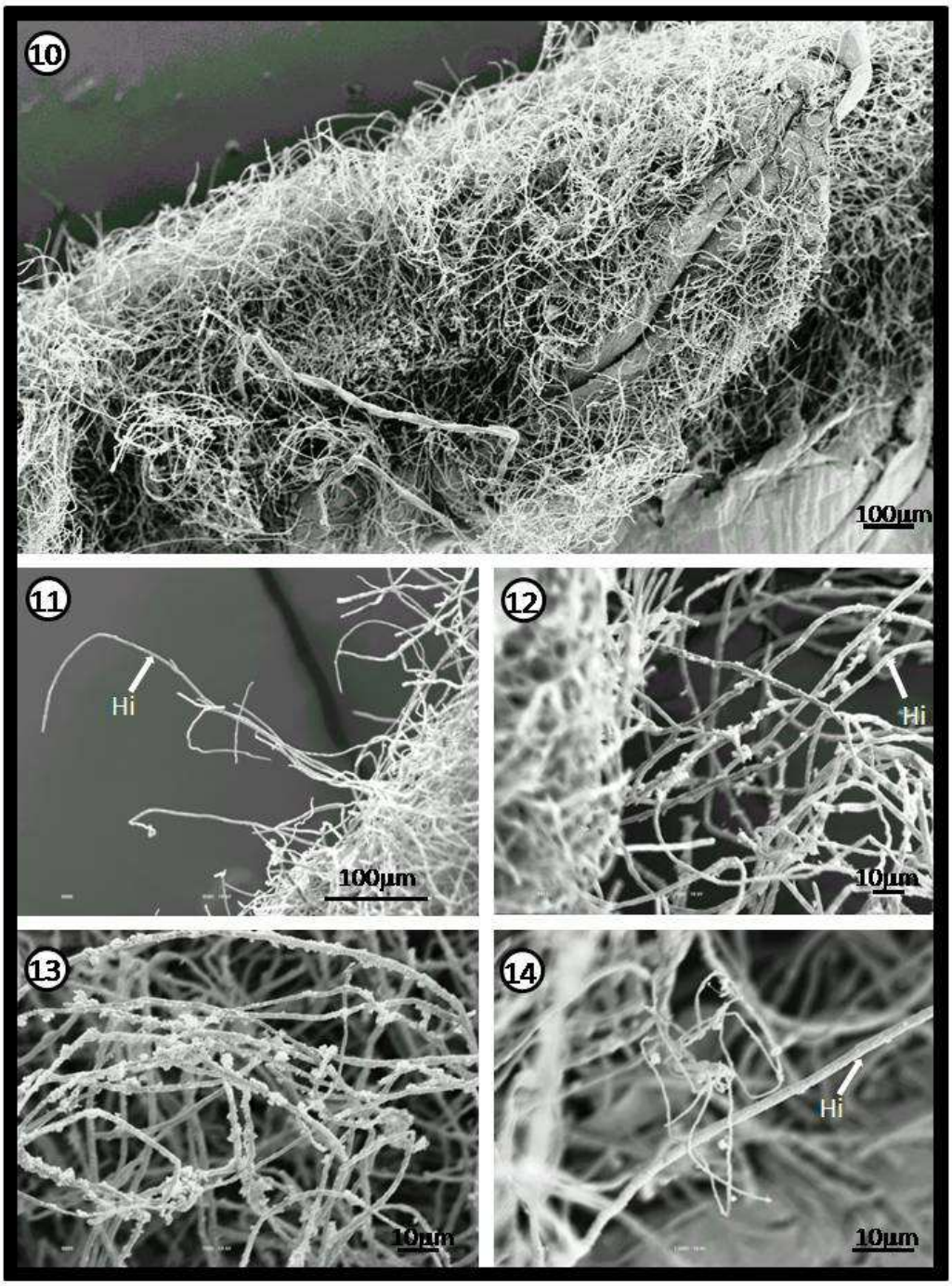

Figure 5 (Sections 10-14) - Scanning electron microscopy (SEM) showing the ultra-morphology of *Fusarium oxysporum* strain grown in the presence of *S*-(–)-limonene during bioconversion process into limonene-1,2-diol. Hi = hyphae.

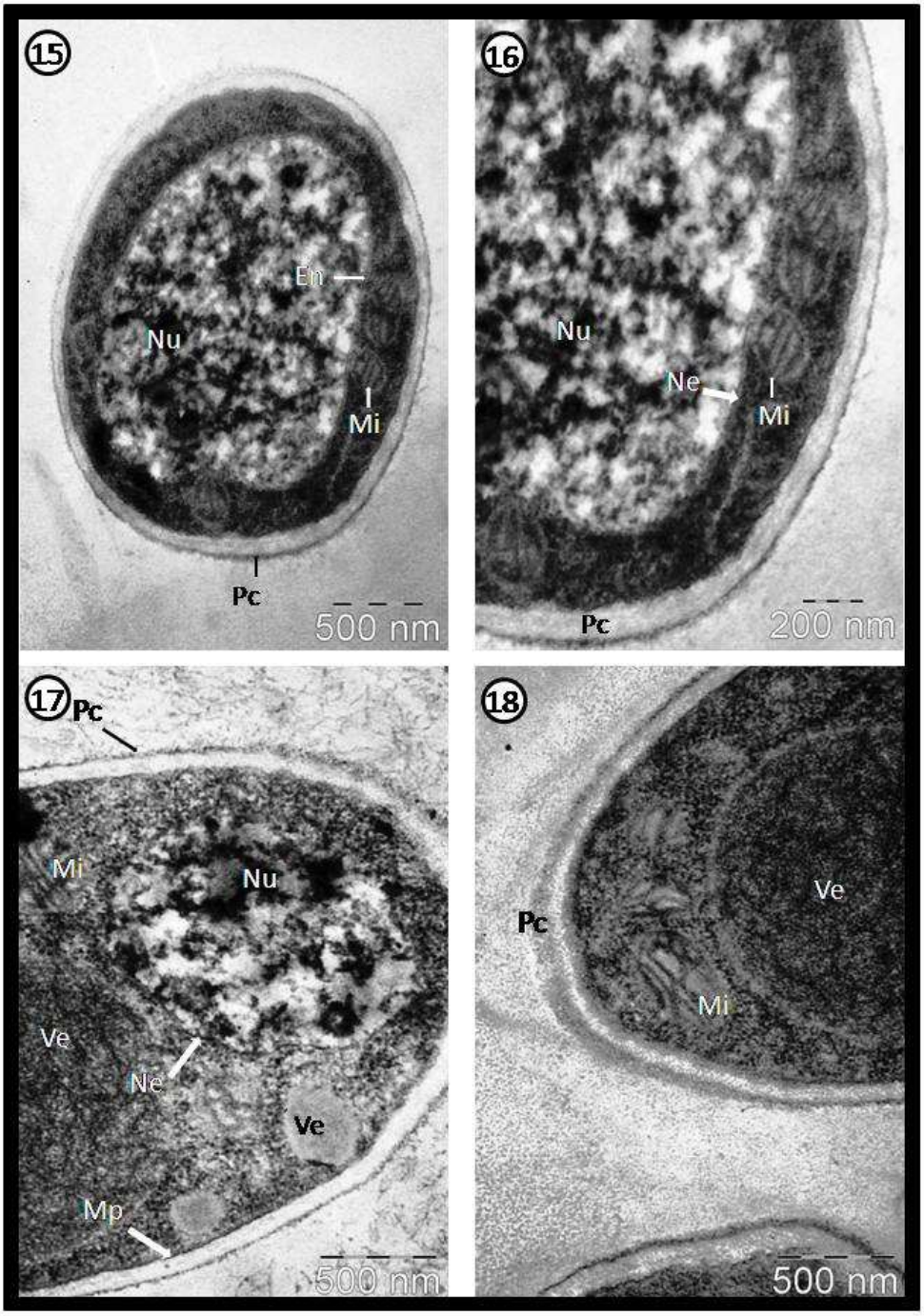

Figure 6 (Sections 15-18) - Electron micrograph showing cross sections of *Fusarium oxysporum* grown in the absence of terpenes using glucose as carbon source (control). En = nuclear envelope; Mp = plasma membrane, Mi = mitochondrion; Nu = nucleus; Pc = cell wall; Ve = vesicle.

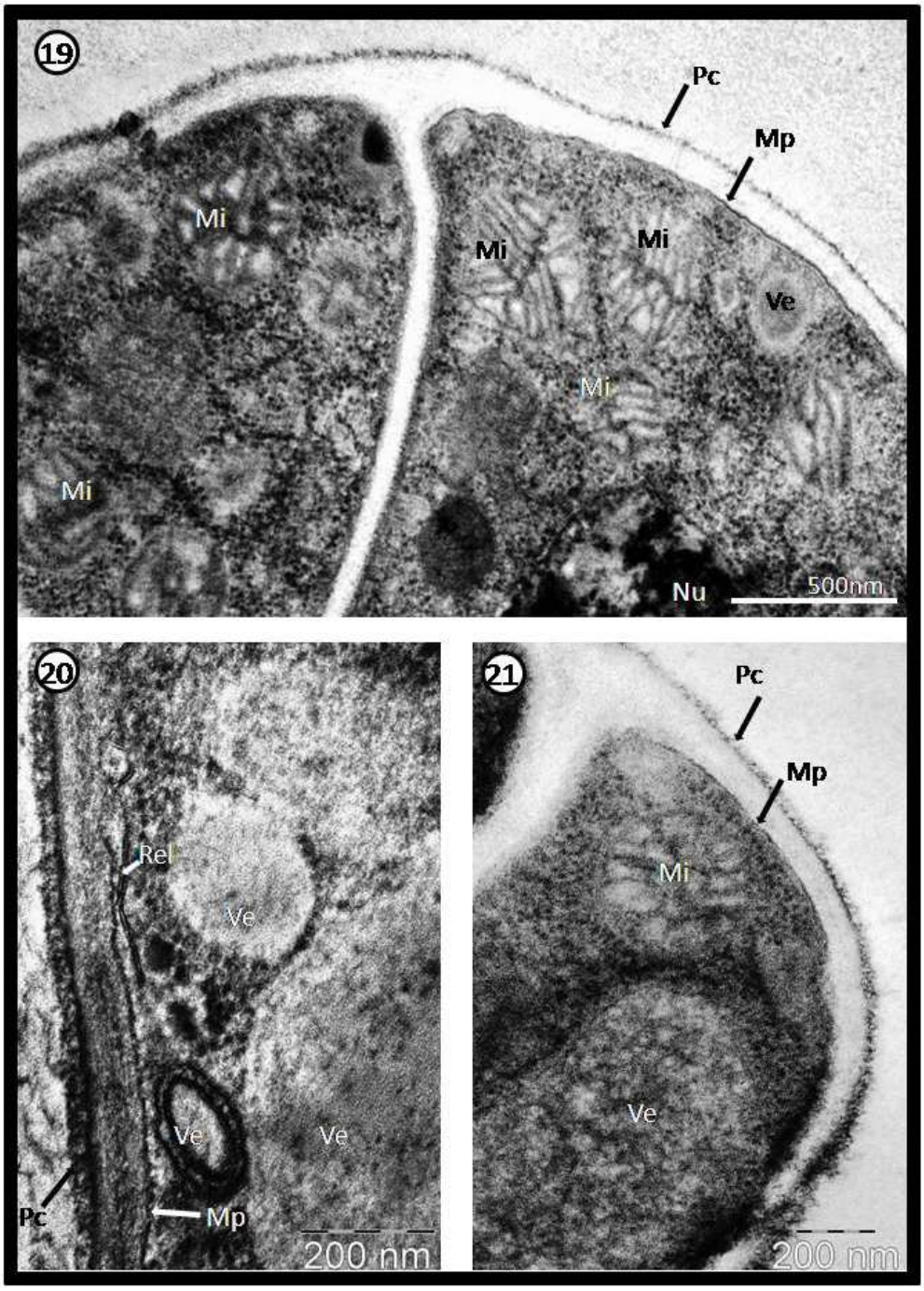

Figure 7 (Sections 19-21) - Electron micrograph showing cross sections of *Fusarium oxysporum* grown in the absence of terpenes using glucose as carbon source (control). Mp = plasma membrane, Mi = mitochondrion, Pc = cell wall; Rel = smooth endoplasmic reticulum; Ve = vesicle.

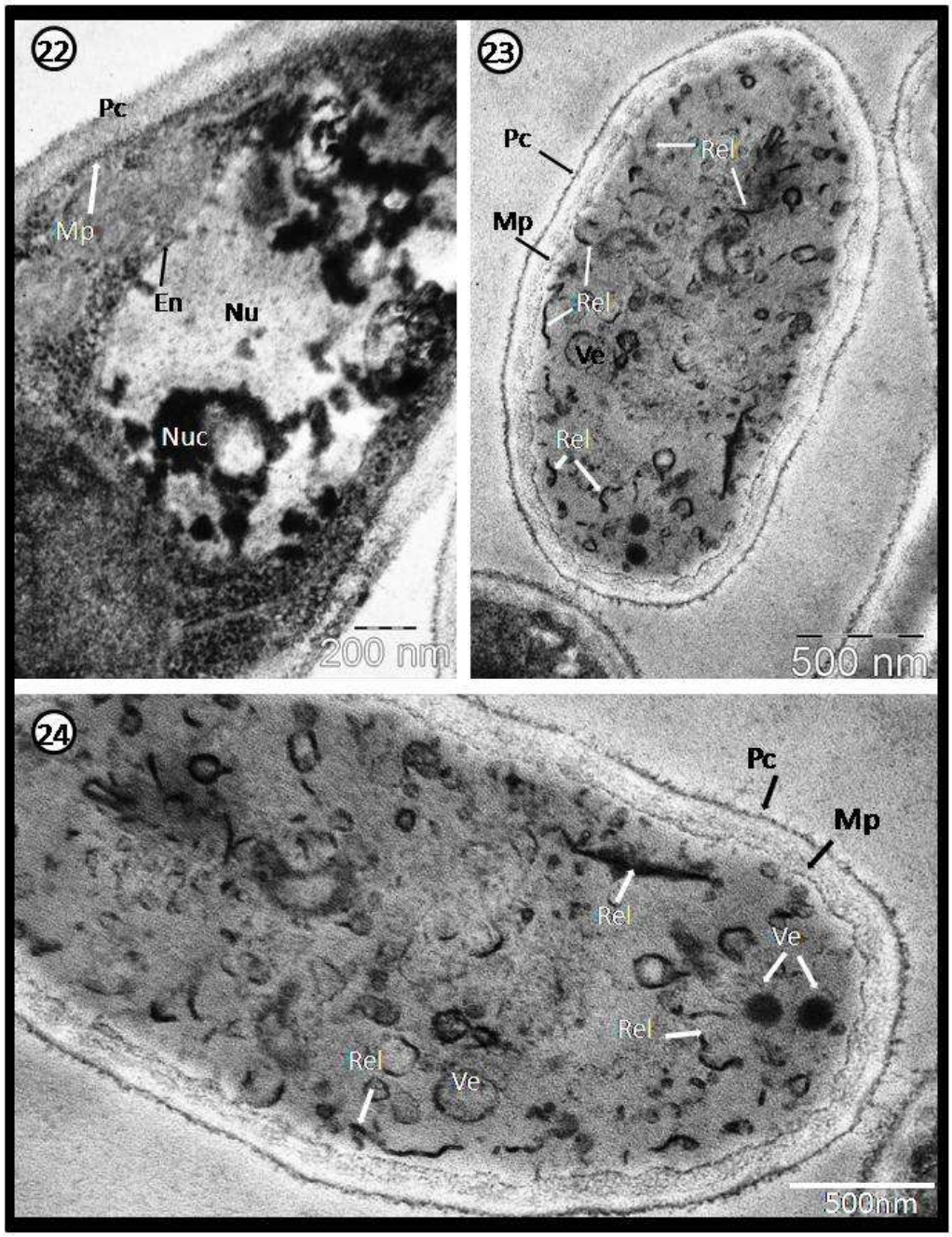

Figure 8 (Sections 22-24) - Electron micrograph showing cross sections of *Fusarium oxysporum* grown in the presence of *R*-(+)-limonene during biotransformation process into α-terpineol. En = nuclear envelope; Mp = plasma membrane, Mi = mitochondrion; Nu = nucleus; Pc = cell wall; Rel-smooth endoplasmic reticulum; Ve = vesicle.

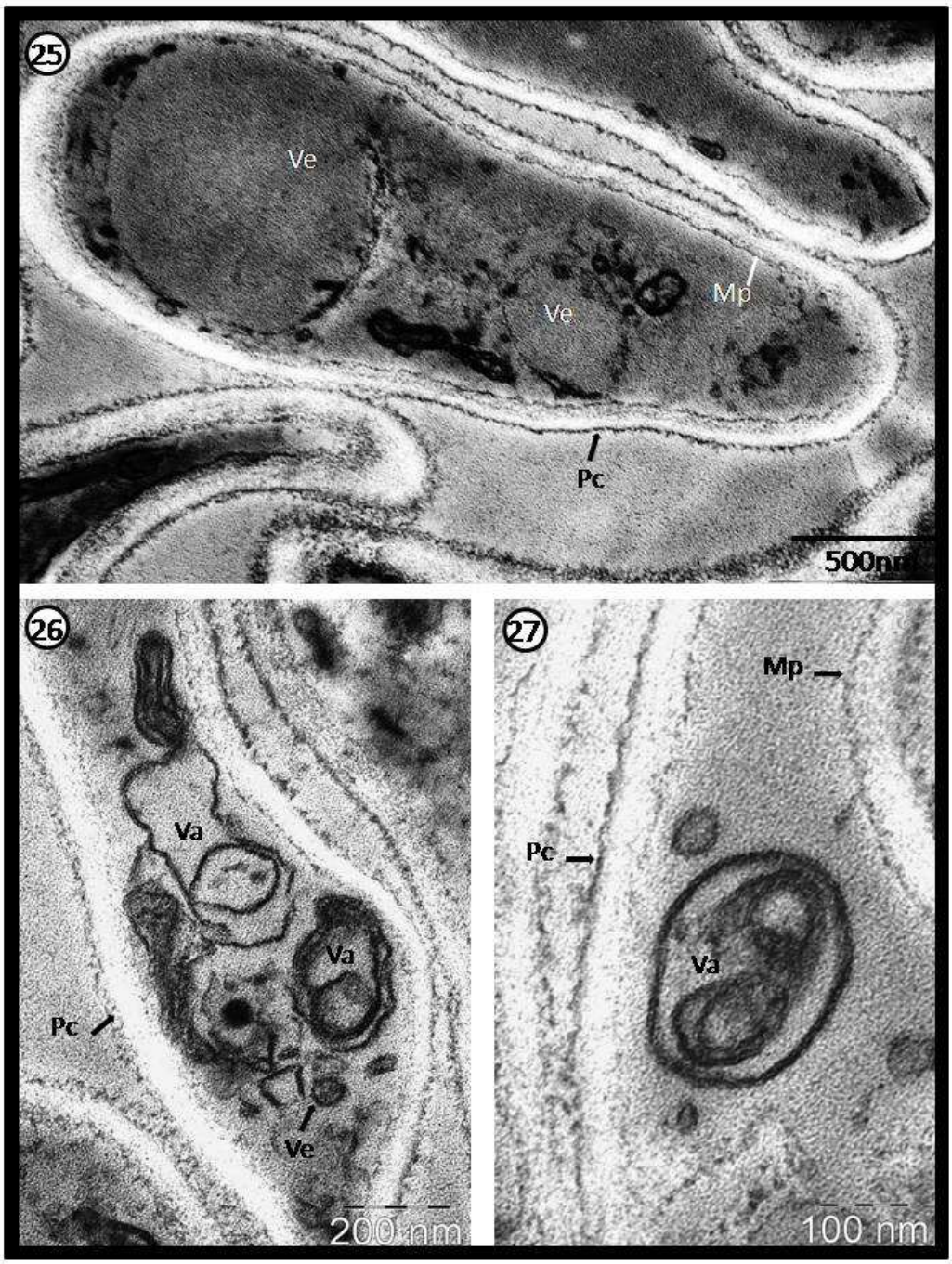

Figure 9 (Sections 25-27) - Electron micrograph showing cross and oblique sections of *Fusarium oxysporum* in the presence of *R*-(+)-limonene. Mp = plasma membrane, Pc = cell wall; Rel = smooth endoplasmic reticulum, Va = autophagic vacuole with Ve = vesicle.

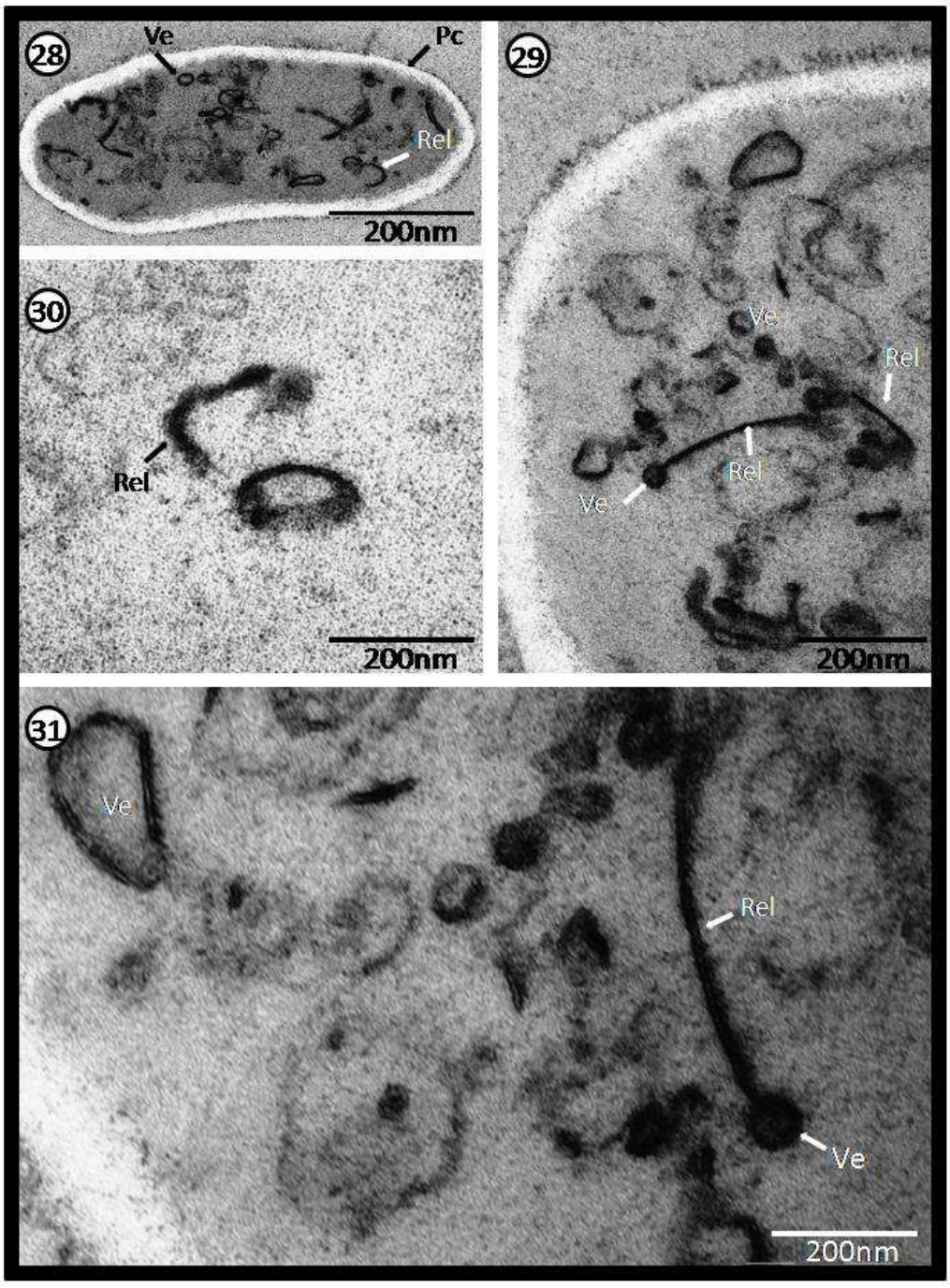

Figure 10 (Sections 28-31) - Electron micrograph showing cross and oblique sections of *Fusarium oxysporum* in the presence of *R*-(+)-limonene. Mp = plasma membrane, Pc = cell wall; Rel = smooth endoplasmic reticulum; Ve = vesicle.

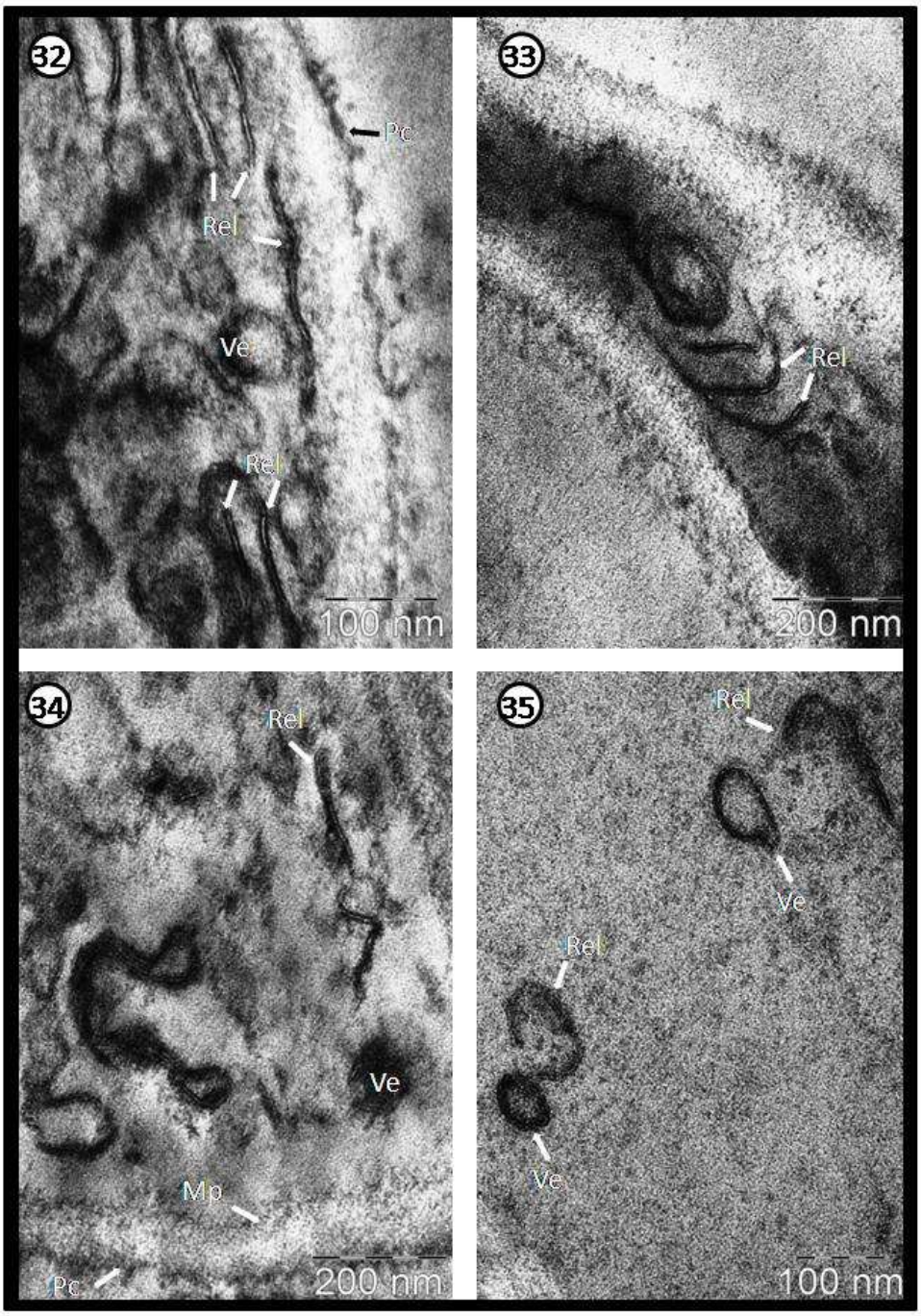

Figure 11 (Sections 32-35) - Electron micrograph showing cross and oblique sections of *Fusarium oxysporum* in the presence of *R*-(+)-limonene. Mp = plasma membrane, Pc = cell wall; Rel = smooth endoplasmic reticulum; Ve = vesicle.

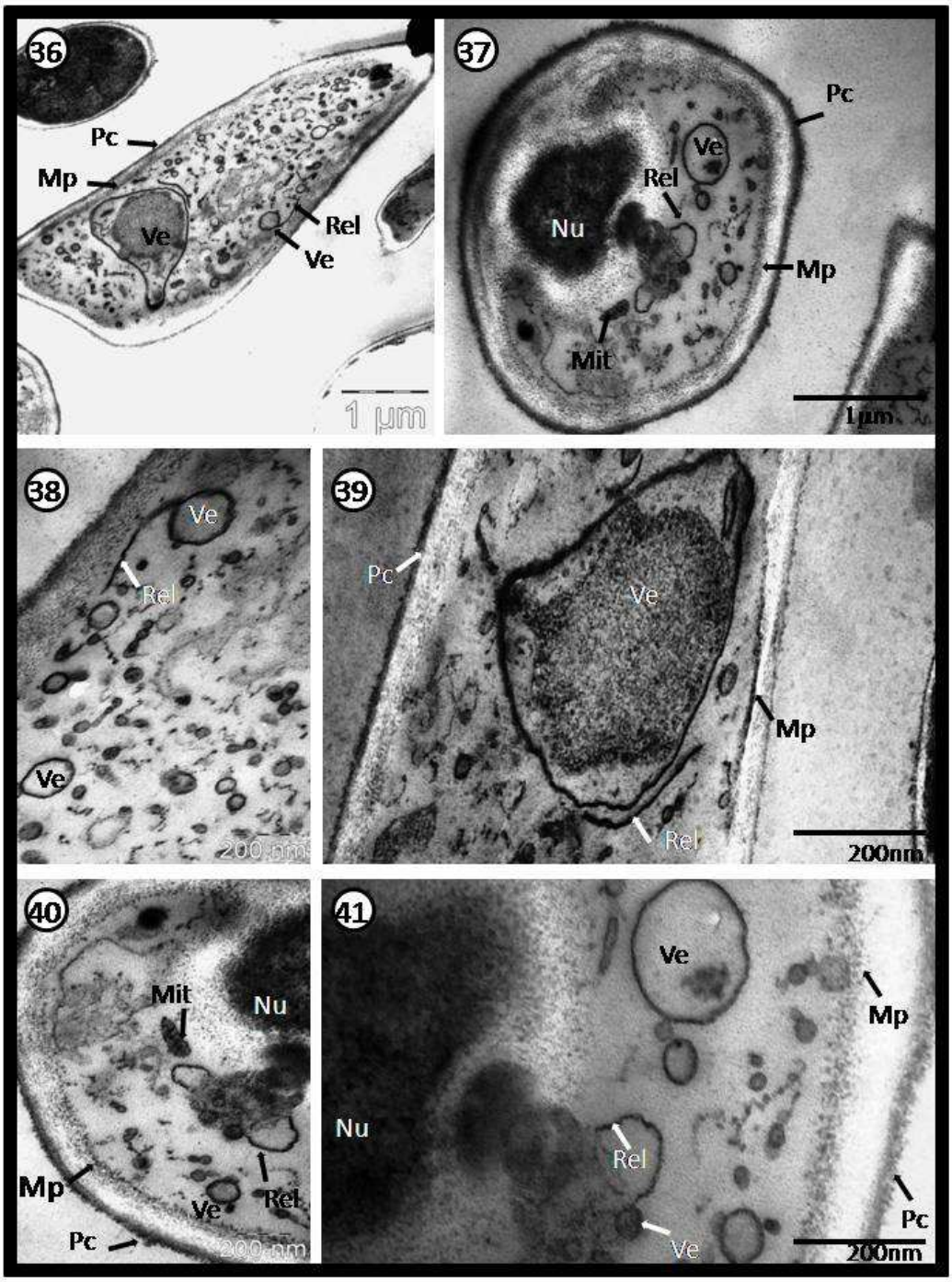

Figure 12 (Sections 36-41) - Electron micrograph showing cross and oblique sections of *Fusarium oxysporum* cultivated in the presence of *S*-(–)-limonene. Mp = plasma membrane, Nu = nucleus; Pc = cell wall; Rel = smooth endoplasmic reticulum; Ve = vesicle.

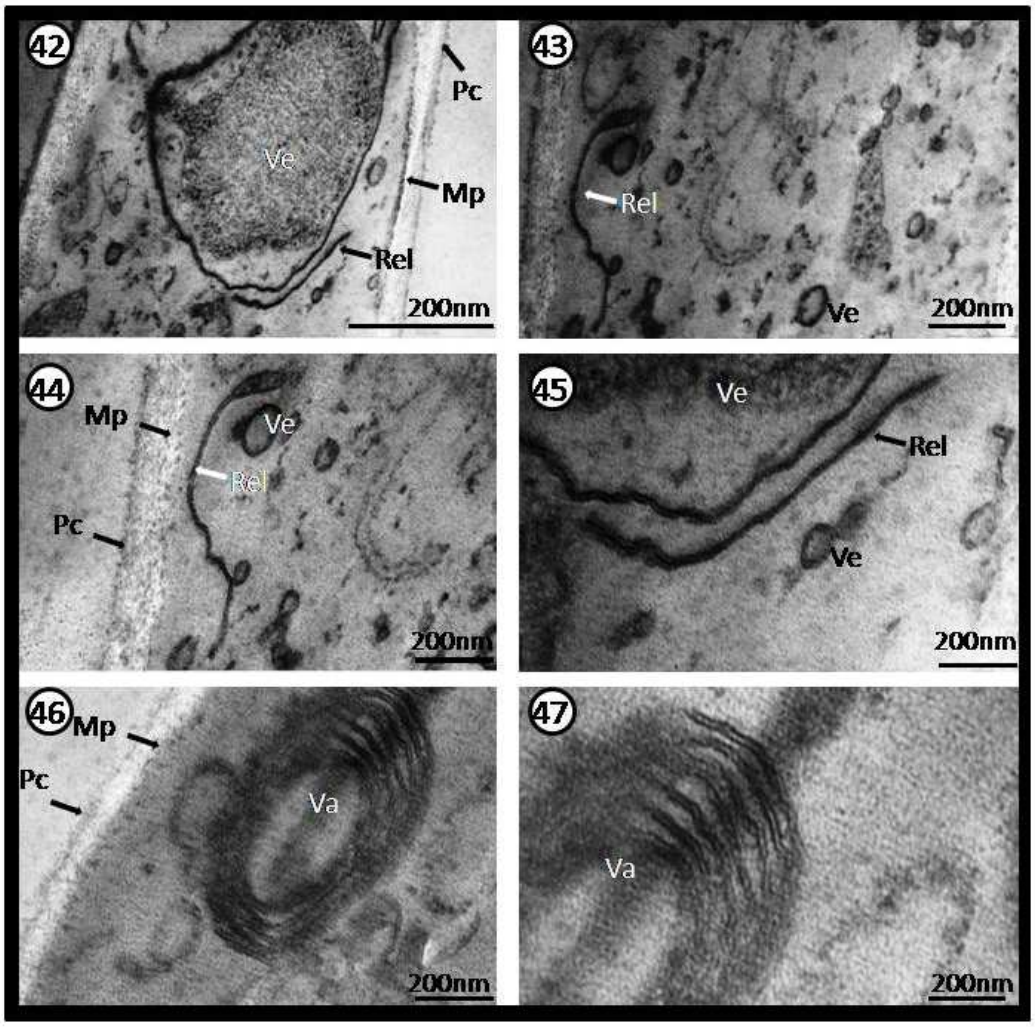

Figure 13 (Sections 42-47) - Electron micrograph showing cross and oblique sections of *Fusarium oxysporum* grown in the presence of *S*-(–)-limonene. Mp = plasma membrane, Pc = cell wall; Rel = smooth endoplasmic reticulum, Va = autophagic vacuole with Ve = vesicle.

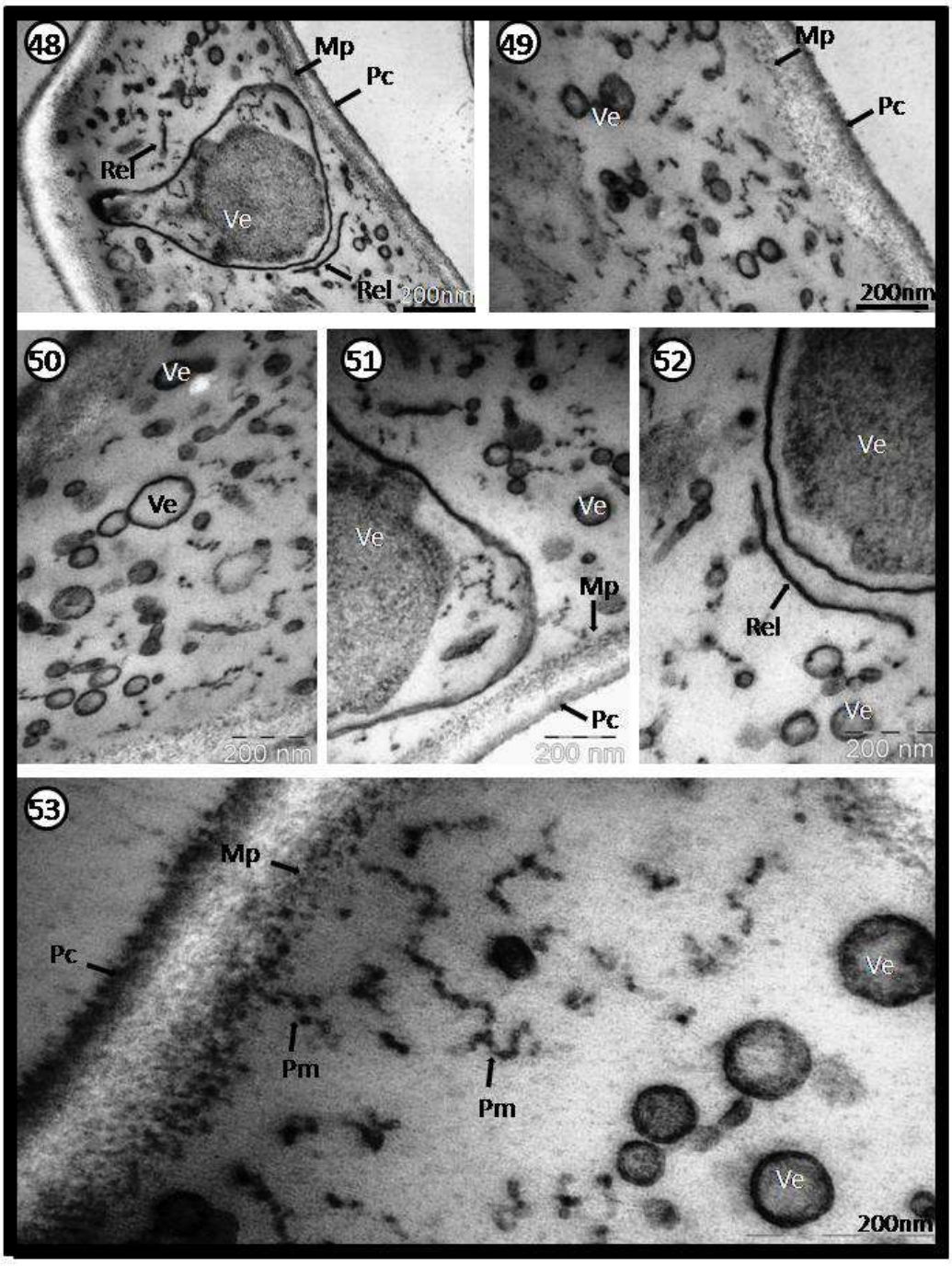

Figure 14 (Sections 48-53) - Electron micrograph showing cross and oblique sections of *Fusarium oxusporum* grown in the presence of *S*-(–)-limonene. Mp = plasma membrane, Pc = cell wall, Pr = membrane protein; Rel = smooth endoplasmic reticulum; Ve = vesicle.

4. Conclusion

The results collected so far with *Fusarium oxysporum* 152B encourage further studies with this biocatalyst and the characterization of its enzyme system, involved in the bioconversion of *R*-(+)-limonene into α-terpineol and *S*-(–)-limonene into limonene-1,2-diol, considering the concentration of products achieved, 4.0 and 3.7 $g.L^{-1}$, respectively. In this sense, an extensive comparative study of the bioconversion processes leading to these products was conducted. The enzyme related to the production of limonene-1,2-diol from *S*-(–)-limonene, limonene-1,2-epoxide hydrolase, has an intracellular nature and it is highly influenced by an aerobic system and seemed to be cofactor dependent, considering that the product was not detected with an anaerobic process. Meanwhile, the results recently published for *R*-(+)-limonene had proved that the enzyme responsible for the production of α-terpineol, a limonene hydratase, by the biotransformation of *R*-(+)-limonene has an intracellular nature and acts in anaerobic conditions. Furthermore, it was conducted an ultra-morphological and ultra-structural analysis, comparing *Fusarium oxysporum* grown on glucose (as control) and the biomass resulted in the biotransformation of *R*-(+)- and *S*-(–)-limonene, exhibiting the cytoplasm mostly occupied by smooth endoplasmic reticulum, vesicles and vacuoles that characterize cells in detoxification state. This is the first report that characterizes the bioconversion of *R*-(+)- and *S*-(–)-limonene by cellular detoxification using ultra-structural analysis.

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Capítulo 6

α-Terpineol bioproduction: Optimization of process conditions

ABSTRACT

In this study, the biotransformation of *R*-(+)-limonene into high concentrations of *R*-(+)-α-terpineol by *Sphingobium* sp. was investigated through a sequence optimization strategy involving the main process variables. This strategy comprised the Plackett-Burman design for variables screening followed by a central composite design to evaluate how process conditions such as the pH medium, inoculum and substrate concentration, temperature, and agitation affected the biotransformation. The statistical analysis of the results showed that the optimal process conditions for α-terpineol production were pH 7.0, limonene at 350 g L^{-1}, agitation at 200 rpm and 28 ^{o}C, while the optimum organic phase/aqueous phase ratio was 12.5:37.5 ($v.v^{-1}$), respectively. Under these conditions, the α-terpineol concentration reached approximately 500 $g.L^{-1}$ which is the highest α-terpineol concentration ever described in biotechnological processes. The experimental design optimization adopted herein was an effective tool for this study.

Keywords: Limonene, biotransformation, natural flavors, monoterpene alcohol, *Sphingobium* sp.

1. Introduction

α-Terpineol is one of the most commercially important monoterpene alcohols in the flavor industry. Its odor resembles that of lilac; its sweet smell is reminiscent of peach. This compound has an aroma threshold of 280 to 350 ppb, and its estimated annual consumption is approximately 9.2 tons (Burdock and Fenaroli, 2010).

α-Terpineol occurs in a large number of essential oils, such as the oils of *Cupressaceae*, *Pinaceae*, and *Lavandin*; it also exists in many other leaves, herbs, and flowers. This monoterpene alcohol may also arise during the fractional distillation of pine oils. Nevertheless, it is commonly synthesized by acid hydration of α-pinene or turpentine followed by partial dehydration (Burdock and Fenaroli, 2010; Surburg and Panten, 2006). More recently, limonene, α-pinene, or β-pinene biotranformation has produced α-terpineol (Bicas et al., 2008a).

Various authors have reported that many microorganisms are able to convert limonene into α-terpineol (Kraidman et al. 1969; Tan and Day 1998a,b; Tan et al. 1998; Adams et al., 2003; Maróstica and Pastore 2007a; Cadwallader et al. 1989; Agrawal and Joseph, 2000). One of the most viable biotransformation processes described so far yielded almost 130 $g.L^{-1}$ of α-terpineol from limonene in a biphasic medium (Bicas et al., 2010a).

The classical optimization method consists of varying the parameters one at a time while maintaining the other variables constant. However, this strategy is usually time-consuming, requires a large number of experiments, does not consider whether the interaction between factors affect the reaction, and rarely aids full understanding of the process (Sen and Swaminathan, 1997). The Plackett–Burman design is a screening approach that helps to statistically select the significant variables of numerous factor-experiments, aiming to reduce the number of trials in the final design. As for the central composite design, it is a statistical methodology that analyzes how the studied variables and their interaction impact a process. This technique culminates in the proposal of a mathematical model that describes the behavior of the analyzed factors and establishes their optimal values (Rodrigues and Iemma, 2005).

Literature papers have already optimized natural aroma compounds production by response surface methodology (Çelik et al., 2004; Melo et al., 2005) as well as *R*-(+)-limonene biotransformation into *R*-(+)-α-terpineol with *Fusarium oxysporum* (Bicas et al., 2008b) and a new isolate strain (Rottava et al., 2011).

Because there is no information about how the main parameters involved in the *R*-(+)-limonene biotransformation into *R*-(+)-α-terpineol using *Sphingobium* sp. influence the reaction, it was conducted an extensive study to define how the medium composition (pH) and the cultivation conditions (temperature and agitation, and substrate and inoculum concentration) affect α-terpineol production. Therefore, this chapter describes the optimization of the main process variables involved in the biotransformation of *R*-(+)-limonene into *R*-(+)-α-terpineol using a Plackett–Burman matrix with 12 assays (PB-12) for the variables screening, followed by the central composite design methodology.

2. Materials and methods

2.1. Microorganism and chemicals

The strain employed in this work used to be known as *Pseudomonas fluorescens* NCIMB 11671, but it was re-assigned as *Sphingobium* sp. by the Pasteur Institute (Paris, France) by means of the classical method of partial sequencing of the 16S RNA gene. *R*-(+)-Limonene (SAFC, ≥ 93% purity), in soybean oil or hexadecane (Sigma-Aldrich, ≥99% purity) as organic phase, was used as substrate. 1-Pentanol (Sigma-Aldrich, ≥99% purity) in ethanol (Ecibra, 99.5% purity) was employed as internal standard. The concentration of the product was measured using α-terpineol standards (SAFC, ≥96% purity). All the solvents were kept under refrigeration temperature.

2.2. Pre-culture preparation

Three full loops of a 48-hour-old culture on a Petri dish were transferred to a 500-mL conical flask containing 0.68 g of glucose, 0.17 g of $(NH_4)_2SO_4$, 3.4 mL of Hutner solution, 6.8 mL of solution A, and 159.8 mL distilled water (Fontanille et al., 2002). Solution A consisted of 5.2 g of K_2HPO_4 and 6.62 g of KH_2PO_4 in 200 mL of distilled water. This pre-culture was incubated at 30 ºC and 200 rpm for 24 h, to reach an optical density close to 5.0 at 600 nm (OD_{600}) (Bicas et al., 2010a).

2.3. Biocatalyst production

The biocatalyst was produced in a bioreactor with a working volume of 4.8 L (Bioflo 310, Bioflo & Celligen, New Brunswick, USA), using 170 mL of the pre-culture as inoculum, 4 L of distilled water, 170 mL of solution A, 85 mL of Hutner solution, 4.25 g of $(NH_4)_2SO_4$, 212.5 mL of hexadecane, and 15 g of *R*-(+)-limonene (70 g L^{-1},

organic phase). Temperature, agitation and aeration were kept at 30 ºC, 500 rpm, and 0.5 slpm, respectively. The pH was set at 6.5; the OD_{600} of the liquid phase and the composition of the organic and aqueous phases (GC-FID, see Section 2.7) were monitored. After 72 h, the biomass was recovered from the bioreactor by centrifuging the culture medium at 10,000 g for 10 min (5 ºC). The supernatant was eliminated, and the resulting biomass was re-suspended in 25 mL of phosphate buffer 20 mM pH 7.0 at three different optical densities (OD_{600} = 8, 13,18) and frozen (-18 ºC).

2.4. Optimization experiments

Because there was no information about how the main parameters involved in *R*-(+)-limonene biotransformation into *R*-(+)-α-terpineol using *Sphingobium* sp. affected the process, an extensive study to define how the medium composition, the cultivation conditions, the substrate concentration, and the inoculum/culture medium ratio influenced *R*-(+)-α-terpineol production was carried out. To this end, a Plackett–Burman screening design (Rodrigues and Iemma, 2005) with 12 experiments (PB-12) and three center points was run to estimate the experimental error and select the main parameters (Tables 1 and 2). The center points of the selected screening design were substrate at 120 g L^{-1}, inoculum with DO_{600} 13 and pH 7, and incubation at 30 ^{o}C/150 rpm, as adapted on the basis of a previous study (adapted from Bicas et al. 2010a).

Table 1 - Variables and levels used on a Placket-Burman matrix (PB-12).

Variables	**Units**	**Level**		
		-1	**0**	**+1**
pH	-	5.3	7.0	8.7
Inoculum	DO_{600}	8	13	18
Limonene	g/L	40	120	200
Temperature	^{o}C	21	28	35
Agitation	Rpm	0	150	300

To determine the influence of the process conditions on α-terpineol production, a central composite design 2^3 that used the parameters selected during the variables screening was performed. Six replicates were conducted at the central point and at the six axial points, totaling 20 experiments (Rodrigues and Iemma, 2005). The dependent variables (responses) were the concentration of α-terpineol obtained at different times (6, 21, 30, 46, 70, and 94 h). Tables 3 and 4 list the experimental design and the coded and real values of the independent variables. The study ranges had been defined in preliminary tests, while the central points and amplitudes of the central composite design parameters were chosen on the basis of previous results.

A second-order model (Eq. (1)) was adopted, to fit the response variables:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i^2 X_i^2 + \sum \beta_{ij} X_{ij},$$

where Y is the dependent variable, Xi and Xj are the coded independent variables, β_0 is the constant, β_i is the linear coefficient, β_i^2 is the quadratic coefficient, and β_{ij} is the interaction coefficient.

2.4.1. Screening design - Plackett-Burman (PB-12)

Tables 1 and 2 respectively present the tested selected variables and levels and the experimental results. Flasks containing frozen biomass (25 mL of phosphate buffer 20 mM pH 7.0 at three different densities (OD_{600} = 8, 13,18)) were re-suspended in acetate buffer 20 mM pH 5.3 or in Tris-HCl buffer pH 8.7. Twenty-five milliliters of the culture with the appropriate optical density and the same volume of soybean oil were transferred to a 250-mL conical flask, followed by addition of the volume of limonene that was necessary to reach the substrate concentration required in each experiment. The flasks were incubated under different conditions of agitation and temperature, according to the variables chosen for the Placket-Burman assay (Tables 1 and 2).

Table 2 - Placket-Burman matrix with codified values (PB-12) and α-terpineol concentration (g/L) for each experiment.

	pH	Inoculum	Substrate	Temperature	Agitation	α-Terpineol Concentration (g/L)					
						6h	21h	30h	46h	70h	94h
1	1	-1	1	-1	-1	0	0	0	0	0	0
2	1	1	-1	1	-1	0	0	0	0	0	0
3	-1	1	1	-1	1	36,70	104,43	138,87	163,74	174,02	178,82
4	1	-1	1	1	-1	0	0	0	0	0	0
5	1	1	-1	1	1	0	0,31	0,33	0,48	0,47	0,47
6	1	1	1	-1	1	0	0	0	0	0	0
7	-1	1	1	1	-1	1,10	2,00	2,47	3,49	3,96	4,55
8	-1	-1	1	1	1	47,34	98,17	100,50	110,01	117,21	125,47
9	-1	-1	-1	1	1	22,35	29,26	30,19	29,26	28,10	35,39
10	1	-1	-1	-1	1	0	0	0	0	0	0
11	-1	1	-1	-1	-1	0,50	0,94	1,18	1,68	2,34	2,88
12	-1	-1	-1	-1	-1	0,39	0,93	0,95	1,02	1,27	1,33
13	0	0	0	0	0	7,85	16,35	20,14	21,58	21,70	22,49
14	0	0	0	0	0	9,24	18,16	20,05	19,40	21,28	23,31
15	0	0	0	0	0	8,02	17,41	20,02	20,35	21,33	21,68

2.4.2. Central composite design

Tables 3 and 4 respectively display the selected variables and levels as well as the experimental data. The variables and levels shown in Table 3 were chosen according to the statistical evaluation of the results collected during the Placket-Burman assay. Some of the flasks containing the frozen biomass were centrifuged; the cells were re-suspended in the required buffer, as depicted in Table 3. Acetate buffer 20 mM (pH 5.3, 4.3, and 3.6) and phosphate buffer 20 mM (pH 6.3 and 7.0) were used. Twenty-five milliliters of the culture with optical density DO_{600} adjusted at 8 and the same volume of soybean oil were transferred to a 250-mL conical flask, followed by addition of the volume of limonene that was necessary to reach the desired substrate concentration in each experiment. The flasks were incubated under different conditions of agitation; the temperature was maintained at 28 ºC.

Table 3 - Variables and levels evaluated in the central composite design 2^3.

Variables	**Units**		**Levels**				
			-1,68	**-1**	**0**	**+1**	**+1,68**
pH	-	X_1	3.6	4.3	5.3	6.3	7.0
Limonene	g/L	X_2	100	200	350	500	600
Agitation	rpm	X_3	50	113	200	287	350

2.4.3. Data analysis

The results and the response surface methodology were analyzed using the Statistica 7.0 software (StatSoft Inc, Oklahoma, USA). A significance level of 10 ($p < 0.1$) and 5% ($p < 0.05$) was considered for the screened variables and the central composite design, respectively.

2.5. Biotransformation procedure

Twenty-five milliliters of the concentrated biomass at a certain optical density (OD_{600} = 8, 13, or 18) and the same volume of organic phase were transferred to a 250-mL conical flask. The substrate was then added, to reach the final concentration per liter of organic phase established in each test (screening design and central composite design). The flasks were also incubated under varying temperature and incubation conditions.

Table 4 – Results of the central composite design for α-terpineol concentration (g/L) and productivity (g/L.h).

				α-Terpineol Concentration (g/L)						α-Terpineol Productivity (g/L.h)					
	pH	Limonene	Agitation	6h	21h	30h	46h	70h	94h	6h	21h	30h	46h	70h	94h
							Y_1	Y_2	Y_3						
1	-1	-1	-1	1,65	5,54	6,40	7,45	8,59	9,43	0,27	0,26	0,21	0,16	0,12	0,10
2	+1	-1	-1	0,49	6,60	10,01	19,25	25,00	24,97	0,08	0,31	0,33	0,42	0,36	0,27
3	-1	+1	-1	1,73	3,25	3,93	5,37	5,50	5,85	0,29	0,15	0,13	0,12	0,08	0,06
4	+1	+1	-1	1,93	12,08	16,22	18,56	24,51	24,82	0,32	0,58	0,54	0,40	0,35	0,26
5	-1	-1	+1	4,76	8,22	8,21	10,54	11,48	12,11	0,79	0,39	0,27	0,23	0,16	0,13
6	+1	-1	+1	12,99	84,28	103,96	115,40	128,35	143,59	2,17	4,01	3,47	2,51	1,83	1,53
7	-1	+1	+1	4,06	5,44	6,42	6,75	7,68	8,19	0,68	0,26	0,21	0,15	0,11	0,09
8	+1	+1	+1	12,88	47,92	70,34	94,46	98,66	111,50	2,15	2,28	2,34	2,05	1,41	1,19
9	-1,68	0	0	3,54	5,19	6,18	6,56	6,92	6,01	0,59	0,25	0,21	0,14	0,10	0,06
10	+1,68	0	0	7,94	38,72	67,49	117,75	153,14	192,83	1,32	1,84	2,25	2,56	2,19	2,05
11	0	-1,68	0	14,67	47,81	53,14	70,03	64,34	65,38	2,44	2,28	1,77	1,52	0,92	0,70
12	0	+1,68	0	13,63	39,87	55,51	68,40	82,63	92,01	2,27	1,90	1,85	1,49	1,18	0,98
13	0	0	-1,68	0,47	1,30	1,53	1,94	2,26	2,95	0,08	0,06	0,05	0,04	0,03	0,03
14	0	0	+1,68	28,82	74,89	85,19	127,40	139,90	139,33	4,80	3,57	2,84	2,77	2,00	1,48
15	0	0	0	11,44	38,24	61,51	77,37	92,65	110,77	1,91	1,82	2,05	1,68	1,32	1,18
16	0	0	0	13,33	45,95	58,31	70,42	87,71	98,77	2,22	2,19	1,94	1,53	1,25	1,05
17	0	0	0	15,78	50,21	67,21	90,28	106,71	108,84	2,63	2,39	2,24	1,96	1,52	1,16
18	0	0	0	16,56	48,74	68,68	74,94	98,09	110,16	2,76	2,32	2,29	1,63	1,40	1,17
19	0	0	0	16,99	53,89	90,20	105,06	101,91	108,01	2,83	2,57	3,01	2,28	1,46	1,15
20	0	0	0	14,81	50,90	78,67	98,21	120,84	110,21	2,47	2,42	2,62	2,14	1,73	1,17

2.6. Organic phase proportion

This experiment aimed to test how the amount of organic phase affected the α-terpineol concentration. The amount of organic phase was tested at three different levels, while the final volume of the biotransformation system was kept as 50 mL of culture medium, as follows: (i) 25 mL of organic phase and 25 mL of aqueous phase (the condition used in the optimization experiments), (ii) 12.5 mL of organic phase and 37.5 mL of aqueous phase, and (iii) 37.5 mL of organic phase and 12.5 mL of aqueous phase. All the other system conditions were maintained under the optimized conditions (28 ^{o}C, pH 7.0, limonene at 350 g L^{-1}, and agitation rate of 200 rpm).

2.7. Analytical conditions

Samples from the organic phase were periodically withdrawn from the reaction system to monitor substrate consumption and product formation. Before being injected (1 µL) into the gas chromatograph, the oil had to be extracted (vortexing for 40 s) with the same volume of ethanol (1:1, v/v) containing 0.2 % (v/v) 1-pentanol as internal standard. The products were analyzed on a HP-7890A (Agilent Technologies) gas chromatograph with flame ionization detector (GC-FID). An HP-5 capillary column of 30-m length x 0.250-mm i.d. x 0.25-µm film thickness was employed. Helium was the carrier gas with a constant flow of 1 mL min^{-1}. α-Terpineol was quantified after adding 0.2% (v/v) of 1-pentanol as internal standard to the ethanol samples; the concentrations were expressed as mass of product per liter of organic phase.

3. Results and discussion

3.1. Screening design - Plackett-Burman (PB-12)

The industry has taken great interest in biotransformation processes; however, a series of drawbacks remain to be overcome to become industrially competitive (Bicas et al., 2009). In this sense, the major challenges scientists have to face during such processes lie on the high cytotoxicity, low solubility, and high volatility of both the substrate and the product, which directly impact the biotransformation rates and process costs (Krings and Berger, 1998). The parameter log P_{ow} (octanol/water partition coefficient) represents how toxic organic solvents are to microorganisms. Substances with log P_{ow} between 1-5 are usually toxic to microbial cells. Overall, monoterpenes have log P_{ow} between approximately 2.0 and 5.0 (4.83 for limonene) (Heipieper et al., 1994).

According to De Carvalho et al. (2005), the carbon source used to grow cells can significantly influence the composition of the cell membrane. Different membrane compositions can lead to cells with distinct hydrophobicity. This affects the ability of cells to absorb hydrophobic/hydrophilic compounds and may culminate in non-specific permeability. Compounds such as terpenoids make microbial cell membranes more fluid. Consequently, the cell loses integrity, the dry matter diminishes, and the dissipation of proton-motive force (electrochemical gradient of H^+ across the membrane) deactivates energy metabolism (Onken and Berger, 1999). In addition, enhanced membrane fluidity can harm the maintenance of enzyme complexes related to the membrane, such as the complex formed between the cytochrome P-450 monooxygenase and the NADPH-cytochrome P-450 reductase, involved in oxidative transformations of terpenes and other lipophilic compounds (Onken and Berger, 1999).

The use of biphasic systems is an efficient strategy to deal with these difficulties: such systems facilitate product recovery and raise yields by reducing substrate and product toxicity and their losses by volatilization (Bicas et al., 2013; Cabral, 2001). The organic phase typically consists of hydrocarbon solvents (e.g., *n*-decane, *n*-hexadecane) with an octanol/water partition coefficient (log P) higher than 4, a commonly accepted requisite for good tolerance by whole microbial cells (Fontanille and Larroche, 2003). Authors have already applied this approach to produce isonovalal from α-pinene oxide by *Pseudomonas rhodesiae* (Fontanille and Larroche, 2003) and patented it to bioconvert some terpenes (Muller et al., 2007). Here, it was employed vegetable oil, an environmentally friendly alternative to organic solvents previously adapted as organic phase by Bicas et al. (2008a) and Bicas et al. (2010a), for limonene biotransformation.

Table 1 describes the levels of the tested variables in the screening design, and these codified values were applied in the PB-12 Plackett–Burman matrix (Table 2). In this case, a P value of 0.1 is currently recommended since it is more conservative and lowers the risk of falsely excluding statistically significant parameters (Rodrigues and Iemma, 2005).

To screen the tested variables, limonene was added to the organic phase at concentrations ranging from 40 (level -1) to 200 (level +1) $g.L^{-1}$. Table 5 shows that the substrate concentration positively affected the tested levels for 30, 46, 70, and 94 h ($p < 0.1$) of biotransformation. In a similar study, Bicas et al. (2008b) observed that limonene exerted a negative effect, probably because *R*-(+)-limonene was toxic to *F. oxysporum*, a difficulty that we overcame by working in a biphasic system. As stated

before, Bicas et al. (2010a) verified that the biocatalyst we used here was quite insensitive to substrate inhibition, so it was possible to employ initial substrate concentrations up to 180 $g.L^{-1}$.

Table 5 - Estimates of the effects of the parameters analyzed after 30, 46, 70 and 94 h of biotransformation.

Factor	Time (h)	Effect	SE	t (9)	*P* value
Mean	**30**	**22,31**	**7,827**	**2,851**	**0,0191**
	46	**24,73**	**9,209**	**2,686**	**0,0250**
	70	**26,11**	**9,833**	**2,656**	**0,0262**
	94	**27,76**	**10,047**	**2,763**	**0,0220**
pH	**30**	**-45,64**	**17,501**	**-2,608**	**0,0284**
	46	**-51,45**	**20,591**	**-2,499**	**0,0339**
	70	**-54,40**	**21,988**	**-2,474**	**0,0353**
	94	**-58,00**	**22,466**	**-2,581**	**0,0296**
Inoculum	30	1,87	17,501	0,107	0,9173
	46	4,85	20,591	0,236	0,8191
	70	5,70	21,988	0,259	0,8011
	94	4,09	22,466	0,182	0,8596
Substrate	**30**	**34,86**	**17,501**	**1,992**	**0,0775**
	46	**40,80**	**20,591**	**1,981**	**0,0789**
	70	**43,83**	**21,988**	**1,994**	**0,0774**
	94	**44,80**	**22,466**	**1,994**	**0,0773**
Temperature	30	-1,25	17,501	-0,072	0,9445
	46	-3,87	20,591	-0,188	0,8552
	70	-4,65	21,988	-0,211	0,8373
	94	-2,86	22,466	-0,127	0,9016
Agitation	**30**	**44,22**	**17,501**	**2,526**	**0,0324**
	46	**49,55**	**20,591**	**2,406**	**0,0395**
	70	**52,04**	**21,988**	**2,367**	**0,0421**
	94	**55,23**	**22,466**	**2,459**	**0,0362**

SE Standard error

Parameter in bold are statistically significant for the response ($p < 0.1$), considering the residual SS

Evaluation of the medium pH revealed that this variable impacted α-terpineol production considering the ranges tested. Three different pH values were investigated by using phosphate buffer 20mM pH 7.0, acetate buffer 20mM pH 5.3, and Tris-HCl buffer pH 8.7. The results in Table 5 demonstrated that the best culture medium for this process relied on a lower pH range. Indeed, the results achieved at maximum level (+1) with pH 8.7 were not significant. This corroborated data observed in practical studies—terpene biotransformation processes normally involve the substrate as the sole carbon

source; authors typically apply mineral media (saline solutions) or buffers as the culture medium (Maróstica Jr. and Pastore, 2007a; Speelmans et al., 1998), even though some authors have proposed that best fungal growth and best bioconversion yield correlate, suggesting that media with other carbon sources should be used (Adams et al., 2003).

It was also examined the variable inoculum concentration, which is an important factor in a fermentation process and considerably affects the subsequent stages (Bicas et al., 2008b). In industrial fermentation processes, the age and density of the inoculum directly influence the *lag* phase duration, the specific growth rate, the biomass yield, sporulation, and the final product quality, consequently impacting the production costs (Sen and Swaminathan, 2004).

Inoculum concentration was measured by the optical density, at three levels (DO_{600} 8, 13, and 18). This variable did not significantly ($p < 0.1$) affect the concentration of α-terpineol. Despite these findings, Fontanille and Larroche (2003) found that biocatalyst content influenced the maximum product recovery positively in the case of α-pinene oxide biotransformation into isonovalal. The use of mycelium concentrates in fungal experiments might enhance the process yield (Bicas et al., 2008b). Taking all this into account, the range studied here allows for application of lower biomass concentrations, which can make the process less costly. In this sense, it was chosen DO_{600} 8.0 as standard for the following trials.

This study also assessed how culture conditions like temperature and agitation system affected α-terpineol production. The medium temperature directly influences biological reactions. Moreover, medium agitation promotes microorganism development and cell-substrate interaction. However, in addition to increasing the process energy costs, high temperatures and agitation rates might enhance substrate and product loss as well as side reactions. Thus, it is essential to seek an ideal balance, to obtain the best results (Bicas et al., 2008b).

As for the agitation rate, it was checked whether it was possible to produce α-terpineol without stirring (level -1, less costly) as compared with stirring at maximum level (+1, 300 ppm). The agitation rate affected the α-terpineol concentration positively ($p < 0.1$, Table 5), probably due to increased cell-substrate contact. Thus, optimal agitation may be situated at values above the maximum value tested in the screening design (300 rpm), suggesting the use of a wider range for this variable.

Considering a confidence interval of 90%, temperature did not impact the biotransformation process significantly (Table 5). This could result from

biotransformation inhibition during microbial growth and enzyme denaturation at high temperature, close to 35 °C. Authors have reported similar results for *Penicillium digitatum* NRRL 1202 (Tan et al., 1998), *Pseudomonas putida* (Chatterjee and Bhattacharyya, 2001), and *Fusarium oxysporum* 152B (Bicas et al., 2008b, 2010b), for which bioconversion decreased dramatically at temperatures above 32, 30, and 28 °C, respectively. Hence, in this experiment it is recommended the adoption of mild temperatures (lower than 30 °C), to obtain interesting performances.

3.2. Central composite design

On the basis of how the screened variables impacted limonene biotransformation into α-terpineol at the tested levels, we found that the medium pH, the substrate (limonene) concentration, and the agitation rate ($p < 0.1$) were worth considering in the further optimization design. In this perspective, it was selected new study ranges for the tested levels (Table 3) and these variables were optimized using a 2^3 central composite design with six center points (see Table 4, which also presents the data analyzed for α-terpineol productivity, given in $g.L^{-1}h^{-1}$). This parameter was not considered in the subsequent statistical evaluation, because we were more concerned about product concentration than productivity. In fact, it was verified that the productivity profile was similar in all situations: it decreased along the biotransformation. This was expected, given that terpenes toxicity was a lesser issue at the beginning of the process, whilst increasing product concentration along bioconversion diminished biocatalyst efficiency and process productivity.

When α-terpineol concentration was evaluated, it was verified that the onset of optimal production profile occurred at 40 h of fermentation, and that the most promising results in quantitative terms emerged at 70 and 94 h (Table 4). Hence, the optimum biotransformation time for the statistical evaluation were considered to be 46, 70 and 94 h. These data were submitted to statistical analyses, including fitting to Eq. (1), followed by an analysis of variance (ANOVA) at 95% confidence level. Only the statistically significant parameters have been used for analysis of the behavior of the fitted mathematical models:

$$Y_1 = 87.00 + 29.62\,X_1 + 28.38\,X_3 - 14.65\,X_1^2 - 12.15\,X_2^2 - 13.76\,X_3^2 + 20.95\,X_1X_3 \quad \text{(Eq. 2)}$$

$$Y_2 = 102.43 + 35.82\,X_1 + 30.32\,X_3 - 14.82\,X_1^2 - 17.13\,X_2^2 - 17.98\,X_3^2 + 21.55\,X_1X_3 \quad \text{(Eq. 3)}$$

$$Y_3 = 99.89 + 42.73\,X_1 + 32.20\,X_3 - 17.41\,X_2^2 - 20.08\,X_3^2 + 25.04\,X_1X_3 \quad \text{(Eq. 4)}$$

where Y is the dependent variable (α-terpineol concentration at different times of the fermentation process, Y_1 = 46 h, Y_2 = 70 h, and Y_3 = 94 h).

The data was treated using the software Statistica 7.0, which generated the regression coefficients and the respective statistical analysis of the examined parameters.

Table 6 summarizes the results of the ANOVA test, including the regression coefficients for the coded second order polynomial equation, the coefficients of determination (R^2), and the *F* and *p* values.

Table 6 – Results for regression coefficients and analysis of variance (ANOVA) for the dependent variables of the central composite design.

Parameter [a]	Coefficient	α-Terpineol Concentration (g/L)		
		46h	**70h**	**94h**
		Y_1	**Y_2**	**Y_3**
Mean	β_0	87,00	102,43	99,89
Linear				
X_1	β_1	29,62	35,82	42,73
X_2	β_2	-	-	-
X_3	β_3	28,38	30,32	32,20
Quadratic				
X_1	β_1^2	-14,65	-14,82	-
X_2	β_2^2	-12,15	-17,13	-17,41
X_3	β_3^2	-13,76	-17,98	-20,08
Interaction				
X_1X_2	β_{12}	-	-	-
X_1X_3	β_{13}	20,95	21,55	25,04
X_2X_3	β_{23}	-	-	-
$F_{calculated}$		14.83	14.05	15.11
F_{listed} [b]		2.92	2.92	2.96
$F_{calculated}$:F_{listed}		5.07	4.81	5.10
p-value		< 0.0001	< 0.0001	< 0.0001
R^2 [c]		0.87	0.87	0.84

[a] X_1 = pH, X_2 = limonene (g/L), X_3 = agitation (rpm)

[b] Values of F_{listed} at $p < 0.05$

[c] R^2 = coeficiente of determination

For the fitted equations to be predictive, they must satisfy a certain criterion based on the F_{test} values ($F_{calculated}$ and F_{listed}), which in turn depends on the F ratio calculated for the regressions related to the residuals ($F_{calculated}/F_{listed}$). The criterion is that this ratio must be higher than that of F_{listed} (Khuri & Cornell, 1996), thereby enabling one to plot the response surfaces.

The results suggested that the fitted models were suitable (significant and predictive) and led to significant regression, low residual values, no lack of fit, and satisfactory coefficients of determination. For example, the results analyzed at 46, 70, and 94 h of the process afforded coefficients of determination (R^2) equal to 0.87, 0.87, and 0.84, respectively, indicating that the models accounted for 87, 87, and 84% of the observed data variation. Although they were not ideal, the R^2 values were perfectly acceptable for biological systems (Rodrigues and Iemma, 2005). The high $F_{calculated}/F_{listed}$ value in relation to F_{listed} proved that the models were valid for these variables, so they were predictive in these cases (Table 6). Indeed, the calculated F value was 14.83, 14.05, and 15.11, respectively, approximately five times higher than the respective listed value; the *p* value of the model was lower than 0.0001 for the three cases. In other words, the ANOVA results evidenced that the quadratic model adjusted for the process responses was satisfactory.

Because the obtained codified models were highly significant, it were constructed contour curves to illustrate how the variables behaved in relation to these models. Figures 1, 2, and 3 display the contour curves for the variable α-terpineol production at three different times—46, 70, and 94 h of biotransformation—which corresponded to the graphical representation of Eq. 2, 3, and 4, respectively.

Figures 1a (limonene x pH), 1b (agitation x pH), and 1c (agitation x limonene) depict the contour curves obtained for α-terpineol production after 46 h of biotransformation. Figure 1b shows how agitation and pH (1b) interacted and thus impacted α-terpineol production, indicating the appropriate condition for this biotransformation process. A rise in both variables gradually increased production to a maximum concentration, yielding more than 100 g L^{-1} α-terpineol. Figure 1a revealed that it was possible to use a wide range of limonene concentrations and pH values: the optimum concentration of α-terpineol remained constant from 240 to 400 g L^{-1} and from 6.0 to 7.0, respectively.

Figures 2a (limonene x pH), 2b (agitation x pH), and 2c (agitation x limonene) bring the contour curves obtained for α-terpineol production after 70 h. The curve profiles were similar to those achieved after 46 h. Figures 2a and 2c showed that the optimal limonene concentration tended to the central coded value 0, or 350 g L^{-1}. In this condition, agitation rate and pH could lie between 200 and 320 rpm and between 6.0 and 7.0, respectively. After 72 h, the highest α-terpineol concentration was approximately 153 g L^{-1} at pH 7.0 (codified value: +1.68), limonene at 350 g L^{-1}

(codified value: 0), and 200 rpm (codified value: 0) (Table 5). These data agreed with the analysis of the contour curve in Figure 2. Most interestingly, it is possible to adapt a minimal stirring of 200 rpm (within the optimal range shown above) and still attain higher α-terpineol production while saving energy.

Regarding Figures 3a, 3b, and 3c, it was clear that the pH had to lie within values close to 7.0 (or +1 coded value) as compared with the other process times. This was because increasing pH was directly related to maintenance of the limonene concentration within the optimal range of 350 $g.L^{-1}$, which kept production at optimum level.

According to Figures 1b, 2b and 3b, high agitation rates gave maximum α-terpineol values. We verified that extremely low pH harmed the microorganism and diminished the concentration of the desired product. This was also true for the agitation rate—low values considerably reduced α-terpineol production (for example, less than 3 $g\ L^{-1}$ α-terpineol after 94 h under stirring at 50 rpm).

Considering that this work aimed to achieve maximum α-terpineol production from limonene biotransformation, it is worth noting that maximum concentration of this compound was 190 $g\ L^{-1}$ after 94 h of process, with maximum productivity of 2.05 $g\ L^{-1}\ h^{-1}$. This is by far the largest α-terpineol concentration that has been obtained by biotechnological means. Hence, we have improved on the previous process, which had furnished maximum α-terpineol concentration of 130 $g\ L^{-1}$ (Bicas et al., 2010a).

In general, the contour curve profiles were ideal: all the figures presented the predicted optimal regions within the studied levels. As cited previously, the medium pH could vary from 5.8 to 7.0 during α-terpineol production, although it was best to maintain the pH from 6.3 to 7.0. An analysis of Eq. 2, 3, and 4 and of the respective contour curves showed that the limonene concentration and agitation could oscillate from 250 to 400 $g\ L^{-1}$ (the best results obtained at 350 $g\ L^{-1}$) and from 200 to 320 rpm, respectively, whilst the α-terpineol production remained close to the maximal value. In fact, it was possible to use a wide range of stirring rates without significantly altering product yield, but controlling the medium pH and the limonene concentration was crucial. Nonetheless, applying the lowest possible stirring rate while keeping production within the maximum range would be desirable, to simplify the process and make it more attractive and competitive from an economic standpoint.

Other authors have used multi-response analysis to establish the optimal process conditions for other systems of limonene biotransformation. For example, Bicas et al.

(2008b) obtained the best results using 0.5% (v/m) *R*-(+)-limonene, medium ratio of 0.25 (m/m), and 72 h cultivation at 26 ^{o}C and 240 rpm when they employed a *Fusarium oxysporum* strain. In these optimized conditions, they attained 2.4 g.L^{-1} product (Bicas et al., 2008b), subsequently reported as being approximately 4 g.L^{-1} (Bicas et al. 2010b). In another study, Rottava et al. (2011) optimized *R*-(+)-limonene biotransformation, to produce α-terpineol in the presence of fungal and yeast strains. The authors reported production of approximately 1.7 mg.L^{-1} α-terpineol in optimized conditions (substrate concentration of 1.75%, mass of inoculum of 2 g, and substrate-to-ethanol volume ratio of 1:1).

Mathematical methods (equation derivation) gave the optimal conditions presented in Table 7, where the predicted values for the production of α-terpineol were 117, 137 and 113 g after 46, 70 and 94 h of process, respectively. The predicted values for each variable were adapted and used for the model validation (validation data following predicted values): pH = 6.3; Limonene = 400 g.L^{-1}; Agitation = 250 rpm, temperature kept at 28 ^{o}C.

Table 7 – Experimental validation under the optimized conditions for the production of α-terpineol from the biotransformation of limonene.

Time (h)	Codified value	Real value	PD[a] (g.L^{-1})	EV[b] (g.L^{-1})	EV[c] (g.L^{-1})	RD[d] (%)	RD[e] (%)
46	X_1= 1.01	X_1= 6.3					
	X_2= 0	X_2= 350	117	92.02	99.46	-27.15	-17.64
	X_3= 1.03	X_3= 290					
70	X_1= 1.2	X_1= 6.5					
	X_2= 0	X_2= 350	137	112.54	131.04	-21.73	-4.55
	X_3= 0.84	X_3= 272					
94	X_1= 0	X_1= 5.3					
	X_2= 0	X_2= 350	113	141.86	183.86	20.34	38.54
	X_3= 0.80	X_3= 268					

Where: X_1 = pH; X_2 = Limonene concentration (g.L^{-1}) and X_3 = Agitation

[a] *PD = Predicted values by the model*

[b] *EV = Experimental values*; Mean values obtained in optimal conditions from model derivation: pH = 6.3; Limonene = 400 g.L^{-1}; Agitation = 250 rpm, temperature kept at 28 ^{o}C

[c] *EV = Experimental values*; Mean values obtained in optimal conditions from contour curves analyses: pH = 7.0; Limonene = 350 g.L^{-1}; Agitation = 200 rpm, temperature kept at 28 ^{o}C

[d,e] Relative deviation = [(experimental value – predicted value)/experimental value] x 100, for experimental value A and B, respectively

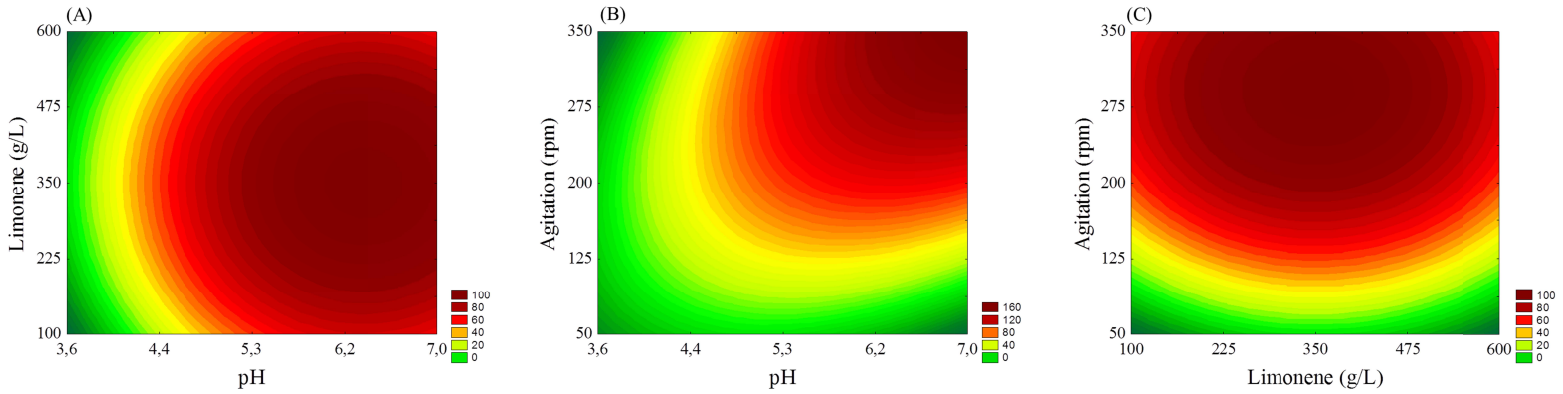

Figure 1 – Contour curves of α-terpineol production after 46 h of biotransformation process as a function of (a) Limonene x pH (Agitation = 200 rpm), (b) Agitation x pH (Limonene = 350 g/L), (c) Agitation x Limonene (pH = 5.3).

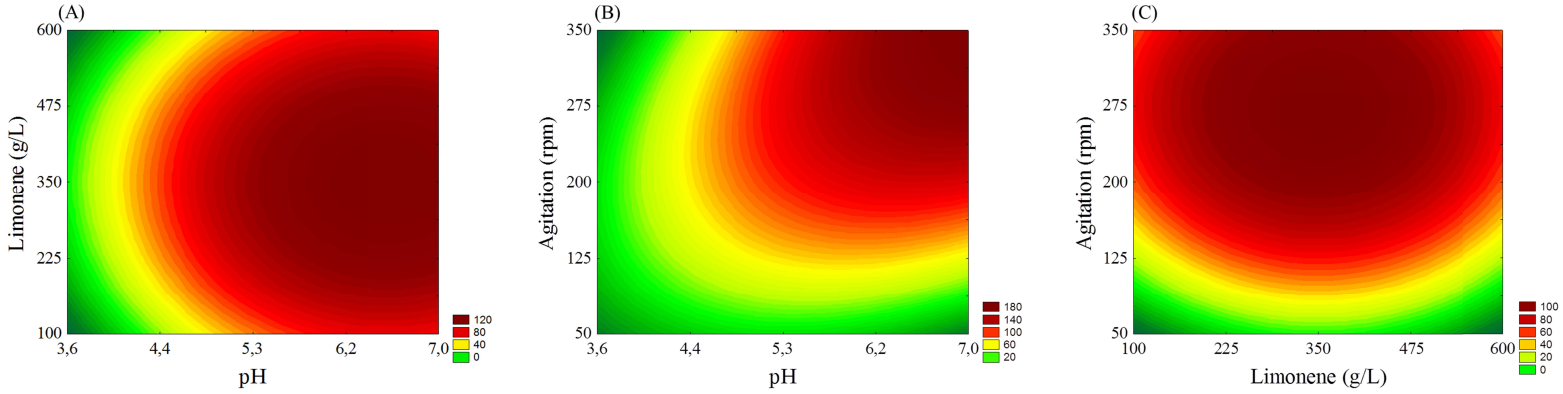

Figure 2 – Contour curves of α-terpineol production after 70 h of biotransformation process as a function of (a) Limonene x pH (Agitation = 200 rpm), (b) Agitation x pH (Limonene = 350 g/L), (c) Agitation x Limonene (pH = 5.3).

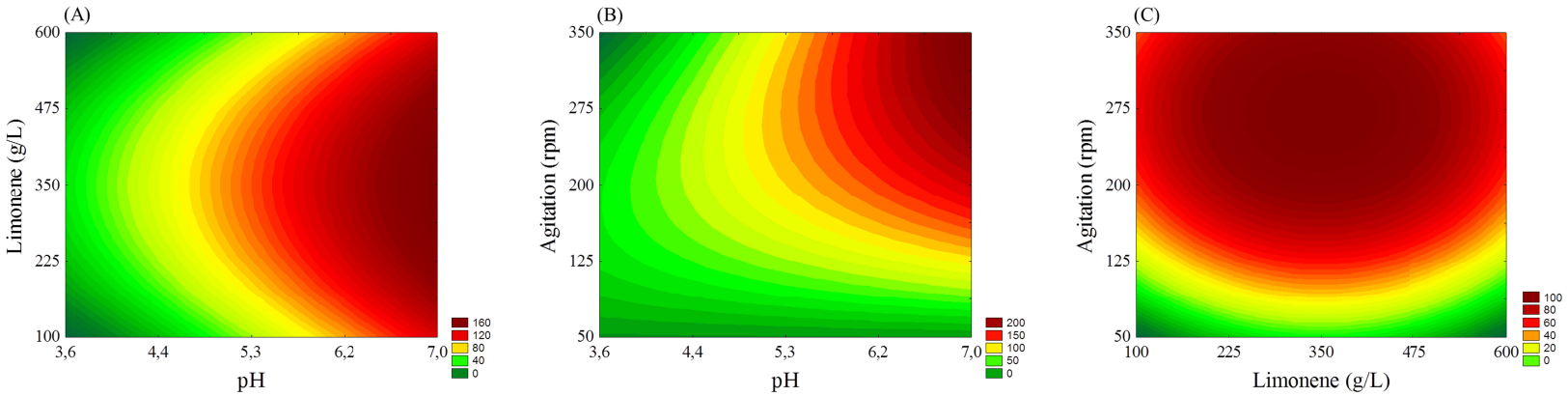

Figure 3 – Contour curves of α-terpineol production after 94 h of biotransformation process as a function of (a) Limonene x pH (Agitation = 200 rpm), (b) Agitation x pH (Limonene = 350 g/L), (c) Agitation x Limonene (pH = 5.3).

Meanwhile, the multi-response optimization of the profiles predicted for the response variables with valid mathematical models (production of α-terpineol after 46, 70, and 94 h of biotransformation) provided the optimal process conditions by analyzing contour curves and adapting the optimal values to practical work, as follows: pH 7.0, agitation speed of 200 rpm, limonene at 350 $g.L^{-1}$, and temperature of 28 ^{o}C.

The experimental validation was conducted in triplicate for both conditions (predicted by the model and analyzed from the contour curves) and Table 7 shows the results as mean values. The relative deviation values obtained for each response variable attested that the optimization methodology employed here was satisfactory. For the variable 94 h, the experimental α-terpineol production was higher than the value predicted by the derived model (eq. 4), while for 70 h the deviation was very close to the values predicted by the model. As may be seen in Table 7, the best results were observed at pH 7.0, agitation speed of 200 rpm, 350 g L^{-1} of limonene at, and temperature of 28 ^{o}C, affording the 183 g of α-terpineol per liter of organic phase. This culminated in a biotransformation process with enhanced α-terpineol formation. In conclusion, the experimental values achieved for this process were very similar to the expected ones, which was highly satisfactory.

3.3. Organic phase proportion

This experiment aimed to test how the amount of organic phase affected the α-terpineol concentration. We assayed the organic phase/aqueous phase ratio at three different levels while maintaining the final volume of the biotransformation system at 50 mL of culture medium, as follows: (a) 25 mL of organic phase and 25 mL of aqueous phase (the condition used in the optimization experiments), (b) 37.5 mL of organic phase and 12.5 mL of aqueous phase and (c) 12.5 mL of organic phase and 37.5 of aqueous phase. All the other optimized system conditions were kept under optimized conditions (limonene at 350 $g.L^{-1}$, pH 7.0, agitation rate of 200 rpm and 28 ^{o}C).

The initial goal using an organic phase in the aqueous reaction system is due the improvement of enzymatic and microbial biotransformations of terpenes compared with the use of pure aqueous media, presenting several positive impacts by allowing continuous removal of the product, decreasing product inhibition and facilitating the recovery of both product and biocatalyst (De Carvalho and Da Fonseca, 2006).

This approach really enhanced α-terpineol production as compared with previous results involving no organic phase (data not shown): 12.5 mL of the organic

phase together with 37.5 mL of the aqueous phase increased the α-terpineol concentration to approximately 500 $g.L^{-1}$ (Figure 4). However, excess organic phase (37.5 mL plus 12.5 mL of aqueous phase) did not produce satisfactory results, probably because the organic solvent interfered with the cell membrane permeability and reduced cell viability (De Carvalho and Da Fonseca, 2006).

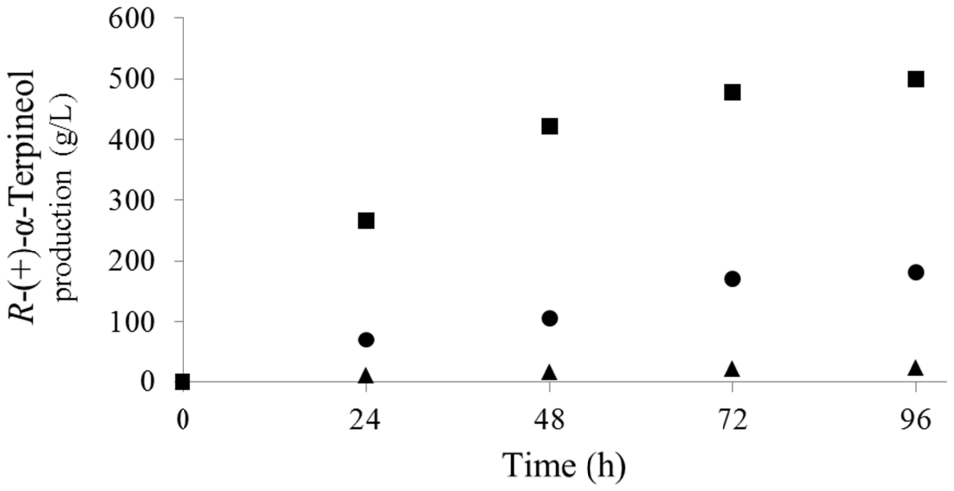

Figure 4 – Biotransformation of *R*-(+)-limonene into *R*-(+)-α-terpineol testing the proportion of organic phase in the system, where: ●: 25 mL of organic phase for 25 mL of aqueous phase, ▲: 37.5 mL of organic phase for 12.5 mL of aqueous phase and ■: 12.5 mL of organic phase for 37.5 mL of aqueous phase. Process conditions: pH 7.0, 350 g/L limonene concentration, 200 rpm of agitation speed and 28 ^{o}C.

4. Conclusion

We have optimized *R*-(+)-α-terpineol production from *R*-(+)-limonene biotransformation by *Sphingobium* sp. To this end, we used a Plackett–Burman matrix with 12 assays (PB-12) to screen the variables, followed by a central composite design methodology. The optimization technique employed here aided full understanding of the process. The models allowed us to experimentally design α-terpineol production with the desired conditions within the studied limits. The optimized biotransformation system consisted of 12.5 mL of organic phase to 37.5 mL of aqueous phase, limonene at 350 g L^{-1}, pH 7.0, agitation at 200 rpm, and temperature of 28 ^{o}C. So far, the process reported here has been the most promising alternative for the biotechnological

production of α-terpineol, generating approximately 500 $g.L^{-1}$ (organic phase) of this monoterpene alcohol.

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Conclusão Geral

O trabalho desenvolvido ao longo do projeto de Doutorado, apresentado nesta tese, visou a estudar a produção biotecnológica de bioaromas a partir da biotransformação de terpenos, focando principalmente na obtenção de limoneno-1,2-diol e α-terpineol, utilizando os biocatalisadores *Fusarium oxysporum* 152B e *Sphingobium* sp., respectivamente.

No estudo inicial, foi apresentado o amplo potencial e versatilidade do fungo *Fusarium oxysporum* para a biotransformação de terpenos, sendo que este biocatalisador foi capaz de utilizar diversos terpenos como fonte de carbono e energia, além de metabolizar os substratos *S*-(–)-limoneno, limonene-1,2-epoxido, α-pineno, linalol, γ-terpineno, em novos compostos derivados que foram identificados e quantificados por cromatografia a gás. Dentre estes produtos, a bioconversão de *S*-(–)-limoneno a limoneno-1,2-diol foi investigada em maiores detalhes, tendo em vista que a concentração obtida, mesmo em condições não otimizadas, foi de 1.2 $g.L^{-1}$. Desta forma, este processo de bioconversão foi caracterizado quando a influência de alguns parâmetros, como a idade do inóculo utilizada, indução das células, presença de cosolventes e adição sequencial de substrato. Além dos resultados coletados, observou-se a sequência na via metabólica deste fungo, que ainda foi capaz de produzir 1-hidroxi-2-oxolimoneno, a partir da metabolização do diol, sugerindo que o fungo *Fusarium oxysporum* 152B possui uma via de degradação de limoneno recentemente descoberta.

Na sequência, o trabalho prático visou ao desenvolvimento de um processo sustentável, com base na utilização de resíduos agroindustriais como meio de cultura alternativos, além de permitir a redução dos custos de processo associados à aquisição de matérias-primas sintéticas, comumente utilizadas nos meios de cultura convencionais. A seleção dos resíduos mostrou o grande potencial da manipueira, resíduo do processamento da mandioca, como meio de cultura, principalmente por ser rica em fonte de carbono e diversos minerais. Quando utilizada em um processo de otimização, a produção de biomassa chegou a aproximadamente 30 $g.L^{-1}$ (base seca), a 30 ^{o}C e 240 rpm de agitação. A biomassa produzida nessas condições foi aplicada no processo de bioconversão de *S*-(–)-limoneno a limoneno-1,2-diol, que por sua vez também foi otimizado seguindo uma estratégia sequencial para a seleção das variáveis e posteriormente validação do modelo por delineamento central composto rotacional. Com base nas análises estatísticas, a produção do limoneno-1,2-diol a partir da

bioconversão de *S*-(–)-limoneno pelo fungo *Fusarium oxysporum* 152B chegou a 3.7 $g.L^{-1}$, utilizando pH 6.5, 5 $g.L^{-1}$ de sustrato, a 28 ^{o}C e 250 rpm de agitação. Trata-se da primeira descrição da integração de um processo de otimização para produção de biomassa em resíduos agroindustriais com a otimização da produção de limoneno-1,2-diol. Além disso, a concentração obtida pode ser considerada como uma das maiores já descritas para este composto por via biotecnológica.

O trabalho ainda visou desenvolver um estudo comparativo entre a biotransformação de R-(+)-limoneno a α-terpineol e a bioconversão de *S*-(–)-limoneno a limoneno-1,2-diol, pela mesma linhagem. Neste enfoque, o trabalho mostrou as diferenças entre cada processo, sendo que no caso da produção do limoneno-1,2-diol constatou-se a necessidade da presença de oxigênio para esse processo, bem como verificar que as enzimas envolvidas nesta bioconversão são intracelulares. Adicionalmente, este foi o primeiro trabalho já descrito que visou analisar as diferenças ultraestruturais causadas no biocatalisador ao longo do processo de bioconversão destes substratos, por meio de microscopia eletrônica de varredura e transmissão, caracterizando mais um objetivo inovador deste projeto de Doutorado.

Finalmente, foi estudada a otimização da produção de α-terpineol a partir da biotransformação de *R*-(+)-limoneno, utilizando o biocatalisador reconhecido como *Sphingobium* sp. A seleção de variáveis foi realizada por meio de um Plackett-Burman (PB-12), seguido de um delineamento central composto rotacional 2^3 com as variáveis mais influentes neste processo (pH, limoneno e agitação). Após o desenvolvimento do trabalho prático e análise estatística dos dados, observou-se que as melhores condições para este processo foram pH 7.0, concentração de limoneno de 350 $g.L^{-1}$, agitação de 200 rpm e 28 ^{o}C. Nestas condições, a produção deste álcool monoterpênico chegou a 500 $g.L^{-1}$, que pode ser considerada como a maior concentração de α-terpineol já relatada na bibliografia de processos biotecnológicos e tornando este processo muito promissor e atrativo do ponto de vista industrial.