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PROSPECÇÃO DE PROCESSOS BIOTECNOLÓGICOS DE INTERESSE INDUSTRIAL

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SUPLENTE

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SUMÁRIO

RESUMO GERAL

O objetivo do presente trabalho foi efetuar estudos de biotransformação de monoterpenos para a obtenção de compostos de aromas naturais, ou bioaromas, enfatizando os processos bioquímicos envolvidos nos procedimentos empregados para possíveis aplicações industriais.

Assim, o estudo se iniciou com 70 linhagens, isoladas em uma planta de processamento de suco de laranja e selecionadas em um estudo prévio pois mostraram-se capazes de utilizar este *R*-(+)-limoneno como única fonte de carbono. Estes micro-organismos foram testados quanto a sua capacidade de biotransformar este monoterpeno em compostos de aroma. Dentre todas as linhagens potencialmente degradantes do *R*-(+)-limoneno, apenas a linhagem LB285JLB mostrou acúmulo significativo de metabólito de interesse em concentração que justifique estudos de otimização.

A seguir, o método de Superfície de Resposta foi empregado para otimizar os principais parâmetros do processo de produção de biomassa desta linhagem, utilizando glicerol como fonte de carbono. Dentre os parâmetros analisados, três (concentração de substrato, temperatura e agitação) influenciaram significativamente ($p < 0,10$) a produção de biomassa da linhagem LB285JLB, dentro das faixas estudadas. A otimização dessas variáveis por um Delineamento Composto Central Rotacional revelou que as condições ótimas para a fermentação foram 6-9 $g.L^{-1}$ de glicerol, temperatura entre 35-40 °C e agitação de 230 a 300 rpm.

Finalmente, estudos preliminares de prospecção do potencial dos micro-organismos endofíticos isolados do Baru (*Dipteryx alata* Vog.), visando o desenvolvimento de processos biotecnológicos de interesse industrial. Até o presente momento os trabalhos conduzidos demonstraram que as linhagens endofíticas podem ter aplicação potencial em processos de produção de bioaromas a partir da biotransformação de monoterpenos, juntamente com a co-produção de enzimas. Esses resultados abrem precedentes para que novas pesquisas sejam consideradas a fim de determinar o potencial deste nicho de micro-organismos.

ABSTRACT

The objective of the present work was to study the biotransformation of monoterpenes to obtain natural flavor compounds (bioflavors), focusing the biochemical processes involved in the procedures investigated for possible industrial applications.

Therefore, the study started with 70 strains, isolated in a previous study from a citrus processing plant and capable of use *R*-(+)-limonene as sole carbon and energy source. These microorganisms were tested for their ability to biotransform this monoterpene on flavor compounds. Among the strains potentially degrading of *R*-(+)-limonene, just the strain LB285JLB showed significant accumulation of interest metabolite concentration which could justify optimization studies.

Subsequently, the Response Surface Methodology was employed to optimize the main parameters of the process of LB285JLB biomass production, using glycerol as carbon source. Only three (glycerol concentration, temperature and agitation) of all parameters tested influenced significantly ($p < 0.1$) the biomass production. The optimization of these variables applying a Central Composite Design revealed that the optimal biotrasformation conditions were 6-9g.L^{-1}, 35-40 °C and 230-300 rpm.

Finally, preliminary propection studies evaluating the potential of endophytes isolated from Baru (*Dipteryx alata* Vog.), for the development of biotechnological processes of industrial interest.

To date, the studies conducted have shown that endophytic microorganisms may have potential application in biotransformation process for bioflavors production, and also should be considered with process of enzymes co-production. These results encourages that further researchs should be considered to determine the potential of these microorganisms.

INTRODUÇÃO GERAL

1. Aromas

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 e ácido, além do mais recente *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 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 baixo peso molecular (em geral menores que 400 daltons) e, apesar de não representarem nenhuma função nutritiva ao alimento, estes compostos são freqüentemente 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).

1.1. Vias metabólicas para a produção de compostos de aroma

Os aromas podem ser extraídos empiricamente de substâncias vegetais ou animais, mas também podem ser produzidos por mecanismos químicos ou biológico. 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, como a conversão de precursores naturais pela biocatálise, síntese *"de novo"* e o 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. 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 (MARÓSTICA JR., 2006; TAN *et al*., 1998).

2. Mercado Mundial: Ascensão x Dificuldades

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; 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; BERGER, 1998).

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; BERGER, 1998).

3. Terpenos

Do ponto de vista econômico, uma possibilidade interessante para a produção de aromas por bioprocessos é a utilização de terpenos como substrato, pois ocorrem largamente na natureza. Assim como o limoneno, α e β-pineno, que são considerados resíduos industriais com valor comercial reduzido (YOO *et al.*, 2002) e representam um substrato ideal para processos biotecnológicos, sendo intensamente estudados em experimentos de conversão microbiana (MARÓSTICA JR, 2006).

Os terpenos são metabólitos secundários de plantas, produzidos, em parte, para defesa contra microrganismos 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 (TEISSEIRE, 1994). Na maioria dos casos, o odor característico e agradável de frutas, ervas e especiarias são resultantes dos terpenos voláteis (KRINGS *et al.*, 2006).

CAPÍTULO 1 - REVISÃO BIBLIOGRÁFICA

1. Introdução

Os terpenos são metabólitos secundários de plantas, produzidos, em parte, para sua defesa contra microrganismos e insetos, exercendo um papel fungicida e responsável por atrair polinizadores (GERSHENZON; DUDAREVA, 2007; LANGENHEIM, 1994). Em mamíferos, podem desempenhar diversas funções, como atuar como reguladores em reações enzimáticas e na estabilização de membranas celulares, além de participarem em diversas vias metabólicas (DE CARVALHO; DA FONSECA, 2006).

Estes compostos contêm estrutura carbônica formada por resíduos de isopreno (C_5H_8), podendo ser classificados quanto ao número de carbonos, em monoterpenóides (dez carbonos), sesquiterpenóides (quinze carbonos), diterpenóides (vinte carbonos), triterpenóides (trinta carbonos) e tetraterpenóides ou carotenóides (quarenta carbonos) (TEISSEIRE, 1994).

Até o presente momento, mais de 30.000 terpenos naturais foram identificados (MODZELEWSKA et al., 2005). Os terpenos mais simples (mono e sesquiterpenos) são encontrados em grande quantidade em óleos essenciais, cujo aroma é atribuído principalmente a presença destes compostos. Além disso, são amplamente empregados como aditivos nas indústrias de perfumaria e de alimentos. Enquanto isso, os di e triterpenos são menos voláteis e obtidos de resinas de plantas (TRUDGIL, 1986). Na maioria dos casos, os terpenos voláteis e seus derivados oxigenados, conhecidos com terpenóides, são responsáveis pelo odor característico e agradável de frutas, ervas e especiarias (KRINGS *et al.*, 2006). Os terpenos têm recebido atenção devido ao esclarecimento de seu papel na prevenção de doenças, de sua atividade como inseticidas naturais e agentes microbianos (DE CARVALHO; DA FONSECA, 2006).

Uma possibilidade para a produção de aromas naturais é a utilização de terpenos, pois diversas características os tornam um substrato ideal para os processos biotecnológicos. Do ponto de vista econômico despertam grande interesse pois ocorrem largamente na natureza, como o limoneno e o α-pineno, que podem ser encontrados por preços baixos e acessíveis, por serem rejeitos industriais gerados em escala industrial.

Além disso, são bons materiais de partida para a síntese de produtos químicos refinados devido à sua similaridade estrutural com os produtos resultantes (BICAS, 2009).

O *R*-(+)-limoneno, por exemplo, monoterpeno mais abundante na natureza, é o componente majoritário do óleo da casca da laranja em uma fração superior a 90%, tornando-se um precursor barato (US$ 1-2 por Kg) e muito promissor (BAUER; GARBE; SURBURG, 2001; MATTHEWS; BRADDOCK, 1987). Este composto tem sido intensamente estudado em experimentos de conversão microbiana visando a produção de derivados oxigenados como, por exemplo, álcool perílico, carvona e α-terpineol. Estes compostos são reconhecidos por seu aroma agradável e, alguns deles, por serem capazes de atuar não apenas na prevenção ou formação de diversos tipos de câncer, mas também na regressão de tumores malignos (CROWELL, 1999; JUN; JEONG; HO, 2006). Além disso, os produtos formados a partir do limoneno possuem valor consideravelmente maior, podendo ser comercializados a US$ 30-60 por Kg (DUETZ et al., 2003).

Enquanto isso, α- e β-pineno são os maiores constituintes do óleo essencial de coníferas, atingindo concentrações entre 75 a 90% em sua composição. Estes compostos são encontrados em grandes concentrações na terebentina, resíduo industrial proveniente da indústria de polpa e papel, representando um substrato ideal para processos biotecnológicos devido a seu preço acessível (YOO; DAY, 2002). Estes monoterpenos bicíclicos são precursores de importantes compostos de aroma, como terpineóis, cânfora, geraniol, mentol, verbenol e verbenona (BAUER; GARBE; SURBURG, 2001).

Atualmente, de aproximadamente 6.500 compostos de aroma conhecidos, apenas 300 são utilizados na indústria. Dentre eles, apenas de 50 a 100 compostos são produzidos através de fermentação microbiana, enquanto a grande maioria a partir de síntese química (SCRAGG, 2007). Atualmente, observa-se uma nova tendência em países desenvolvidos, como por exemplo na Alemanha, onde 70% de todos os compostos de aroma utilizados em alimentos são considerados "naturais" (DEMYTTENAERE; VAN BELLEGHEM; KIMPE, 2001). Todos estes fatores, aliados ao interesse por aromas naturais frente aos sintéticos, levaram a um aumento nos estudos que visam a produção biotecnológica dos "bioaromas" (MARÓSTICA JR, 2006).

Devido à grande relevância e diversidade dos compostos de aroma e fragrância, a literatura científica está repleta de revisões que abrangem temas como a química dos

terpenóides (GRAYSON, 2000), as reações químicas de terpenos para a produção de aromas (SWIFT, 2004) e outros produtos químicos (MONTEIRO; VELOSO, 2004), biotransformação do limoneno (DUETZ *et al.,* 2003; MARÓSTICA JR.; PASTORE, 2007a) e outros terpenos (DE CARVALHO; DA FONSECA, 2006; DEMYTTENAERE, 2001; TRUDGILL, 1990; VAN DER WERF; DE BONT; LEAK, 1997) e a produção de aromas naturais empregando a biocatálise (GIRI *et al.*, 2001; LOMASCOLO *et al.,* 1999; SCHRADER *et al.*, 2004; SERRA; FUGANTI; BRENA, 2005; WELSH; MURRAY; WILLIAMS, 1989).

1.1.Métodos para a produção de compostos de aroma

Tradicionalmente, os compostos de aroma são obtidos basicamente por dois processos distintos: síntese química e extração de fontes naturais.

A extração de aromas naturais de vegetais apresentam algumas desvantagens, pois está sujeita às instabilidades advindas da sazonalidade, de fatores políticos e climáticos, além dos problemas ecológicos relacionados ao extrativismo. Outro problema deste método é a baixa concentração dos compostos de interesse, que ocasiona uma elevação considerável no custo das extrações (BICAS, 2009; MARÓSTICA JR, 2006).

Os estudos de transformação de terpenos por síntese química são pioneiros, sendo que os primeiros relatos desta atividade ocorreram há mais de 130 anos, empregando cloreto de nitrosila (TILDEN; LOND, 1875). Já na década de 50, Royals e Horne Jr. (1951) aplicaram este método para produção de carvona a partir de *R*-(+)-limoneno, com um rendimento variando entre 56 e 60%. A partir destes trabalhos, novos estudos surgiram ao longo dos anos, adaptando e refinando a metodologia que emprega cloreto de nitrosila, sendo hoje uma importante metodologia para a produção industrial de carvona (BICAS, 2009).

Outro mecanismo investigado para a oxidação de olefinas consiste no uso de dióxido de selênio (TRACHTENBERG; CARVER, 1970; TRACHTENBERG; NELSON; CARVER, 1970). Com a utilização desta metodologia, a oxidação de diversos terpenos foi estudada, como o caso do limoneno (SAKUDA, 1969; THOMAS; BUCHER, 1970), α-pineno (LI, 2000; ZHENG; LU, 1995), β-pineno (COXON; DANSTED; HARTSHORN, 1970), canfeno (HIRSJARVI, 1956) e ainda alguns sesquiterpenos (SATHE *et al.*; 1966).

Diversos estudos compreendem a utilização de metais como catalisadores na oxidação de terpenos, podendo ser um método promissor para produção de derivados oxigenados (GONÇALVES; BUENO; GUSEVSKAYA, 2006; GUSEVSKAYA; ROBLES-DUTENHEFNER; FERREIRA, 1998). Recentemente, Lima *et al.* (2006) avaliaram os principais parâmetros capazes de afetar a reação de oxidação alilíca ou epoxidação do limoneno utilizando o catalisador metal(salen). Além disso, os mesmos autores investigaram a utilização de CO_2 supercrítico ao invés dos solventes orgânicos usuais. Apesar de ser um sistema similar ao obtido com os solventes orgânicos, mostrou-se mais seletivo na formação do epóxido (LIMA et al., 2005).

As novas tendências na química orgânica, visando a produção de compostos de aroma em escala industrial, ocorrem mediante reações de fotooxidação via oxigênio singleto, empregando corantes como fotosensibilizadores. Esta abordagem pode ser reconhecida como "química verde", sendo mais atrativa quando comparada aos métodos tradicionais de síntese química, por ser limpa, não gerar resíduos e favorecer a tecnologia sustentável. Entretanto, a demanda de grande quantidade de energia das fontes artificiais é um dos desafios a serem transpostos (OELGEMÖLLER; JUNG; MATTAY, 2007). Esta metodologia já foi empregada na oxidação de citronelol para a produção de óxido de rosa em larga escala (WOOTTON; FORTT; DE MELLO, 2002).

A maioria dos aromas utilizados no mundo (aproximadamente 80%) são obtidos por processos químicos. Embora a síntese química seja responsável pelas maiores escalas de produção retratadas até o momento, pode resultar na criação de altos impactos ambientais, por emitirem carga considerável de resíduos não-biodegradáveis, muitas vezes não apresentam régio- ou enantioseletividade ao substrato, resultando em mistura de produtos (BICAS, 2009). Para revisões mais detalhadas sobre a transformação de terpenos por síntese química visando a produção de aromas, recomenda-se a leitura de Swift (2004) e Monteiro e Veloso (2004).

2. Biotecnologia: bioprocessos para a produção de compostos de aroma

Durante os últimos anos a indústria visa adaptar seus processos e produtos às recentes tendências mundiais. A preocupação com as questões ambientais forçaram o desenvolvimento de processos mais limpos, como o conceito conhecido como 3R

(Reduza, reuse e recicle), que impulsionou a demanda por produtos naturais e funcionais, contendo os compostos bioativos (BICAS, 2009).

Neste enfoque, a biotransformação surge como uma alternativa promissora para a produção de aromas a partir da oxidação de terpenos, pois ocorre em condições brandas de reação, possui elevada régio- e enantioseletividade, não gera resíduos tóxicos e resulta na produção de compostos classificados como "naturais". Além disso, muitos processos de biotransformação podem resultar na produção de compostos dificilmente obtidos por métodos químicos (BICAS, 2009; GIRI *et al*., 2001; JANSSENS *et al.*, 1992).

Segundo Kieslich (1984), a biotransformação pode ser definida como a reação química catalisada por microrganismos ou enzimas. Estas reações são usualmente desenvolvidas por culturas em crescimento, previamente formadas, células imobilizadas, enzimas purificadas ou sistemas multifásicos (LEUENBERGER, 1990). Em geral, os compostos voláteis produzidos por microrganismos são metabólitos secundários, isto é, não são essenciais para seu metabolismo, mas podem contribuir para sua sobrevivência, podendo inibir competitivamente outras espécies ou diminuir a toxicidade de determinado solvente presente no meio (BERGER, 1995).

De acordo com De Carvalho e Da Fonseca (2006), apenas 7% dos artigos científicos publicados na última década, voltados a biotransformação de terpenos, utilizam enzimas purificadas como biocatalisador, enquanto células vegetais, fungos, leveduras e bactérias representam 11%, 33%, 2% e 41%, respectivamente.

2.1. Problemas encontrados no processo de biotransformação

A grande maioria dos estudos de biotransformação de terpenos descritos até o momento é de caráter acadêmico, sendo inviáveis para aplicação industrial devido a problemas encontrados durante o processo, como principalmente os baixos rendimentos devido à volatilidade do produto e substrato, além da toxicidade do terpeno aos microrganismos em geral (CHATTERJEE; BHATTACHARYYA, 2001).

Além disso, a multiplicidade dos metabólitos originados da biotransformação resultam em baixas concentrações dos produtos finais e intermediários, elevando os custos de recuperação dos compostos gerados. Para que o acúmulo dos produtos possa ser maior deveria haver um longo tempo de fermentação, o que contrasta com o interesse de pequenos curtos de processo, para minimizar a instabilidade e volatilidade do substrato (SPEELMANS; BIJLSMA; EGGINNK, 1998).

Na maioria dos casos, os compostos de aroma estão presentes em baixas concentrações nos sistemas fermentativos, o que também acarreta em um alto custo de processos de recuperação, o que muitas vezes é compensado pelo custo elevado dos aromas naturais, que chegam ser de 10 a 100 vezes superior ao dos sintéticos (JANSSENS et al., 1992).

Muitos estudos abordam o efeito inibidor de terpenos aos microrganismos. Isto pode ser explicado pelo fato de que o composto pode diminuir a velocidade do processo de fosforilação oxidativa nas células (CHATTERJEE; BHATTACHARYYA, 2001). O nível de toxicidade dos solventes orgânicos, imiscíveis em água, aos micro-organismos pode ser medido pelo valor de *log* P (coeficiente de partição octanol-água). As toxicidades mais extremas foram observadas em valor de log P entre 1 e 5. Compostos como o limoneno (log P = 4,83) aumentam a fluidez das membranas dos fungos filamentosos e a sua permeabilidade não específica, perda da integridade celular, decréscimo de matéria seca e inativação da energia metabólica devido à dissipação da força próton motriz (gradiente eletroquímico de H^+ através da membrana) (ONKEN; BERGER, 1999b).

Além disso, os processos de biotransformação ainda enfrentam problemas relacionados à recuperação dos produtos. A volatilidade e baixa solubilidade de muitos compostos de aroma em água dificultam sua recuperação. Além do fato de que, concentrações muito elevadas do produto no meio podem ter efeito inibidor sobre os microrganismos (JANSSENS *et al.*, 1992).

Desta forma, observa-se a necessidade do desenvolvimento de técnicas mais apuradas e sensíveis, permitindo a produção e recuperação do produto em níveis satisfatórios.

3. Biotransformação do limoneno

Nos últimos anos, muitos trabalhos destinaram-se aos estudos de biotransformação do limoneno, sendo que este moterpeno monocíclico é considerado como o terpeno mais distribuído na natureza (DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001), fazendo parte da estrutura de mais de 300 vegetais (BURDOCK, 1995).

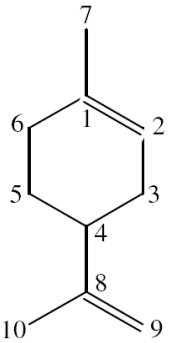
O *R*-(+)-limoneno, o principal componente de óleos essenciais de diversas frutas cítricas (BAUER; GARBE; SURBURG, 1990). Especialmente no caso do Brasil, que é

considerado como o maior produtor mundial de frutas cítricas e destaca-se por sua expressiva produção de laranjas, o óleo de laranja e *R*-(+)-limoneno são exportados pelo Brasil em um volume de 30 a 40 mil toneladas anualmente, como subproduto da indústria citrícola (ABECITRUS, 2008).

Desta forma, torna-se um substrato interessante para os processos de bioconversão, pela alta disponibilidade e preço baixo e acessível (KRASNOBAJEW, 1984). O limoneno é separado do óleo essencial obtido no suco de laranja pela sua baixa solubilidade em água, alta tendência a autoxidação, polimerização e formação de *off-flavors*, tornando-se um subproduto industrial adequado e atrativo para processos de bioconversão, podendo ser transformados em compostos de alto valor agregado (VAN DER WERF; DE BONT; LEAK, 1997; BERGER; KRINGS; ZORN, 2002). Enquanto isso, o isômero S-(-)-limoneno é encontrado em uma grande diversidade de plantas, como *Mentha* spp (DEMYTTENAERE; DE KIMPE, 2001).

Assim sendo, a utilização do limoneno para síntese de compostos de aroma e funcionais pode ser muito promissora do ponto de vista econômico, pois a partir deste monoterpeno podem ser obtidos diversos compostos, como principalmente seus derivados oxigenados, α-terpineol, álcool perílico, carveol, carvona e mentol (DUETZ *et al*., 2003).

Devido a dificuldade da oxidação química em introduzir regiospecificamente grupos carbonilas ou hidroxilas nos carbonos 7 e 10 e nos grupos metilas presentes nos carbonos 3 e 6 (Figura 1), o processo leva a uma mistura de produtos (ONKEN; BERGER, 1999b). Sendo assim, por empregar o uso de reações enzimáticas, a biotecnologia apresenta vantagens quando comparada a síntese química, devido a sua enantio e regioespecificidade.



Limoneno

Figura 1 - Estrutura do limoneno (MARÓSTICA JR, 2006).

Neste intuito, os estudos de oxidação enzimática do limoneno tiveram início na década de 60 por Dhavalikar e Bhattacharyya (1966). Os autores isolaram uma linhagem de *Pseudomonas* de amostras do solo capaz de utilizar limoneno como única fonte de carbono, que converteu este substrato a uma grande diversidade de produtos, como compostos perílicos, carveol, carvona e dihidrocarvona. Após estes relatos, Cadwallader *et al.* (1989) selecionaram uma linhagem capaz de utilizar limoneno como única fonte de carbono e produzir ácido perílico e α-terpineol. Recentemente, Bicas e Pastore (2007) realizaram um extenso trabalho para isolamento de microrganismos de diferentes pontos de uma indústria de processamento de laranjas. Os autores visaram selecionar microrganismos pré-resistentes e adaptados a ambientes drásticos. Na sequência, 70 bactérias foram selecionadas pela sua capacidade em resistir a 2% de limoneno e utilizá-lo como única fonte de carbono.

Este substrato tem sido intensivamente estudado quanto a biocatálise conduzida por microrganismos. A Figura 2 compreende as seis rotas metabólicas de conversão do limoneno, descritas até o presente momento:

1. oxidação do substituinte metila em compostos perílicos;
2. conversão da dupla ligação do anel ao diol correspondente;
3. oxidação alílica a *cis*, *trans* carveóis e carvona;
4. epoxidação da ligação dupla na unidade isoprenil a α-terpineol;
5. oxidação alílica a isopiperitenol;
6. epoxidação da dupla ligação 8,9 a limoneno-8,9-epóxido.

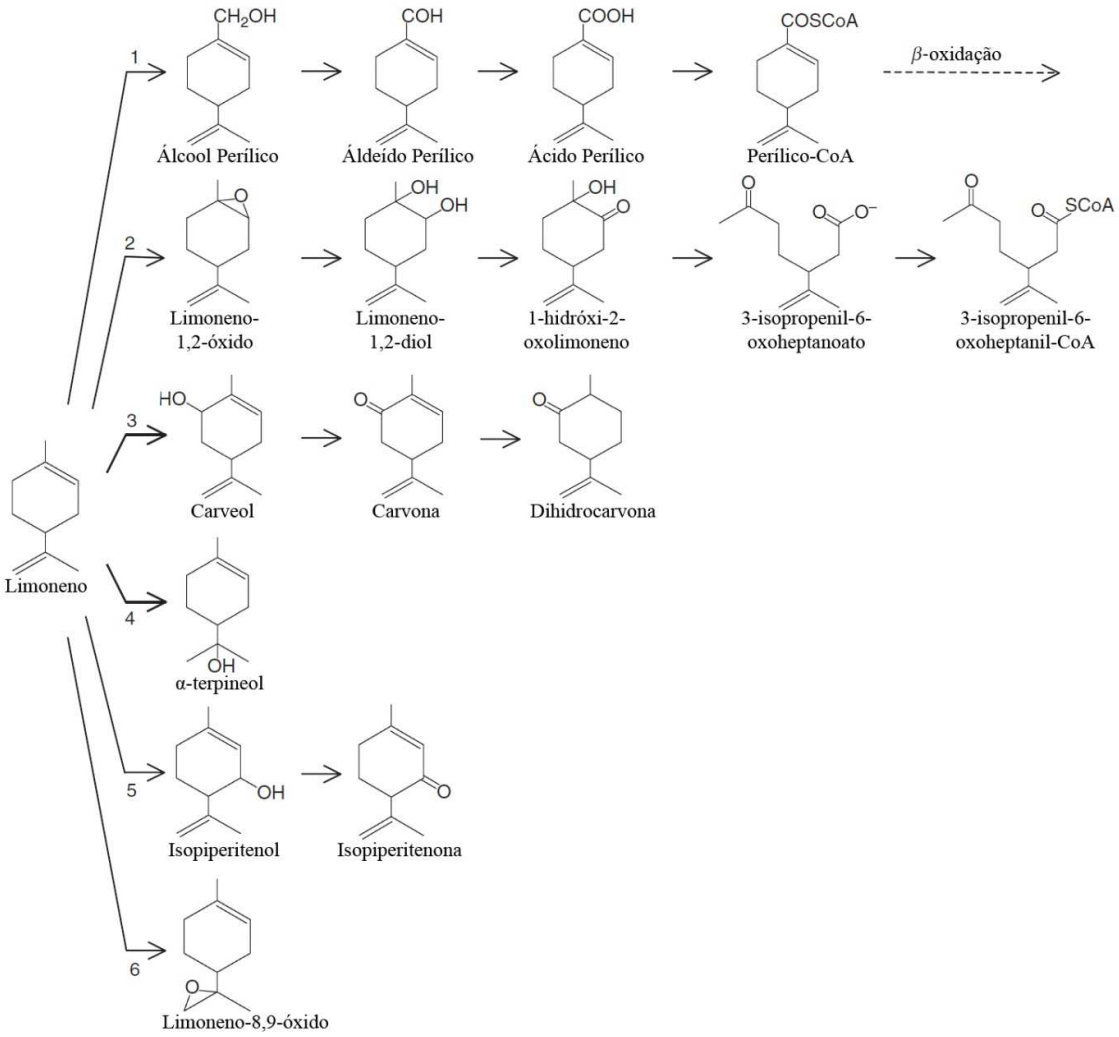


Figura 2 - Principais rotas envolvidas na biotransformação do limoneno (MARÓSTICA JR, 2006; BICAS et al., 2008a; VAN DER WERF; SWARTS; DE BONT, 1999).

3.1.Produção de compostos perílicos

Três substâncias de grande interesse industrial podem ser obtidas a partir do ataque da posição 7 do limoneno (rota metabólica 1 da Figura 2), resultando em álcool perílico, aldeído perílico e ácido perílico. Estudos relatam a biotransformação do limoneno a seus derivados perílicos por bactérias, fungos filamentosos (CHATTERJEE; BHATTACHARYYA, 2001; SPEELMANS; BIJLSMA; EGGINK, 1998; CADWALLADER *et al.*, 1989; CHANG; ORIEL, 1994; CHEONG; ORIEL, 2000; MENÉNDEZ *et al.*, 2002) e leveduras (VAN RENSBURG et al., 1997).

Após os estudos pioneiros de Dhavalikar e Bhattacharyya (1966), que obtiveram compostos perílicos em seu processo, Cadwallader e colaboradores (1989) selecionaram

uma linhagem de *Pseudomonas gladioli* capaz de produzir 1861 ppm de ácido perílico e α-terpineol. Entretanto, os autores relatam que, ao final de sete dias de fermentação, o composto perílico não podia mais ser detectado no meio devido a seu rápido desaparecimento. Uma linhagem de *Bacillus stearothermaphilus* BR388, isolada da casca da laranja, mostrou-se resistente ao limoneno e foi capaz de metabolizá-lo como única fonte de carbono. Observou-se a produção de álcool perílico em concentração máxima de 200 mg/L e α-terpineol e aldeído perílico como produtos minoritários (CHANG; ORIEL, 1994). Posteriormente, esta linhagem ainda foi estudada com base na engenharia genética (CHEONG; ORIEL, 2000).

A linhagem de *Pseudomonas putida* GS1 metabolizou limoneno a ácido perílico em altas concentrações, chegando a 3 $g.L^{-1}$ (SPEELMANS; BIJLSMA; EGGINK, 1998). Outra linhagem de *Pseudomonas putida* MTCC 1072 foi capaz de oxidar o limoneno a álcool perílico e sobrerol, com rendimentos de 36 e 44%, respectivamente. Os autores ainda estudaram outros parâmetros importantes no meio fermentativo, relatando que a concentração de 0,2% de substrato mostrou-se melhor e, quando empregado concentrações superiores de limoneno, a produção foi influenciada negativamente. Além disso, a atividade de biocatálise mostrou-se mais eficaz ao redor de pH 5 (CHATTERJEE; BHATTACHARYYA, 2001).

Trytek e Fiedurek (2005) relataram a biotransformação de limoneno pelo fungo psicrotrófilo, *Mortierella minutissima*, selecionada por mostrar-se capaz de resistir à alimentação do substrato via fase gasosa. O processo de bioconversão resultou em álcool e ácido perílico em concentrações significativas, além de compostos minoritários como carveol e carvona. Aproximadamente 120 $mg.L^{-1}$ de álcool perílico foram obtidos após 120 horas de fermentação, a 15 ^{o}C e pH 6,0. Os autores concluíram que o uso de baixa temperatura favoreceu o processo de biotransformação, minimizando perdas de substrato e produto por volatilização. Aparentemente, este foi o único trabalho a empregar um microrganismo psicrotrófilo em processos de bioconversão.

Além disso, diversos estudos têm relatado que estes compostos possuem propriedades preventivas contra diversos tipos de câncer, como de fígado, mama e pulmão (CHATTERJEE; BHATTACHARYYA, 2001). Imagawa e Ming-Sing (2000) evidenciaram que o uso de álcool perílico, quando utilizado separadamente ou em combinação com imunodepressivos, foi responsável pela redução no nível de rejeição em pacientes submetidos a transplante de órgãos.

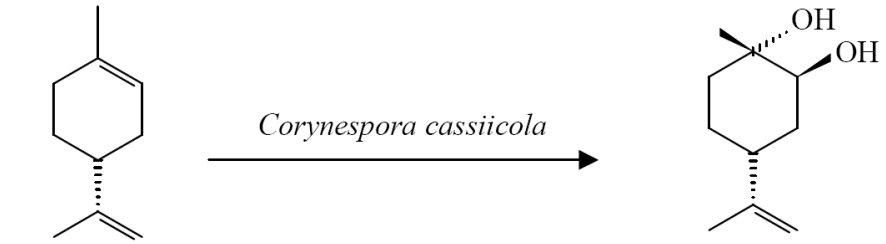
Ensaios *in vivo* relatam que o álcool perílico também possui ação quimiopreventiva contra cânceres quimicamente induzidos em fígado e pâncreas de ratos (MILLS *et al.,* 1995) e é muito eficaz na prevenção de tumores recorrentes ou cânceres secundários em animais tratados por quimioterapia (HAAG; GOULD, 1994).

3.2. Bioconversão da dupla ligação do anel ao diol correspondente

Alguns autores relatam que a formação de dióis, a partir do limoneno, é uma rota comum no metabolismo de monoterpenos de alguns fungos (MUKHERJEE; KRAIDMAN; HILL, 1973).

O primeiro relato da obtenção de dióis ocorreu com o emprego de uma linhagem de *Cladosporium* sp, capaz de atacar o limoneno na ligação 1,2, resultando em *cis*- e *trans*-diol em concentrações finais de 0,2 e 1,5 $g.L^{-1}$, respectivamente (KRAIDMAN; MUKHERJEE; HILL, 1969). Uma linhagem de *Aspergillus* sp., *i.e. A. cellulosae*, foi capaz de converter os dois isômeros do limoneno em limoneno-*trans*-1,2-diol como produto principal (NOMA; YAMASAKI; ASAKAWA, 1992).

Um dos trabalhos mais significativos na área envolvendo a produção de dióis, foi descrito por Abraham *et al.* (1985). Os autores descrevam um processo de recuperação com elevado rendimento de *1S,2S,4R*-limoneno-1,2-diol a partir de *R*-(+)-limoneno utilizando uma linhagem de *Corynespora cassiicola*, com alimentação contínua de substrato em um bioreator com capacidade de 100L contendo 70L de meio de cultura. Quando 1.300 g de substrato foram utilizadas, 900 g de *1S,2S,4R*-limoneno-1,2-diol e pequenas quantidades de *1R,2R,4R*-diasteroisômero foram recuperados após 96 horas de processo, representando uma alternativa econômica para a preparação de dióis (Figura 3) .



R-(+)-limoneno
1.300 g

1S,2S,4R-limoneno-1,2-diol
900 g

Figura 3 - Alternativa econômica para o preparo de 1*S*,2*S*,4*R*-limoneno-1,2-diol a partir de *R*-(+)-limoneno em bioreator de 100L (ABRAHAM et al., 1985).

Observou-se que a linhagem de bactéria *Rhodococcus erythropolis* DLC14 inicia a biotransformação do limoneno com epoxidação na posição 1,2 catalisada por uma enzima limoneno monooxigenase, cuja atividade mostrou-se não dependente do sistema citocromo P450 (VAN DER WERF; SWARTS; DE BONT, 1999). No ano seguinte, verificou-se que as células deste microrganismo, quando desenvolvidas na presença de terpenos, exibe atividade de limoneno epóxido hidrolase (LEH), que permite converter *cis*-limoneno-1,2-epóxido em limoneno-1,2-diol. O isômero *trans* só foi convertido quando o *cis* não estava disponível. Os autores ainda desenvolveram um processo para recuperação do produto, baseado na afinidade do limoneno epóxido pela fase orgânica e do diol pela fase aquosa (DE CARVALHO; VAN KEULEN; DA FONSECA, 2000). Além destas descobertas, diversos estudos foram realizados visando o isolamento e caracterização da enzima limoneno-hidrolase do microrganismo *Rhodococcus erythropolis* DCL14 (VAN DER WERF; OVERKAMP; DE BONT, 1998; VAN DER WERF *et al.*, 1999; BARBIRATO *et al*., 1998).

Recentemente, um estudo investigou a capacidade de mais de 60 linhagens de microrganismos em biotransformar *R*-(+)- e *S*-(-)-limoneno, utilizando a metodologia de microextração em fase sólida para verificar a produção de compostos de aroma. Uma linhagem de *Corynespora cassiicola* mostrou-se capaz de produzir (1*S*,2*S*,4*R*)- e (1*R*-2*R*-4*S*)-limoneno-1,2-diol a partir do isômero *R*-(+)- e *S*-(-)-, respectivamente (DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001).

3.3.Oxidação alílica a cis, trans carveóis e carvona

A oxidação na posição 6 do limoneno pode gerar substâncias de grande relevância industrial como carveol e carvona. Este último ocorre nas formas *R*-(+) e *S*-(-) e seus isômeros estão presentes em diversos óleos essenciais, diferindo significativamente em suas propriedades sensoriais. *R*-(+)-carvona é o principal componente do óleo de cominho, sendo encontrado em concentrações de aproximadamente 60%, enquanto sua forma *S*-(-) ocorre amplamente em óleos de hortelã, em concentrações entre 70 e 80% (BAUER; GARBE; SURBURG, 1990). Ambos são amplamente utilizados como compostos de aroma em diversos produtos alimentícios, bebidas, produtos de higiene e higiene bucal (OHLOFF, 1994).

O primeiro relato do ataque da posição 6 do limoneno foi feito em 1966, quando os autores indianos obtiveram carveol, carvona e dihidrocarvona utilizando linhagens de

microrganismos isolados de frutas cítricas (DHAVALIKAR; BHATTACHARYYA, 1966).

Uma linhagem de basidiomiceto *Pleurotus sapidus* mostrou-se capaz de biotransformar o *R*-(+)-limoneno em carveol e carvona, como produtos majoritários. Após 12 horas de cultivo, a concentração dos produtos chegaram a 70 e 30 mg/L, respectivamente, com alimentação de substrato via fase gasosa. Os autores observaram que a presença de limoneno no pré-cultivo na presença afetou negativamente a capacidade de crescimento do microrganismo, embora a taxa de conversão dos produtos tenha sido mais de duas vezes maior para o carveol e três vezes para a carvona, quando comparado com o teste sem a adaptação ao substrato (ONKEN; BERGER, 1999b).

Duetz *et al.* (2001) descreveram a capacidade das células de *Rhodococcus opacus* PWD4, após crecerem em tolueno com única fonte de carbono, hidroxilaram *R*-(+)-limoneno na posição 6, resultando na formação de *trans*-carveol enantiomericamente puro. Posteriormente, a conversão de (+)-*trans*-carveol a (+)-carvona, com altos rendimentos, tornou-se possível pela linhagem *Rhodococcus globerus* PWD8, demonstrando um grande potencial industrial para a produção desta cetona (Figura 4).

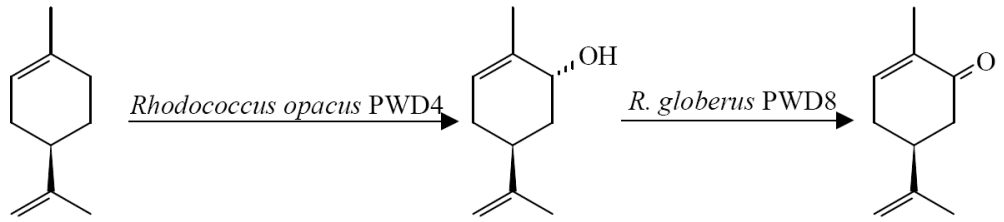


Figura 4 - Processo de bio-oxidação do *R*-(+)-limoneno a *trans*-carveol e posteriormente a carvona, utilizando linhagens de *Rhodococcus* PWD4 e PWD8 (DUETZ et al., 2001).

A biotransformação de alguns terpenos foi testada em outro trabalho, dentre eles o *S*-(-)-limoneno. Os autores observaram que uma linhagem de *Aspergillus niger* foi capaz de biotransformar somente (-)-limoneno com conversão satisfatória, produzindo carveol e dihidrocarveol com rendimentos de 18% e 15%, respectivamente (DIVYASHREE; GEORGE; AGRAWAL, 2006).

No mesmo ano, um grupo português descobriu a capacidade da linhagem *Rhodococcus erythropolis* DCL14 transformar (-)-trans-carveol a (-)-carvona. As

autoras detectaram que o uso de sistemas bifásicos melhoraram as taxas de conversão (TECELÃO; VAN KEULEN; DA FONSECA, 2001). O mesmo grupo publicou um artigo evidenciado as possíveis aplicações desta cetona. Para uma revisão completa sobre o assunto, recomenda-se a leitura De Carvalho e Da Fonseca (2006b).

Os compostos formados a partir desta via metabólica são produtos muito visados, principalmente pelo seu grande potencial de aplicação e perfil de aroma, além dos últimos trabalhos relacionarem estes compostos a diversas atividades funcionais. A carvona é um importante agente antimicrobiano contra bactérias e fungos patogênicos, com potencial aplicação em alimentos e produtos anti-sépticos. Possui também atividade inseticida, atuando contra moscas, larvas de insetos, inclusive sobre *Aedes aegypti*, o vetor da dengue hemorrágica. Além disso, exibe ação quimiopreventiva contra diversos tipos de câncer (DE CARVALHO; DA FONSECA, 2006b).

3.4. Epoxidação da ligação dupla na unidade isoprenil a α-terpineol

O α-terpineol é um produto comercial muito importante, empregando nos mais diversos setores da indústria, como em sabonetes e cosméticos. Este composto está presente em diversos óleos essenciais, como de bergamota e lima, em concentrações variáveis. Pequenas concentrações podem ser isoladas, por destilação fracionada, de óleos de pinho. *S*-(-)-terpineol possui notas de aroma conífero, enquanto o *R*-(+)-terpineol possui um aroma floral intenso (BAUER; GARBE; SURBURG, 1990).

Este composto é considerado como um dos produtos mais importantes produzidos a partir do limoneno, sendo que este processo já foi descrito com uma grande diversidade de microrganismos como catalisadores. O primeiro trabalho envolvendo a produção de *R*-(+)-terpineol a partir do *R*-(+)-limoneno foi relatado em 1969, utilizando uma linhagem de *Cladosporium* sp. (KRAIDMAN, MUKHERJEE; HILL, 1969).

A partir de então, diversos trabalhos envolvem a produção deste composto, sendo que um dos gêneros de fungos mais bem documentados é de *Penicillium* sp. (ABRAHAM *et al.*, 1985; DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001).

Ainda utilizando um microrganismo deste gênero, observou-se que a biotransformação do limoneno por uma linhagem de *P. digitatum* ocorreu nos primeiros instantes da fase *log* e que a capacidade de conversão foi consideravelmente melhorada pela adição de substrato durante o crescimento do microrganismo (TAN; DAY;

CADWALLADER, 1998). Os mesmos autores ainda testaram as diferenças entre a biotransformação com as células livres e imobilizadas do *P. digitatum* (TAN; DAY, 1998a), além da utilização de 22 co-solventes ao meio de conversão, para verificar sua influência na taxa de conversão. Os autores observaram que a atividade da linhagem utilizada aumentou significativamente após indução pela adição seqüencial de substrato, resultando em um rendimento de 3,2 $g.L^{-1}$ de α-terpineol, após 96 horas de processo (TAN; DAY, 1998b).

A mesma metodologia foi seguida recentemente em outro estudo (ADAMS; DEMYTTENAERE; DE KIMPE, 2003). As linhagens deste experimento foram selecionadas em um trabalho anterior, realizado por Demyttenaere e colaboradores (2001), onde investigaram a capacidade de mais de 60 linhagens de fungos filamentosos em biotransformar o limoneno. Os autores utilizaram a tecnologia de SPME, microextração em fase sólida, e realizaram a otimização de diversos parâmetros envolvidos no experimento, como o tipo de fibra de SPME, tempo de extração e temperatura. Os autores observaram que as melhores condições de análise ocorreram utilizando fibra de polidimetilsiloxano revestida com divinil-benzeno/carboxeno, por 30 minutos a uma temperatura de 25 ^{o}C (DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001). Posteriormente, as linhagens foram investigadas em cultura submersa. Uma linhagem de *P. digitatum*, isolada de amostras de poncã, exibiu elevada enantioseletividade ao converter 93% de *R*-(+)-limoneno a *R*-(+)-terpineol, na presença de etanol como co-solvente (ADAMS; DEMYTTENAERE; DE KIMPE, 2003).

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 utilização de manipueira foi muito eficiente para obtenção de biomassa, resultando em 29,5 $g.L^{-1}$ (peso seco) após 3 dias de fermentação. Além disso, a biotransformação de *R*-(+)-limoneno resultou em 450 $mg.L^{-1}$ de *R*-(+)-α-terpineol (MARÓSTICA JR; PASTORE, 2007b).

Na sequência, Bicas *et al.* (2008a) 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 co-substrato, condições de cultivo e ainda a relação entre inoculo 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 $g.L^{-1}$, nas condições de 30 °C, 270 rpm e 0,5% de *R*-(+)-limoneno.

Em outro trabalho, Bicas *et al*. (2008b) estudaram a habilidade das linhagens *P. rhodesiae* CIP 107491 e *P. fluorescens* NCIMB 11671 em crescer e converter diferentes fontes terpênicas em meio bifásico. Os resultados indicaram que a *P. rhodesiae* é especialista para a bioconversão de pinenos, como α- e β-pinenos. Enquando isso, a linhagem *P. fluorescens* mostrou-se capaz de metabolizar o limoneno em duas vias distintas, sendo que a produção de α-terpineol alcançou a concentração de aproximadamente 11 $g.L^{-1}$. Além disso, os autores detectaram a isomerização de β- para α-pineno, descrita anteriormente apenas por Kashi, Fooladi e Bayat (2007) na bibliografia científica.

3.5.Oxidação alílica a isopiperitenol e isopiperitona

A linhagem de *Aspergillus celulosae* M-77 realizou a hidroxilação alílica e desidrogenação da posição C-3 do limoneno. A partir de *R*-(+)-limoneno obteve-se (+)-piperitona como principal produto da biotransformação, mas observou-se a presença de compostos minoritários como isopiperitenol, limoneno-1,2-*trans*-diol e álcool perílico. Entretanto, a partir de *S*-(-)-limoneno obteve-se a formação de pequenas concentrações de isopiperitona (NOMA; YAMASAKI; ASAKAWA, 1992).

O primeiro relato da obtenção de *trans*-isopiperitenol foi feito em 1998. Os autores utilizaram uma linhagem de levedura, identificada por *Hormonema* sp., capaz de produzir 0,5 $g.L^{-1}$. Este composto pode ser facilmente convertido a (-)-mentol por meio de hidrogenação. Entretanto, os autores relatam que houve falta de reprodutibilidade dos dados e resultados, relacionada às mutações fisiológicas observadas na cultura utilizada (VAN DYK; VAN RENSBURG; MOLELEKI, 1998).

De uma forma geral, os microrganismos oxidam o limoneno não especificamente, produzindo uma grande diversidade de metabólitos secundários e, algumas vezes, detectando a presença de compostos indesejáveis. Entretanto, a conversão é sempre catalisada por enzimas catabólicas que permitem que o limoneno seja utilizado como fonte de energia (MARÓSTICA JR, 2006). No caso das plantas, enzimas de algumas espécies mostraram regioespecificidade ao atacar o limoneno em sua posição C-3 (LUPIEN *et al.*, 1999; HAUDENSCHILD *et al*., 2000).

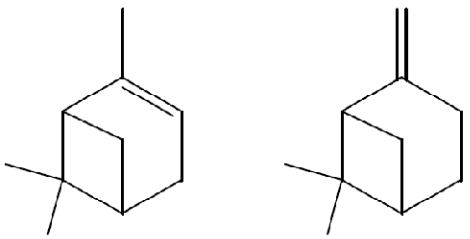
3.6. Epoxidação da ligação 8,9 a limoneno-8,9-epóxido

O limoneno-8,9-epóxido, apesar de ser um composto pouco estudado, tem sido encontrado na fração volátil de muitos alimentos, como poncã e gengibre (BELL; SWODEN; WONG, 2001).

O primeiro relato da obtenção biotecnlógica de limoneno-8,9-epóxido, a partir do ataque da ligação C-8,9 do limoneno, foi observado por uma linhagem de *Xantobacter* sp. C20, isolada de sedimentos de um rio. Este microrganismos foi capaz de converter os enantiômeros do limoneno a limoneno-8,9-epóxido utilizando ciclohexano como fonte de carbono, sugerindo o envolvimento de monooxigenases dependentes do citocromo P-450. O melhor resultado obtido foi a produção de 0,8 $g.L^{-1}$ do epóxido, utilizando uma concentração de 12 mM de substrato (VAN DER WERF; KEIJZER; VAN DER SCHAFT, 2000).

4. Biotransformação de α- e β-pineno

Os α- e β- pinenos (Figura 5), assim como o limoneno, são monoterpenos amplamente distribuídos na natureza. Estes compostos podem ser gerados como resíduos industriais em alguns processos, podendo ser encontrados por um preço comercial reduzido (YOO; DAY, 2002).



α-pineno β- pineno

Figura 5 - Estrutura de α- e β- pinenos.

Estes pinenos são os compostos majoritários do óleo essencial de coníferas, em concentrações que variam de 75 a 90%. Dados estimam que, do montante total de óleo de coníferas produzidos nos EUA, aproximadamente 90% é proveniente da indústria de polpa e papel, produzidos mediante processos químicos ou termomecânicos (YOO; DAY, 2002). A terebentina, rejeito industrial da indústria de polpa e papel, consiste em

uma mistura de terpenóides voláteis originados durante o processo para a produção de papel. Atualmente, é aplicada como solvente em tintas, verniz, aditivos para perfumes e fármacos. Os principais componentes encontrados na terebentina são α- e β-pinenos, presentes em concentrações de 50 a 70% e 15 a 30%, respectivamente (LINDMARK-HENRIKSSON, 2003).

Estes compostos podem ser utilizados como precursores em sínteses químicas. Aproximadamente 25% da produção mundial de α-pinenos (160.000 t) e β-pineno (26.000 t) é utilizada para síntese química de compostos de aroma e fragrâncias (OHLOFF, 1994).

Devido a estas vantagens e, principalmente pela abundância e baixo custo, α- e β-pinenos representam um substrato ideal para processos biotecnológicos. O metabolismo de pinenos por microrganismos pode seguir a uma séria de rotas de degradação, resultando em uma ampla variedade de produtos (MIKAMI, 1988; MARÓSTICA JR, 2006).

As vias metabólicas a partir de α- e β-pineno está compreendida na Figura 6, proposta por Bicas *et al*. (2008b).

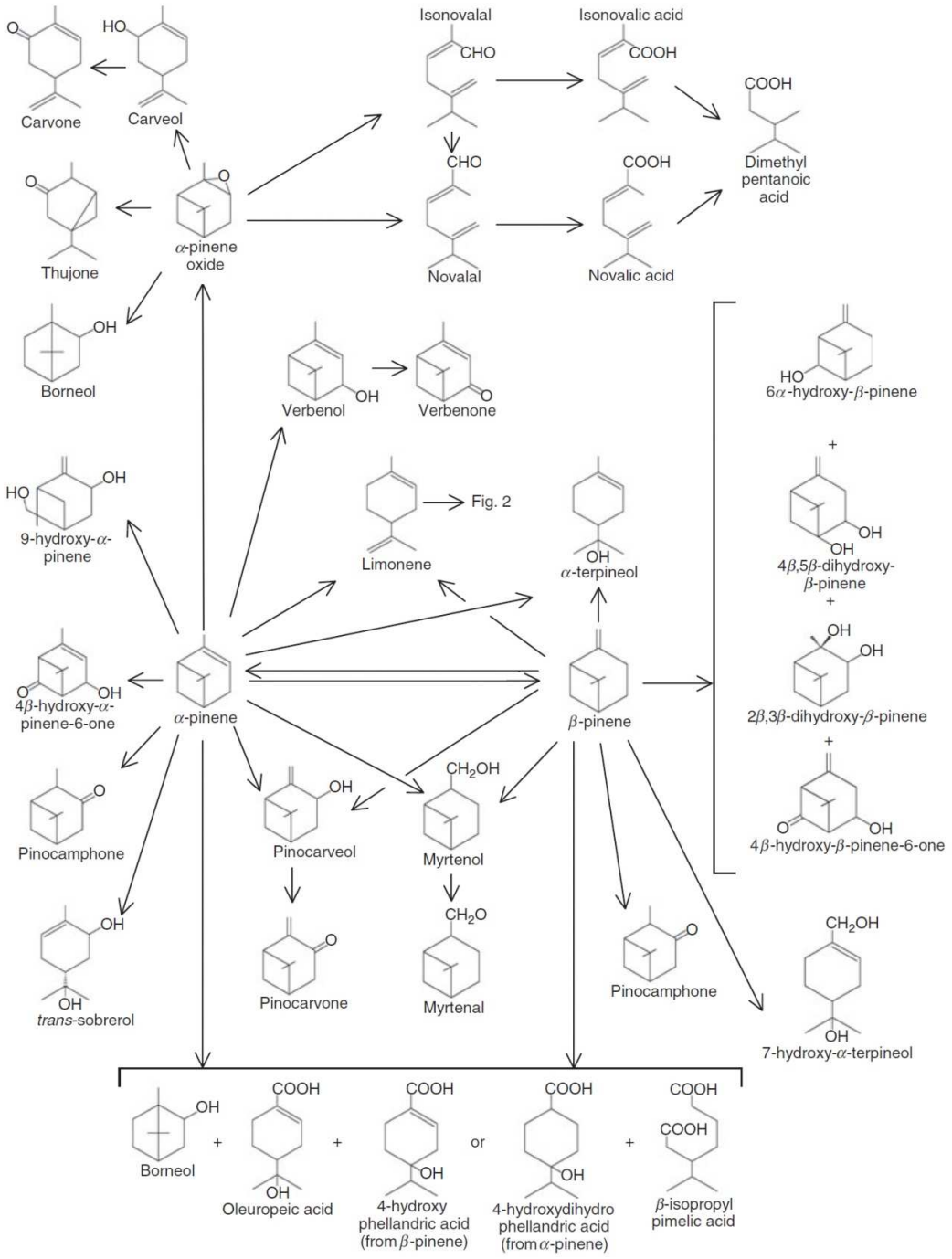


Figura 6 - Rotas metabólicas descritas para a biotransformação de α- e β-pinenos (BICAS et al., 2008b).

O primeiro relato envolvendo a biotransformação de α-pineno foi mediado por uma linhagem de *Aspergillus niger* capaz de metabolizar o α-pineno a produtos oxigenados (BHATTACHARYYA *et al.*, 1960). A concentração de 0,6% ($v.v^{-1}$) de substrato a temperatura variando entre 27 e 28 ^{o}C, por 8 horas de reação, maximizaram

a produção dos metabólitos verbenol, verbenona e *trans*-sobrerol (PREMA; BHATTACHARYYA, 1962b).

Os microrganismos deste gênero foram muito estudados em processos de biotransformação de monoterpenos. Uma linhagem de *A. niger* isoladas do solo de árvores cítricas, mostrou-se capaz de transformar o α-pineno em verbenona. Os autores realizaram uma otimização das condições de processo, variando um parâmetro por vez. As condições ótimas foram observadas quando o microrganismo foi incubado por 6 horas com 200 $mg.L^{-1}$ de substrato e 6 $g.L^{-1}$ de glicose, em tampão fosfato a pH 7,0. Concentrações elevadas de substrato e tempo prolongado de incubação ocasionaram uma diminuição no rendimento da bioconversão, indicando a toxicidade do α-pineno e degradação do produto. Apesar da concentração máxima obtida ter sido 328 $mg.L^{-1}$, ainda é considerado como um rendimento baixo (AGRAWAL; JOSEPH, 2000). O produto obtido, verbenona, é um dos componentes majoritários do aroma de morango e framboesa, sendo considerado como um produto de grande importância comercial para a indústria de alimentos (MARÓSTICA JR, 2006).

Outro estudo envolveu a utilização de uma endobactéria, *Serratia marcescens*, que demonstrou capacidade em biotransformar o α-pineno. O principal produto obtido foi *trans*-verbenol, na presença de compostos minoritários como verbenona e *trans*-sobrerol. Observou-se que alterações nas condições de cultivo (como utilização de outras fontes de nitrogênio e a inclusão de glicose ao meio) alteraram o perfil dos produtos, formando em maior quantidade o α-terpineol (WRIGHT *et al.*, 1986).

Autores testaram o potencial da linhagem de levedura *Hormonema* sp., em biotransformar o α-pineno. Os produtos obtidos foram *trans*-verbenol e verbenona, que atingiram concentrações de 0,4 e 0,3 $g.L^{-1}$, após 96 horas de experimento (VAN DYK; VAN RENSBURG; MOLELEKI, 1998).

Agrawal, Deepika e Joseph (1999) estudaram a capacidade de linhagens de *Aspergillus* sp. and *Penicillium* sp. em transformar α-pineno em verbenol. Posteriormente, verificaram que a transformação de verbenol em verbenona foi inibida com o tratamento da linhagem por luz UV. Entretanto, os autores conseguiram um aumento na eficiência da biotransformação de α-pineno por *Aspergillus niger,* quando o microrganismos foi submetido a tratamento com luz UV.

Observou-se a capacidade da linhagem *Pseudomonas fluorescens* NCIMB 11671 em degradar completamente o α-pineno. Além de utilizá-lo como fonte de carbono e energia, a partir de seu metabolismo origina-se dois aldeídos conhecidos

como isonovalal e novalal, que são posteriormente oxidados formando os ácidos isonoválico e noválico (BEST *et al.*, 1987). Entretanto, outros autores sugerem outra via metabólica envolvida nesta formação, explorando a produção de novalal pela isomerização de isonovalal (LARROCHE; FONTANILLE; LARROCHE, 2003; LINARES *et al.*, 2008). Estes estudos levaram ao desenvolvimento de uma forma otimizada de produção de isonovalal a partir de α-pineno óxido por *Pseudomonas rhodesiae* CIP 107491 (FONTANILLE; LARROCHE, 2003; FONTANILLE; LE FLÈCHE; LARROCHE, 2002).

Pesquisadores descreveram a capacidade de uma linhagem de *Pseudomonas putida* de biotransformar (+)- e (-)-limoneno, (+)- e (-)-α-pineno e (-)-β-pineno. Os compostos de aroma mais importantes obtidos neste experimento foram a partir de α-pineno, resultando em verbenol e verbenona, com rendimentos de 35% e 10%, respectivamente (DIVYASHREE; GEORGE; AGRAWAL, 2006).

Uma linhagem identificada como *Bacillus pallidus* BR425, isolada de amostras de pinheiros, foi capaz de degradar α- e β-pineno, bem como o limoneno. Obteve-se quantidades significativas de pinocarveol, pinocarvona, carveol, carvona e quantidade menores de mirtenol e mirtenal, por exemplo (SAVITHIRY *et al.,* 1998).

Yoo, Day e Cadwallader (2001) isolaram um microrganismos de amostras de solo, identificada como pertencente ao gênero *Pseudomonas* sp., capaz de metabolizar α- e β-pinenos, além de resistir a concentrações superiores a 10% destes substratos. As possíveis vias metabólicas para a degradação destes monoterpenos por este microrganismo foi descrita posteriormente pelo mesmo grupo de pesquisa (YOO; DAY, 2002).

Um grupo brasileiro de pesquisas investigou 5 monoterpenos ((+)- e (-)-limoneno, α- e β-pineno e cânfora) como substrato para a conversão microbiana, visando a produção de aromas e fragrâncias pela linhagem *A. niger* IOC-3913. O estudo foi feito com células em crescimento, previamente crescidas e imobilizadas, em meio líquido ou sólido, com alimentação de substrato via fase gasosa. A transformação de α- e β-pineno gerou verbenona e α-terpineol, respectivamente, como produtos majoritários (ROZENBAUM *et al.*, 2006).

Busmann e Berger (1994) relataram a biotransformação de β-pineno por linhagens de basidiomicetos. Os autores obtiveram produtos importantes, como verbenol, verbenona, mirtenol e *trans*-pinocarveol. A biotransformação de β-pineno por *Hormonema* sp. resultou na produção de pinocanfona e da hidroxicetona 3-hidroxi-

pinocanfona, em concentrações de 0,1 e 0,2 $g.L^{-1}$, respectivamente (VAN DYK; VAN RENSBURG; MOLELEKI, 1998).

Aspergillus niger ATCC 9462 foi investigada para a conversão de (-)-α-pineno, (-)-β-pineno e (+)-limoneno, mas apenas o segundo composto foi transformado por esta linhagem. Aproximadamente 4% de de (-)-β-pineno foi convertido a α-terpineol, atingido quando o substrato foi adicionado em cinco adições seqüenciais, em solução 1:1 de etanol. Os autores ainda relatam que a indução das células não afetou o rendimento da reação (TONIAZZO *et al.*, 2005).

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CAPÍTULO 2

STUDY OF THE BIOTRANSFORMATION OF *R*-(+)-LIMONENE TO AROMA COMPOUNDS BY LIMONENE-RESISTANT MICROORGANISMS

ABSTRACT

Background: This work is focused on the biotransformation of *R*-(+)-limonene to bioflavor production by 70 limonene-resistant microorganisms. To carry out the present study, these microorganisms were isolated in a previous study where authors collected samples from strategic places of a citrus processing plant, to obtain microorganisms resistant to environment containing limonene. From the total of 238 isolated strains, about half were resistant to this monoterpene in concentrations up to 2% in the medium broth. Amongst them seventy were able to grow in mineral medium containing limonene as sole carbon source.

Results: Among 70 strains, it was observed that the most recurrent metabolites for the biotransformation of limonene as α-terpineol, and the highest production reached 29 $mg.L^{-1}$. It was also detected carveol, carvone and limonene-1,2-diol. The strain LB285JLB was used to formulate an alternative culture medium, using glycerol as carbon and energy source for biomass. According to the contour plots and surface responses provided from the central composite design experiment, the best conditions for the microorganism growth measured by its absorbance recovery were: 96 h-reaction in, temperature between 35-40 °C, agitation of 230 to 300 rpm and glycerol concentration of 6-9 $g.L^{-1}$.

Conclusion: The research described in this paper is the initial step for the exploration of aroma compounds production *via* biotransformation of *R*-(+)-limonene, a non-expensive by-product of citrus industry.

Key-words: Limonene; Biotransformation; Terpenes; Aroma compounds.

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; 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; DA FONSECA, 2006a).

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), constituing suitable precursor substrates (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 know at present, constituing the largest group of natural products (DEMYTTENAERE; DE KIMPE, 2001, DE CARVALHO; DA FONSECA, 2006a). This terpenoids have, in general, more characteristic flavour properties than their terpene hydrocarbon counterparts.

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 (DEMYTTENAERE; BELLEGHEM; DE KIMPE, 2001).

The monoterpene hydrocarbon limonene is a popular starting product for bioconversions because of its widespread and cheap availability (KRASNOBAJEW, 1984; MARÓSTICA; PASTORE, 2007a). *R*-(+)-limonene is the main compound in essential oils of citrus fruits, where it occurs in a concentration of more than 90% and in enantiomerically pure form (BAUER *et al.*, 2001; ADAMS *et al.*, 2003).

The screening of microorganisms is of particular interest since there is a large diversity of metabolic process and enzymes involved and an unlimited number of microorganisms present in nature. Microorganisms can modify and degrade a variety of organic molecules and complexes, and so it can be expected that one of them may be able to catalyze a specific reaction of interest (DE CONTI *et al.*, 2001).

There have been many reports concerning the biotransformation of limonene with a view towards potential production of more valuable natural flavor compounds (ABRAHAM *et al.*, 1985; CHANG; ORIEL, 1994; DHAVALIKAR;

BHATTACHARYYA, 1966; KIESLICH et al., 1986; KRAIDMAN, 1969; MUKHERJEE et al., 1973; NOMA et al., 1992; VAN DER WERF et al., 1999).

Bicas and Pastore (2007) isolated 238 strains from samples collected in strategic places of a citrus processing plant, to obtain microorganisms resistant to environment containing limonene. From the total 238 isolated strains, about half were resistant to this monoterpene in concentrations up to 2% in the medium broth. Amongst them seventy were able to grow in mineral medium containing limonene as sole carbon source. Authors noticed that about one half of the isolated microorganisms resist limonene concentrations of up to 2%, except for the Gram positive Cocci, which appear to be more sensitive to limonene, and the fungi, that were not able to grow at high concentrations (2%) of limonene.

Therefore, these 70 strains isolated from a citrus processing plant and resistant to *R*-(+)-limonene were screened for their ability to bioconvert this substrate under liquid phase fermentation in aroma compounds, represented the main objective of this work.

MATERIALS AND METHODS

Chemicals

Substrates used in this research were *R*-(+)-limonene (~98%, Fluka). As reference and standard compounds were (-)-carveol (97%, Aldrich), *S*-(-)-carvone (>99%, Merck), α-terpineol (90%, Aldrich) and *S*-(-)-perillyl alcohol (96%, Aldrich) were used. All of the products were kept under refrigeration (5°C). All other chemicals or solvents were of the best available commercial grade.

Microorganisms, Strain Maintenance and Growth

Seventy microorganisms resistant to 2% of limonene were obtained from Laboratory of Bioflavors Culture Collection, São Paulo, Brazil. The strains were grown on agar plates with yeast and malt agar medium (YMA) consisting in w/v of glucose 1%, malt extract 0,3%, yeast extract 0,3%, bacteriological peptone 0,5% and agar 2%, pH was not adjusted.

Preculture preparation

Three full loops of a 24-h-old culture on a petri dish were transferred to a 250 ml conical flask that contained 50 mL of YM medium (without agar). The flasks were incubated at 30 °C and 150 rpm for 24 h, to reach an optical density close to 3 at 600 nm (OD_{600}).

Cell culture preparation

Twenty milliliters of the preculture were aseptically transferred to a 500 ml conical flask with 180 mL mineral medium (in g/L, $NaNO_3$: 3.0; K_2HPO_4: 1.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5; Fe_2SO_4: 0.01) (MARÓSTICA; PASTORE, 2007b). Cell induction consisted in the addition of 1% of limonene as sole carbon source. The flasks were left at 30 °C and 150 rev min^{-1} until satisfactory growth.

Recovery of the biomass

After centrifuging the culture at 2600 g for 10 min, the supernatant was eliminated and the resulting biomass was suspended in 20 mL of 20 mmol l^{-1} of phosphate buffer pH 7,5.

Biotransformation procedure

The substrate was added to reach a final concentration of 5 $g.L^{-1}$. The flasks were incubated at 30 °C and 150 rev min^{-1}. In the same way, chemical blanks were performed, but without mycelium, to ensure the absence of chemical transformation reactions. Samples were periodically taken from the flasks to follow substrate consumption and product formation, were extracted with the same volume of ethyl acetate (1:1, v/v) and directly analysed by gas chromatography/flame ionisation detection (GC/FID) (decane was the internal standard).

Analytical conditions

The products obtained were analyzed in a HP 7890 gas chromatograph with a flame ionization detector (GC-FID). A HP-5 MS (J&W Scientific, EUA) capillary column of 30 m x 0,250 mm x 0,25 μm i.d. was employed. Helium was the carrier gas, with a constant pressure in the head of the column of 0.8 bar and split ratio of 1:10. The temperature program used was as follows: initial temperature of 80 °C for 3 min, rising

at 20 °C min^{-1} until 200 °C, then held for 4 min. The temperatures in the injector and detector were both 250 °C.

Positive identifications were made by matching sample retention indices (RI) and mass spectra of the samples with those of the standards, analysed under identical conditions. Products quantification were performed with a calibration curve at different concentrations of standards.

1. Experiments done with the strains able to biotransform limonene: LB285JLB, LB249JLB, LB263JLB, LB14JLB, LB192JLB

1.1. *Grown rate in presence of limonene as sole carbon source x Limonene consumption (Carbon source consumption rate)*

Cells were grown as described before (see preculture and culture preparation). After the terpene addition, cells were incubated at 30 °C and 150 rpm and samples were taken at different time intervals (0-96h). At each sampling time the whole contents collected was used for biomass growth measurement by spectrophotometer at 600 nm (DO_{600}) and determination of carbon source (limonene) consumption by gas chromatography (GC).

1.2. Biotransformation without cell induction to substrate

The biotransformation process without cell induction to limonene occured when the material collected on the item "preculture cell preparation" was directly centrifuged (2600 g for 10 min) and applied in the biotransformation. The resultant biomass was transferred to biotransformation medium consisting of 20 mL of 20 $mmol.L^{-1}$ of phosphate buffer at pH 7,5, followed by substrate addition. Production of new compounds and limonene consumption were monitored by gas chromatography during 96 hours and analytical conditions were the same as described before.

1.3. Quantification and identification of the volatile compounds

The quantification of products was performed using the external calibration curve, obtained by distributing different concentrations of standard samples of products. The volatiles products obtained were identified by a HP 7890 gas chromatograph coupled with a HP 5975 mass selective detector (GC-MS). Helium was the carrier gas and the split ratio was 1:5. The capillary column and the temperature program was the

same as described before. The mass spectrometer was operated in the electron impact mode with electron energy of 70 eV and mass range of 35 - 350 m/z. The temperatures of the quadrupole, the ionic source and the GC-MS interface were 150 °C, 230 °C and 250 °C, respectively.

The tentative identification of components was made by comparing spectra with NIST 2005 mass spectral database libraries, with similarities higher than 85 % and supported by retention index data. Positive identifications were made by matching sample retention indices (RI) and mass spectra of the samples with those of the standards, analyzed under identical conditions.

2. Experiments performed with the strain LB285JLB

2.1. Mineral medium formulation and use of glycerol as carbon source for biomass production

In biotechnology-based industrial processes, the formulation of cultivation media is of critical importance because their composition affects product concentration, yield and volumetric productivity. It is also important to reduce the costs of the medium as this may affect the overall process economics (SOUZA et al., 2006). A suitable choice for biomass production, to be applied in biotransformation process, would be the utilization of a low cost source of carbon, as glycerol. Moreover, the use of minimal medium to assess the potential of glycerol as a substrate, it is also important to verify the necessity of minerals and trace elements.

Strain LB285JLB was used to formulate a complete mineral medium, formulated by the combination of two known mineral resources (MARÓSTICA; PASTORE, 2007; VISHNIAC; SANTER, 1957) and the use of glycerol as carbon source.

The strategy was to perform a selection among twelve variables, including media composition and cultivate conditions, employing a Plackett-Burman matrix (RODRIGUES, IEMMA, 2005) with 16 experiments (PB-16) and five central points to determine the parameters that can interfere on the process. Table 1 displays variables and levels evaluated in the screening design.

Table 1 - Variables and levels evaluated in the screening design

X	Unit	Variables	Level (-1)	0	Level (+1)
x_1	$g.L^{-1}$	K_2HPO_4	0,2	1,0	1,8
x_2	$g.L^{-1}$	NaH_2PO_4	0,4	0,8	1,2
x_3	$g.L^{-1}$	NH_4Cl	1,0	2,0	3,0
x_4	$g.L^{-1}$	$MgCl_2.6H_2O$	0	0,1	0,2
x_5	$g.L^{-1}$	$(NH_4)_2SO_4$	1,0	4,0	7,0
x_6	$g.L^{-1}$	$MgSO_4.7H_2O$	0,1	0,5	0,9
x_7	$g.L^{-1}$	NaCl	0,5	1,0	1,5
x_8	$g.L^{-1}$	KCl	0,2	1,5	2,8
x_9	$g.L^{-1}$	$NaNO_3$	0	1,0	2,0
x_{10}	rpm	Agitation	50	150	250
x_{11}	°C	Temperature	20	25	30
x_{12}	$g.L^{-1}$	Glycerol	1	3	5

After the variables selection it was employed a central composite design 2^3 (CCD), with 6 axial points and 4 central points, resulting in 18 experiments (RODRIGUES, IEMMA, 2005). The main objective was to define the optimal conditions for the process. The central points and amplitudes of the parameters applied in CCD, presented in Table 2, were chosen based on preliminary results (PB-16).

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

Variables	Levels				
	-1,68	-1	0	+1	+1,68
Glycerol ($g.L^{-1}$)	3	5,5	9	12,5	15
Temperature (^oC)	20	24	30	36	40
Agitation (rpm)	100	140	200	260	300

2.2. Data analysis

The results were analyzed by the software STATISTICA 7.0 (Statsoft). A significance level of 10% ($P < 0,1$) was considered for the variables screened and 5% ($P < 0,05$) for the central composite design.

RESULTS AND DISCUSSION

1. Biotransformation experiments, quantification and identification of the volatile compounds

The strains tested in this study were obtained from a previous and extensive study where Bicas and Pastore (2007) isolated 238 microorganisms from samples collected in different places of a citrus processing plant. The authors noticed that about half of the Gram positive and Gram negative bacilli and yeasts and some Gram positive Cocci were resistant to limonene concentrations up to 2% in the medium broth. Amongst them seventy were able to grow in mineral medium containing limonene as sole carbon source and was selected to biotransformation experiments. According to Horikoshi (1995), the isolation of microorganisms from extreme environment will enable innovations in the field of fermentation and might bring several benefits to industries. And also, the fact that the strains were able to withstand large concentrations of limonene and to use it as the sole source of carbon, could provide evidences of its ability to convert this substrate into aroma compounds.

From the 70 strains tested in this research, biotransformation processes revealed that 5 strains were able to biotransform limonene in flavor compounds. Results obtained are presented in Table 3, with the main products accumulated after 72 h of bioconversion and their respective approximate yields. All other microorganisms were capable of using limonene as sole of carbon source for growth, but not showed accumulation of metabolites, suggesting the complete degradation of this substrate to CO_2. Even without positive results for the biotransformation, it emphasizes the potential its use in the degradation of limonene in bioremediation processes.

Table 3 - Microorganism and products obtained in the biotransformation of limonene

Strains	Identification	Products	Concentration ($mg.L^{-1}$)
LB285JLB	Gram positive bacilli	α-terpineol	29
LB177JLB	Gram positive bacilli	α-terpineol	18
		cis-p-mentha-2,8-dien-1-ol	t.r.*
LB14JLB	Gram negative bacilli	α-terpineol	16
		Limonene-1,2-diol	t.r.*
LB249JLB	Gram positive bacilli	Carvone	12
		Dihydrocarvone	t.r.*
LB192JLB	Yeast	Carveol	t.r.*

* *t.r.*: trace amounts

The majority of producing microorganisms were Gram positive bacilli, what was expected given that were also majority in the screening results (BICAS; PASTORE, 2007). Among 70 strains, only one cocci was tested, though not proved capable of biotransform limonene. However, the only strain of yeast present in the sample group resulted in the production of carveol. There are few research that describes the biotransformation of terpenes by yeasts.

It was observed that the most recurrent metabolites for the biotransformation of limonene were α-terpineol. This indicates that the microorganisms showed a similar pathway to that already reported and studied by many authors (KRAIDMAN *et al.*, 1969; TAN; DAY 1998a,b; TAN *et al.*, 1998; ADAMS *et al.*, 2003; MARÓSTICA; PASTORE, 2007b; CADWALLADER *et al.*, 1989, BICAS *et al.*, 2008a,b).

There is considerable progress in the study of the biotransformation of limonene. Extensive research on the biotransformation of limonene has resulted in the elucidation of new metabolic pathways, displayed in Figure 1.

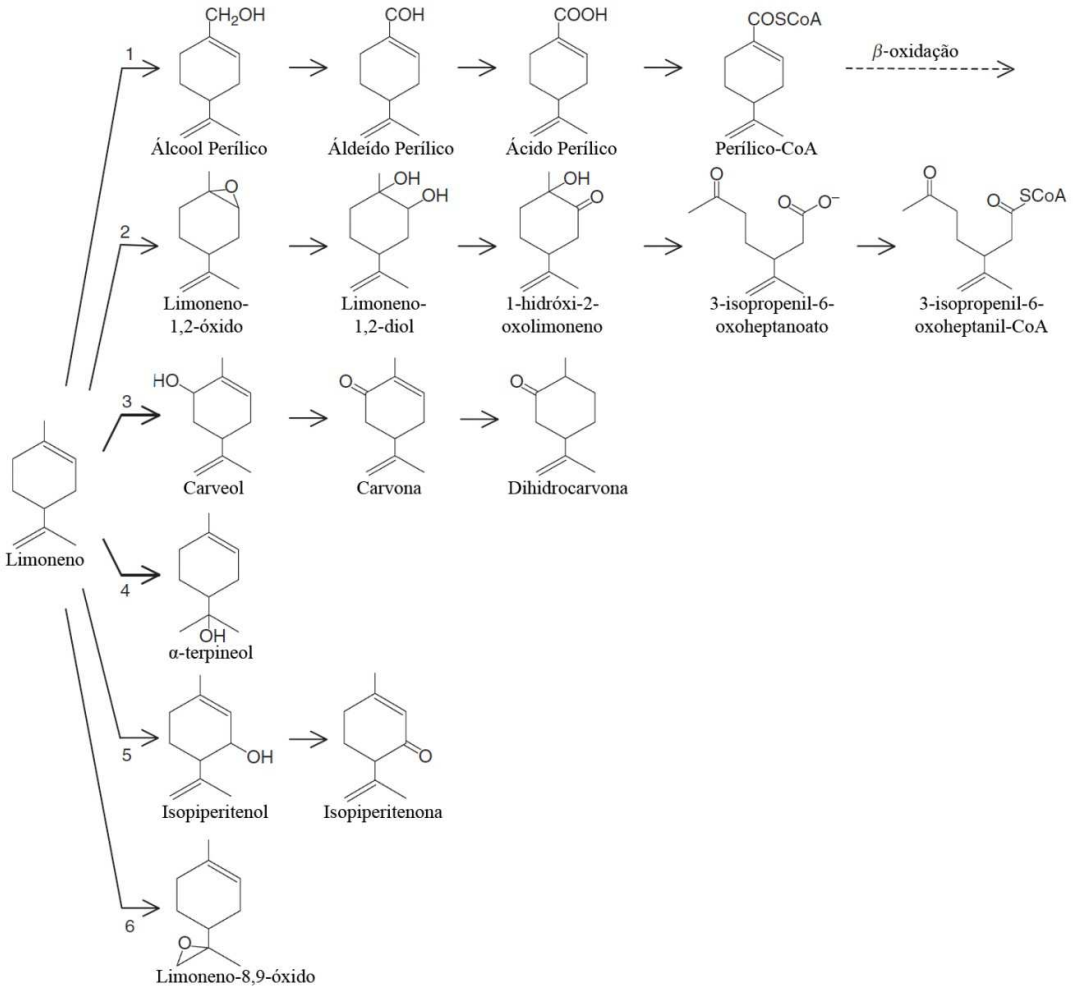

Figure 1 – The six main metabolic pathways for limonene (VAN DER WERF et al., 1999; MARÓSTICA; PASTORE, 2007a; BICAS et al., 2008b).

It was observed that all products obtained from bio-oxidation of limonene in this research belongs to pathway 4 (LB285JLB, LB177JLB and LB14JLB), yielding α-terpineol, pathway 2 (LB14JLB) with an accumulation of limonene-1,2-diol and pathway 3 (LB249JLB and LB192JLB) to obtain aroma compounds with a huge importance and potential application in industry, such as carveol and carvone.

It is worth noticing that it was obtained the highest concentration of α-terpineol derived from the bio-oxidation of limonene, when compared with other products. Strain LB285JLB was responsible for the best yields collected, approximately 29 $mg.L^{-1}$, after 72 hours of process. Figure 2 shows the results of chromatography and mass spectra of α-terpineol production by the strain LB285JLB that seemed capable to oxidize limonene in C8 position.

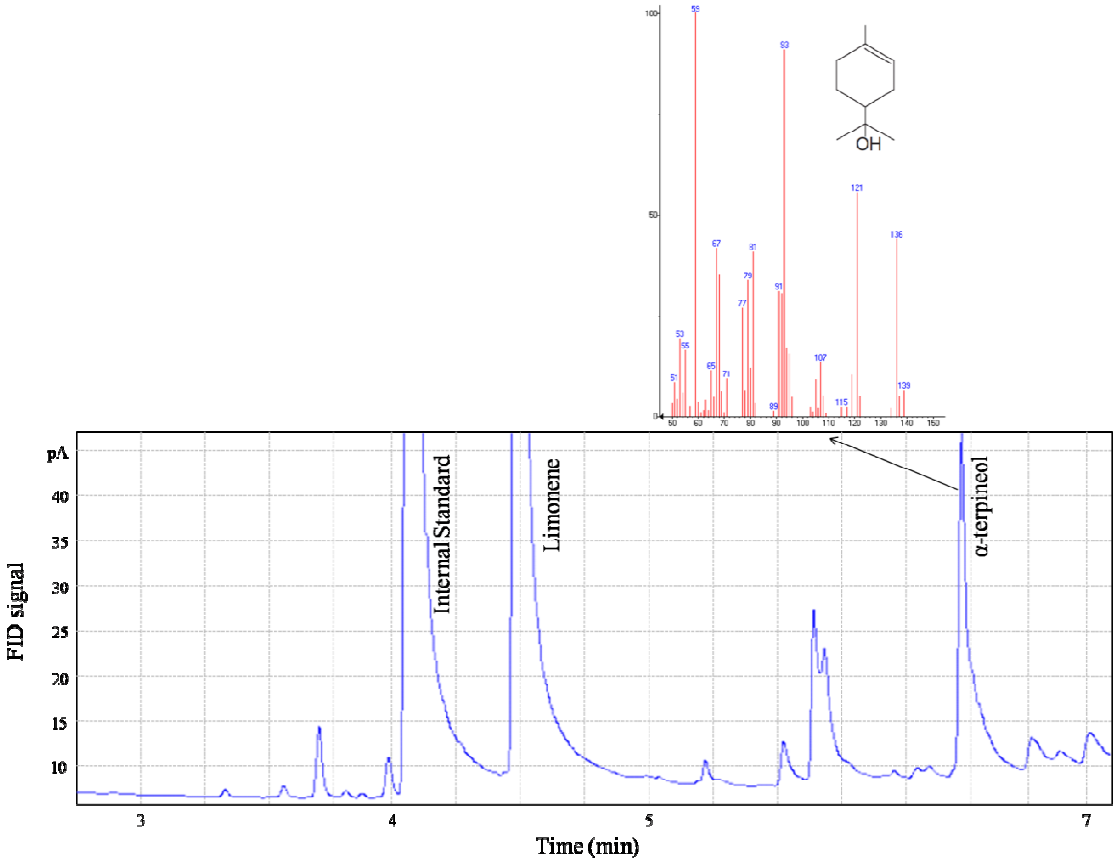

Figure 2 – Chromatogram and mass spectra of the product observed in the biotransformation of *R*-(+)-limonene by the strain LB285JLB, after 72 hours of process.

The same product was observed when limonene was submitted as substrate to the strain LB177JLB. Interestingly, it was noticed that whole cells of this microorganism were also able to accumulate an intermediate labeled *cis*-*p*-mentha-2,8-dien-1-ol, only in trace amounts (mass spectra and structure are shown in Figure 3). It is important to notice that this product was obtained by the action of the microorganism in the substrate and not by limonene self-degradation, as observed by comparing the profile of chemical blank and the resultant biotransformation profile. The presence of such limonene intermediate has been found in a great diversity of essential oil compositions (BASER et al., 2009; JIROVETS et al., 2007). However, its biotechnological production and the metabolic pathway involved have not been extensively cited in the literature.

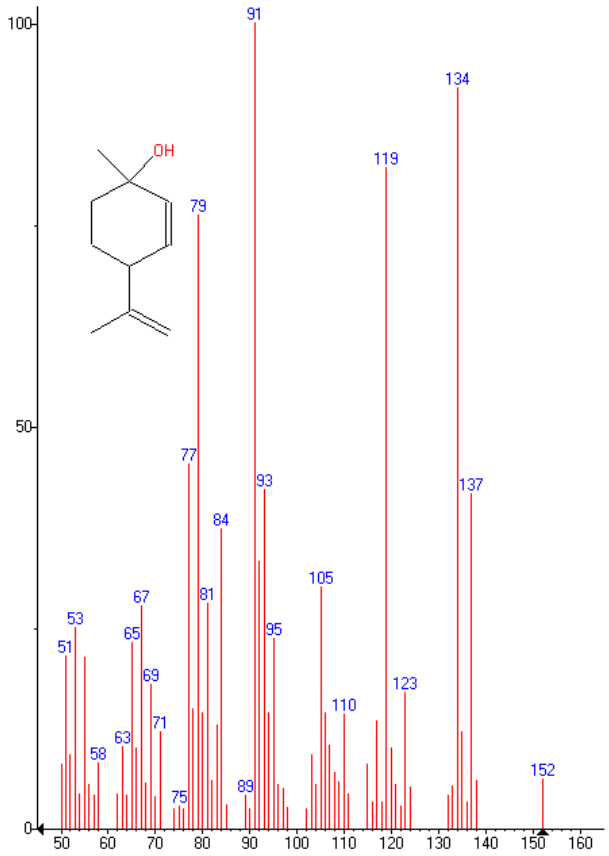

Figure 3 – Mass spectra and structure of *cis-p*-mentha-2,8-dien-1-ol.

As shown in Table 3, limonene-1,2-diol it was also obtained as result from the biotransformation of *R*-(+)-limonene by strain LB14JLB. This gram negative bacillus produced both α-terpineol with an amount of 16 $mg.L^{-1}$ and the diol in trace amounts. These observations were consistent with an already reported hypothesis, where *P. fluorescens* presented two parallel metabolisms for limonene breakdown, one giving rise to limonene-1,2-diol to produce energy and another pathway leading to the formation of α-terpineol (BICAS et al., 2008b). These authors noticed that trace amounts of limonene-1,2-diol were detected throughout the growing phase (<40 h) while traces of α-terpineol were evidenced after, during the stationary phase. It was not possible detect the presence of the correspondent epoxide, suggesting that the rate of diol formation is most intense. The accumulation of limonene-1,2-diol and 1-hydroxy-2-oxolimonene during the limonene-1,2-oxide bioconversion was already described by Van der Werf *et al.* (1999).

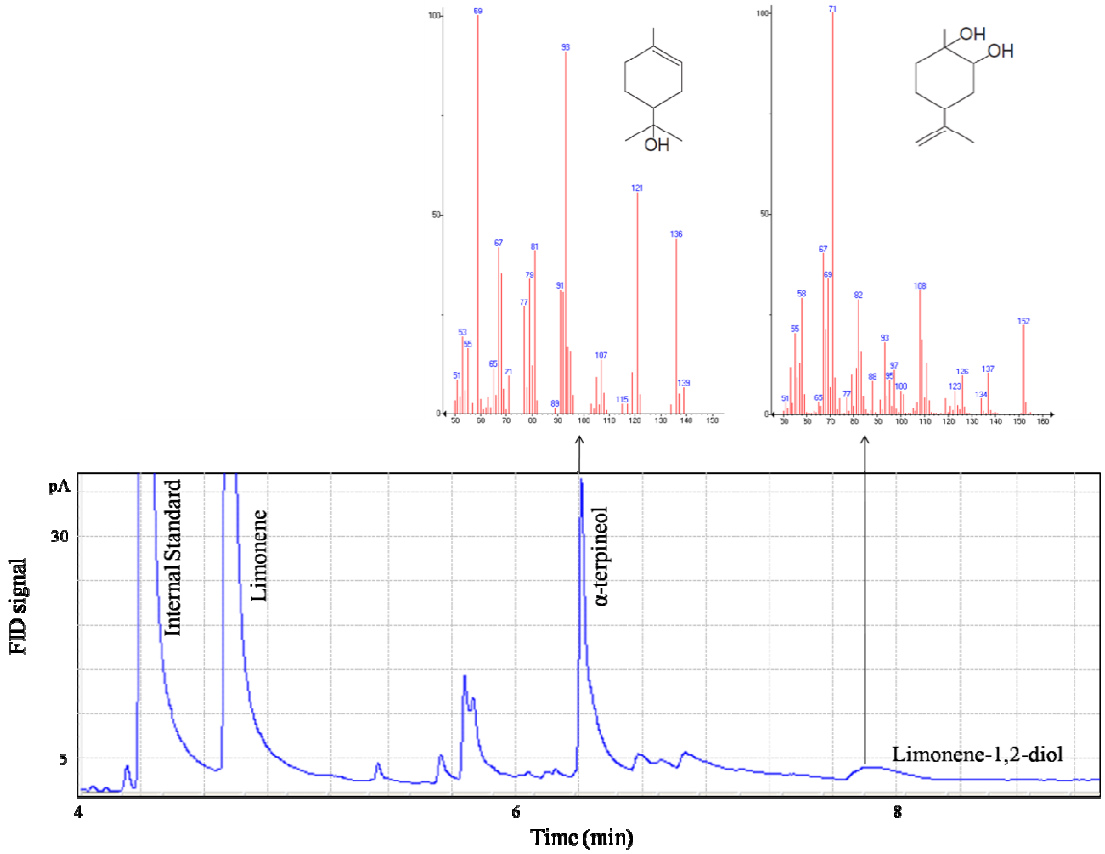

Figure 4 - Chromatogram and mass spectra of the products observed in the biotransformation of *R*-(+)-limonene by the strain LB14JLB, after 72 hours of process.

Concerning the biotransformation of limonene by LB249JLB (a Gram positive bacilli strain), the production of an important compound labeled carvone was evidenced, and traces amounts of dihydrocarvone, an intermediate of the same pathway (BICAS *et al*., 2008b). Carvone has several applications as fragrance and flavor compounds, potato sprouting inhibitor, antimicrobial agent, building block and biochemical environmental indicator, along with its relevancy in the medical field, justify the interest in this monoterpene. For a complete review discussing the importance of producing this compound, the reading of De Carvalho and Da Fonseca (2006b) is suggested. The same authors carried out an experiment to overcome the relatively low water solubilities of both carveol and carvone using an organic:aqueous system (DE CARVELHO; DA FONSECA, 2002a) and the biotransformation was carried out with whole cells of *R. erythropolis* DCL14. Authors noticed that *n*-dodecane was the solvent that allowed the highest retention of cell activity and viability. With the system proposed, two products were obtained at the end, (*4R*)-(-)-carvone and isomerically resolved (-)-*cis*-carveol.

With an air-driven column reactor, after adapting the cells to the presence of solvent, substrate and product a relatively high productivity was achieved (0.164 mg carvone/h mL organic phase) and the yield (0.68 g carvone/g carveol) were attained (DE CARVALHO; DA FONSECA, 2002b).

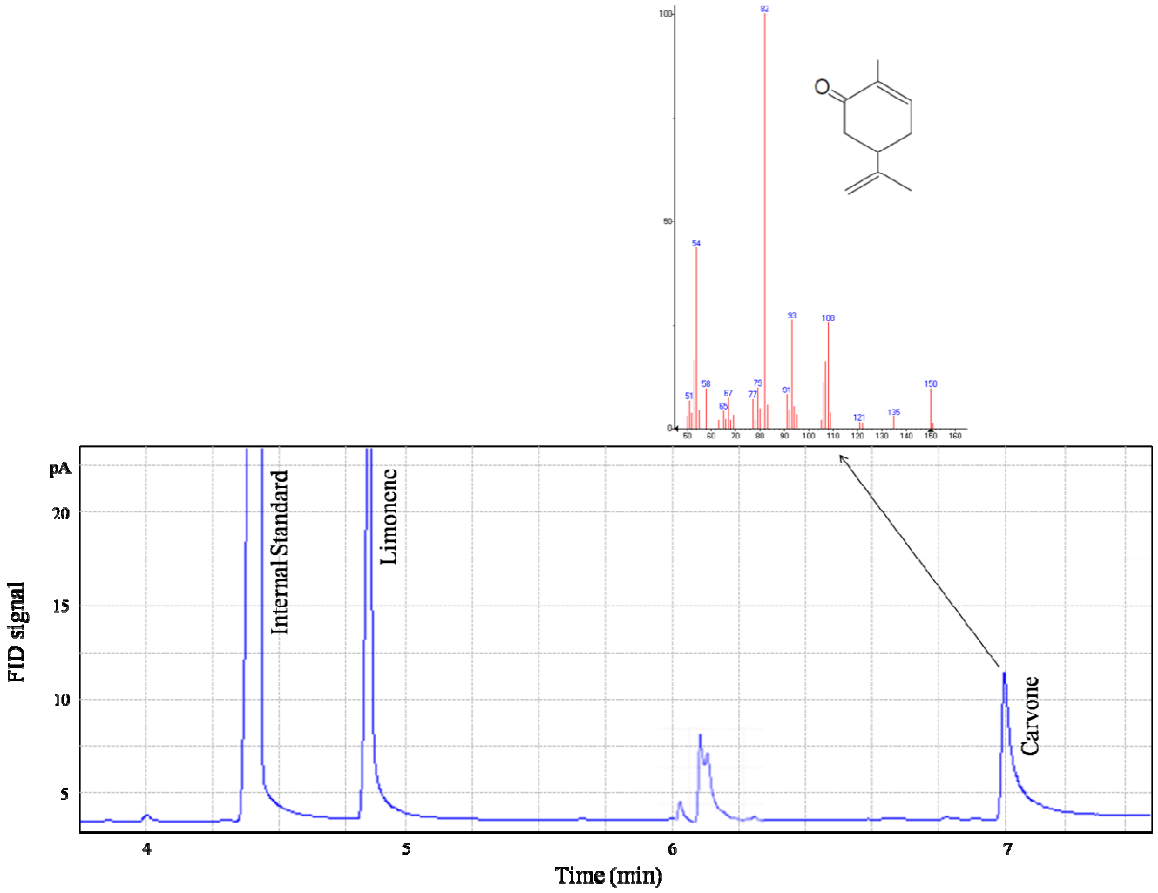

Figure 5 - Chromatogram and mass spectra of the product observed in the biotransformation of *R*-(+)-limonene by the strain LB249JLB, after 72 hours of process.

It was detected the production of carveol after the bio-oxidation of limonene by the strain LB192JLB, whose chromatogram is represented in Figure 5. Interestingly, there are few references that deal with the biotransformation of this substrate using yeasts as catalyst. Recently, Rottava and co-workers (2009) isolated a total of 405 microorganisms and found that 193 strains were able to use the substrate limonene in the prescreening step. Among the total, they concluded that eight strains were able to convert *R*-(+)-limonene, being six strains belong to yeast class and yielded α-terpineol as main product. Carveol is a fragrance ingredient used in decorative cosmetics, fine

fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents (BHATIA *et al*, 2008). The amounts obtained so far, a few milligrams per litre range, of biotechnological production of these products are insufficient for industrial applications. Only few microorganisms have been reported to transform limonene into *cis*- *trans*-carveol and carvone (DHAVALIKAR; BHATTACHARYYA, 1966; BOWEN, 1975; RHODES *et al*., 1985; KIESLICH *et al*., 1986). Low rates were also obtained with the basidiomycete *Pleurotus sapidus*, which converts (+)-limonene into *cis*- and *trans*-carveol and carvone (ONKEN; BERGER, 1999). Duetz et al. (2001) presented a toluene-degrading strain, *Rhodococcus opacus* PWD4, which is able to produce enantiomerically pure *trans*-carveol with traces of carvone by hydroxylation of R-(+)-limonene exclusively at the 6-position. Authors noticed that PWD4 cells grown on glucose were unable to carry out the formation of *trans*-carveol or any other product resulting from the oxidation of limonene. After this research, a portuguese group concluded that the production of such products as result of limonene metabolism by *R. opacus* PW4 cells was found to be dependent on both the type and concentration of the carbon source used for cell growth (CARVALHO; FONSECA, 2003).

Grown with limonene as carbon source x limonene consumption

Microorganisms were tested for their ability to grow on limonene as sole carbon and energy source. The kinetic of growth, monitored by spectrophotometer (600 nm), and substrate consumption, measured by gas chromatography, were used to plot an individual curve behavior of each organism in the presence of limonene, as may seen in Figures 6-10.

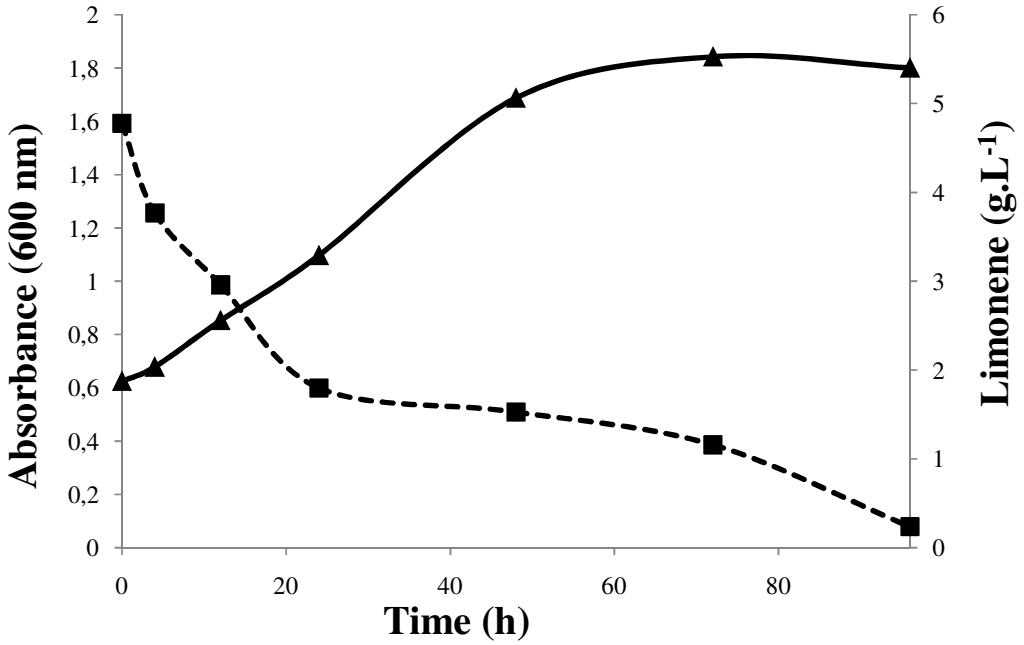

Figure 6 – Limonene (--■--) consumption and kinetic of growth (-▲-) for the strain LB285JLB.

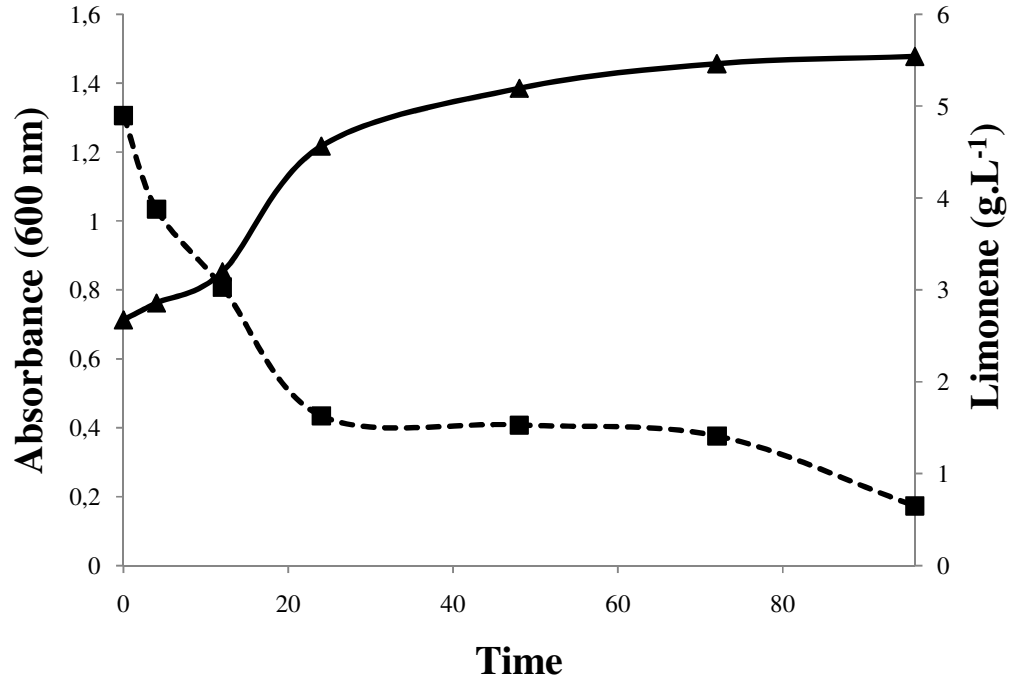

Figure 7 – Limonene (--■--) consumption and kinetic of growth (-▲-) for the strain LB177JLB.

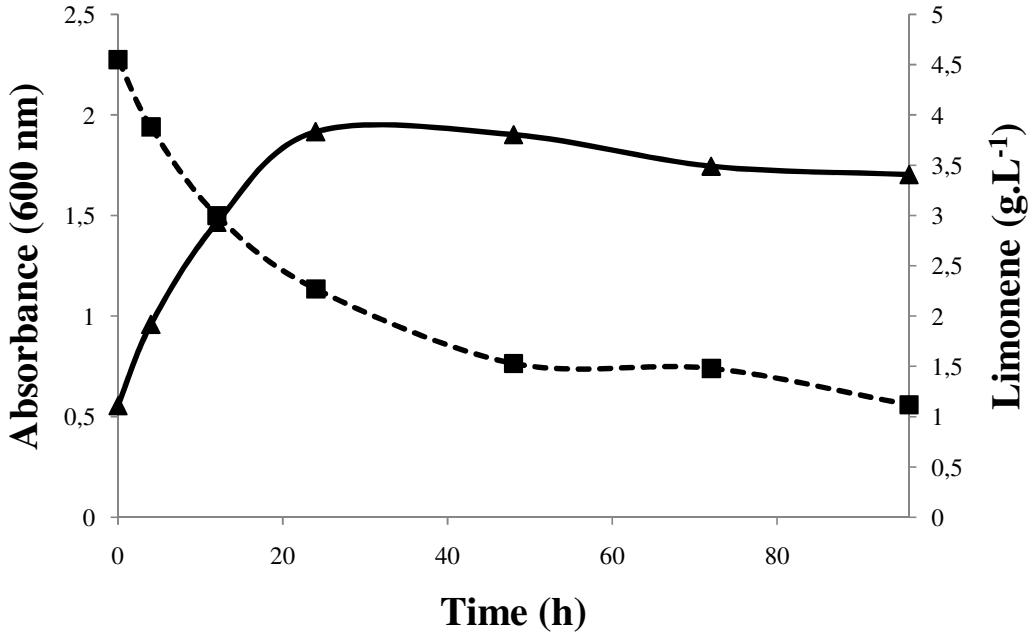

Figure 8 – Limonene (--■--) consumption and kinetic of growth (-▲-) for the strain LB14JLB.

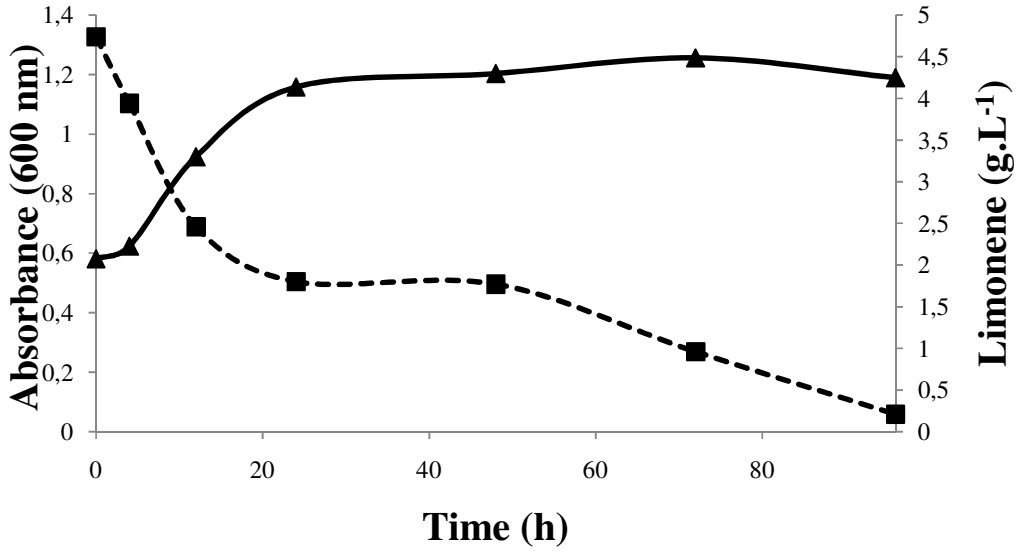

Figure 9 – Limonene (--■--) consumption and kinetic of growth (-▲-) for the strain LB245JLB.

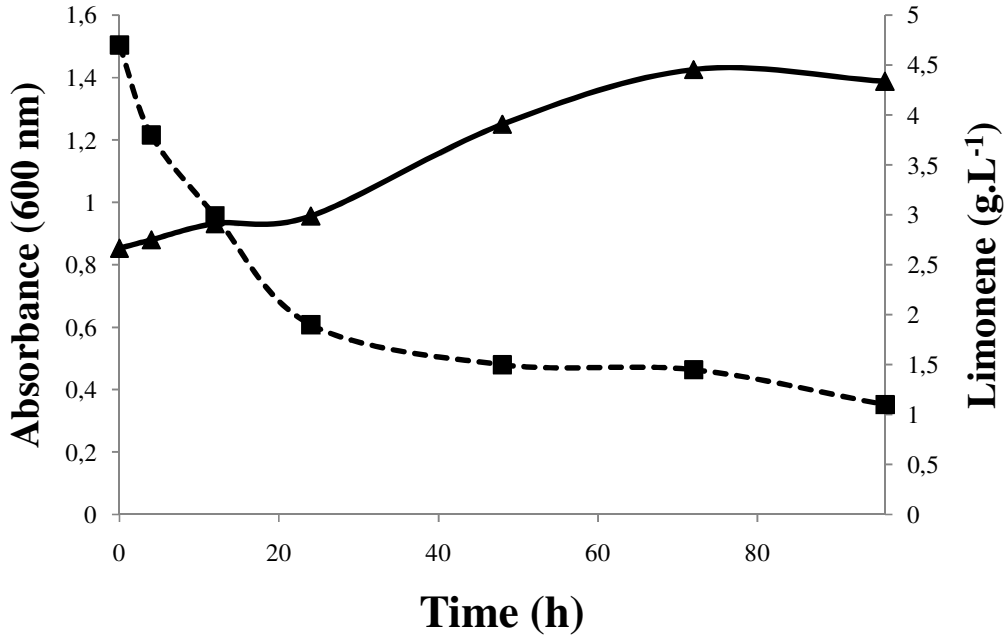

Figure 10 – Limonene (--■--) consumption and kinetic of growth (-▲-) for the strain LB192JLB.

2. Biotransformation without cell induction to substrate

The experiment aimed to verify the strains inducible to the substrate. It was built the kinetic curves with induced and non-induced cells. It could be observed that among the three strains producers of α-terpineol, only LB177JLB seemed to be inducible. These results demonstrated that just like *Penicillium digitatum* (TAN *et al*., 1998a) the enzyme was inducible. Meanwhile, enzymes from LB285JLB and LB14JLB involved in the biotransformation of limonene seemed non inducible, in accordance with previous results collected from *Fusarium oxysporum* (MARÓSTICA; PASTORE, 2007b) and *Pseudomonas fluorescens* (BICAS *et al*., 2010). On the last one, authors observed that the enzyme hydratase involved in this process was found as being cofactor independent, non-inducible and able to perform the transformation of both *R*-(+) and *S*-(-)-limonene.

Figure 11 represents the kinetic of α-terpineol production for LB177JLB, where the highest production occured on the third day of reaction, with a concentration of approximately 18 and 24 mg/L for non-induced and induced cells by limonene, respectively. From these, it could be observed that the cell induction during the inoculum growth by limonene leads to an enhancement of 38% in α-terpineol

conversion. This parameter is important for optimizing the reaction production. Tan and Day (1998b) related the activity of the microorganism enhanced by a factor of 12 after the induction of a sequential addition of the substrate in the conversion of the racemic mixture of limonene to *R*-(+)-α-terpineol. Yoo *et al*. (2001) studied the formation of major bioconversion products *versus* time by β-pinene-grown cells using 1% of substrate as sole carbon source. The accumulation of the *p*-menthene derivatives, *p*-cymene, limonene, and α-terpinolene increased to a maximal concentration, 198 mg/L of *p*-cymeme, 98 mg/L of *α*-terpinolene, and 64 mg/L of limonene after 24 h of fermentation. Toniazzo et al. (2005) obtained higher α-terpineol production for the biotransformation of (−)β-pinene from *A. niger* ATCC 9642, adding the substrate in five steps without cell induction and using ethanol as co-solvent.

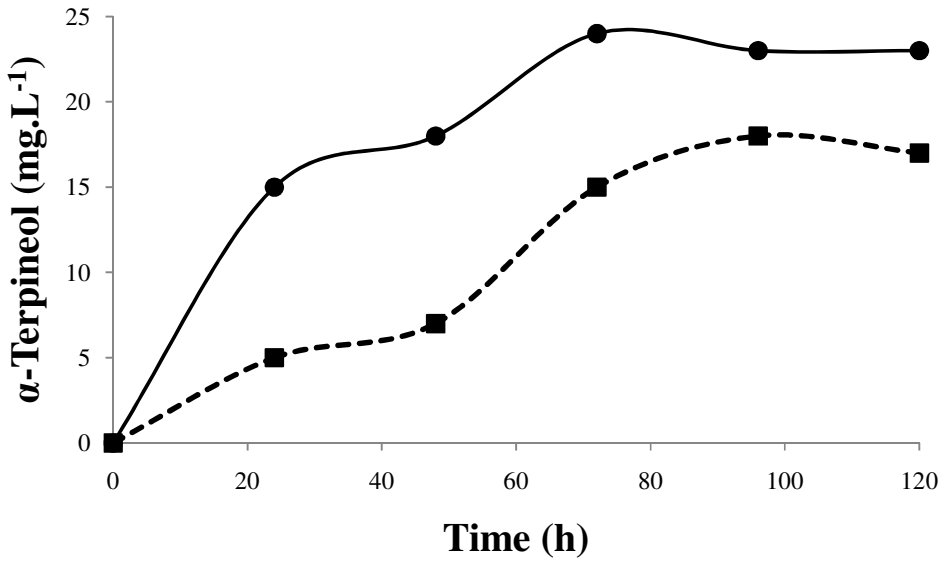

Figure 11 – Kinetic of α-terpineol production by LB177JLB induced (-●-) and non-induced (--■--) cells.

After five days of reaction allows verifying a reduction on substrate bioconversion (data not shown). Takahashi et al. (2000) showed that this reduction could be related to a possible product metabolization. The substrate concentration diminished until the tenth day for both substrates for induced and non-induced cells. This fact may be results of the lower levels of nutrients in the culture medium, making the microorganisms use the substrate as carbon source or by the enzyme inhibition by

the presence of the product in the reaction medium. The literature points out that a common problem in monoterpene biotransformation is the toxicity of these compounds for the microorganisms (DEMYTTENAERE, 2001). To reduce this effect, the method of cell induction by the substrate could be employed, allowing the microorganism adaptation with lower amounts of substrate as performed by Onken and Berger (1999).

Besides, another strain that seemed to be inducible was LB192JLB, though the kinetic profile between non-inducible and inducible was very similar. It was possible to observe that, although the production has reached the same average concentration for both of them, the ratio of production was faster for the enzymes induced by the presence of limonene in the culture media. Hence, non-induced biomass was shown to exhibit a longer lag period on terpineol synthesis than the induced cells. However, the biocatalytic behaviour was the same after this period. In this case, it was evidenced that the carbon source acted as an inducing agent and had a major effect on the yields and rates achieved during the bioconversion phase. Bicas and co-workers (2008b) discussed that the use of α-pinene oxide for cell growth considerably increased isonovalal accumulation in fresh *P. rhodesiae* and lowered novalal accumulation in whole cells of *P. fluorescens*, in comparison with data observed with α-pinene used as carbon source. Authors demonstrated that turpentine (source of α-pinene) could be used as carbon source for biocatalyst production for further bioconversion of α-pinene oxide. This feature opened a possibility to lower the cost of biocatalyst production (BICAS *et al*., 2008b).

3. Experiments performed with the strain LB285JLB

The bioconversion of *R*-(+)-limonene with the strain LB285JLB resulted on an interesting end product, a monoterpene alcohol labeled α-terpineol. This compound has a lilac odour and is one of the most commonly used fragrance compounds, representing an important commercial product that is typically applied in soaps, cosmetics and flavor preparations (BAUER *et al*., 2001). It is mainly produced chemically, starting from pinene or crude turpentine oil by acid hydration to terpine, followed by partial dehydration (TEISSERE, 1994). In this way, α-terpineol is commercially available at relatively low price (ADAMS et al., 2003).

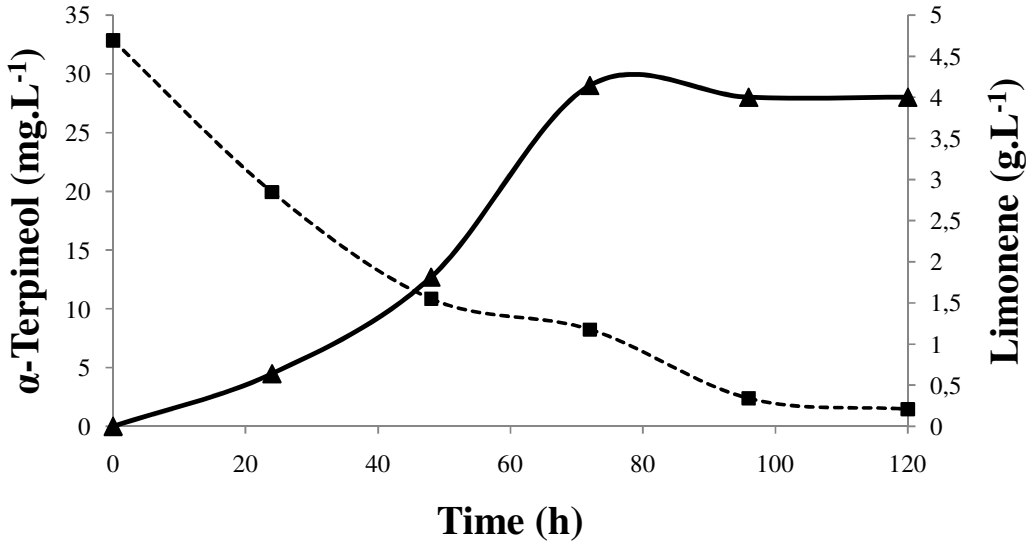

Figure 12 – Biotransformation of limonene (--■--) and time course of α-terpineol (-▲-) production by LB285JLB during 120 hours, monitored by gas chromatography.

As reviewed by Adams *et al*. (2003), bioconversion of limonene to α-terpineol as the main end product has been described using a wide range of microorganisms as catalyst: a *Cladosporium* strain (KRAIDMAN *et al.*, 1969), a *Penicillium* sp. isolated from orange peel (MATTINSON, 1971), *Penicillium digitatum* (ABRAHAM *et al*., 1985; TAN; DAY, 1998ab; TAN *et al*., 1998), *Pseudomonas gladioli* (CADWALLADER; BRADDOCK, 1992; CADWALLADER *et al*., 1989) and *Escherichia coli* expressing a thermostable limonene hydratase (SAVITHIRY *et al.*, 1997).

Maróstica Jr. and Pastore (2007b) conducted a study with a fungal strain identified as *Fusarium oxysporum* 152B in the bioconversion of *R*-(+)-limonene. Authors used agroindustrial residues cassava wastewater and orange peel oil, as substitutes for the fungal cultivation medium and substrate, respectively, and they could abserve that *R*-(+)-α-terpineol accumulated in the medium.

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 400 $mg.L^{-1}$ (MARÓSTICA JR; PASTORE, 2007b) to 2,44 $g.L^{-1}$.

Recently, Bicas and co-workers (2010) described a bioprocess with a high conversion rate of limonene to α-terpineol. The system used consisted of a biphasic medium in which the aqueous phase contained a concentrated resting cells of *Sphingobium* sp. and the organic phase was sunflower oil. After 30 h at 30 °C, ca. 25 g of R-(+)-α-terpineol per liter of organic phase were obtained from *R*-(+)-limonene Authors also performed the bioconversion in a bioreactor, which increased the production rate with no changes in yield and maximal *R*-(+)-α-terpineol concentration.

The bioconversion of *R*-(+)-Limonene by the strain LB285JLB is given in the scheme below (Figure 13).

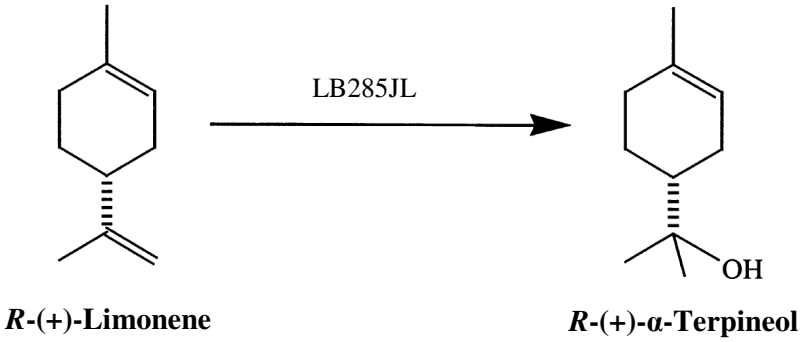

Figure 13 – Conversion of limonene in α-terpineol by strain LB285JLB.

The strain LB285JLB was chosen to further studies due its α-terpineol production had been the largest among those obtained in the present study. Besides, as discussed before this production involves non-inducible enzymes, allowing the use of alternative culture medium for biomass production, which can be used in biotransformation process.

2.1. Mineral medium formulation and use of glycerol as carbon source for biomass production

In previous experiments it was observed that the enzyme responsible for the conversion of *R*-(+)-Limonene to *R*-(+)-α-terpineol seemed non inducible, representing similar behavior to that presented by *Fusarium oxysporum* 152B (MARÓSTICA JR.; PASTORE, 2007b) and *Pseudomonas fluorescens* (BICAS et al., 2008b), because α-terpineol was still produced by a biocatalyst grown on medium rich in any other carbon sources. Therefore, the only need for the culture medium was to support biomass

growth (BICAS et al., 2010). This feature suggested that agricultural by-products such as cassava wastewater (MARÓSTICA; PASTORE, 2007b) and glycerol (SILVA *et al*., 2009; AMARAL *et al*., 2009) might be used as culture medium for biocatalyst production, an approach which would meet requirements for the use of cheap raw materials in microbial processes (PANDEY *et al*., 2000a; PANDEY *et al*., 2000b). This behaviour was markedly different from, for example, that observed for the highly inducible α-pinene oxide lyase of *Pseudomonas rhodesiae* (FONTANILLE; LARROCHE, 2003).

The medium composition usually displays an important role in biotransformation processes, being one of the main factors responsible for alterations in yield (TAN; DAY, 1998b). Productivity of microbial metabolites can be increased by manipulating nutritional requirements, physical parameters and genetic makeup of the producing strain. Also, development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium and trace element sources (GREASHAM, 1983).

Glycerol is abundant in nature, since it is the structural component of many lipids. As reviewed by Silva *et al* (2009), due to its ample occurrence in nature, many known microorganisms can naturally utilize glycerol as a sole carbon and energy source. These microorganisms have attracted attention to the potential use of abundant glycerol produced from biodiesel (BARBIRATO; BORIES, 1997; MENZEL *et al*., 1997).

Glycerol has long been used as a major carbon source in culture medium for the cultivation of microorganisms in industrial fermentation. Less expensive carbon sources, such as glucose, have limited the use of glycerol in fermentations. The increased in biodiesel production generates a huge offer of glycerol on the market, and new applications for this byproduct has become attractive (FERRARI et al., 2005; TANG et al., 2009). Nowadays, glycerol may substitute traditional carbohydrates, such as sucrose, glucose and starch, in some industrial fermentation processes (TANG et al., 2009). This compound has also been considered as a feedstock for new industrial fermentations in the future (WANG et al., 2001).

Screening of the variables

Studies on the fermentation step, using a mineral medium with a carbon source, is of great importance. This step should consider what the ideal composition of the medium and the appropriate concentration of the components employed, to provide the necessary conditions for the microorganisms maintenance.

The traditional one-at-a-time optimization strategy is simple and useful for screening, and the individual effects of medium components can be seen on a graph without the need to revert to more sophisticated statistical analyses. Unfortunately, this simple method frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account in such procedures. It was reported that the complexities and uncertainties associated with large-scale fermentation usually come from a lack of knowledge of the sophisticated interactions among various factors affecting fermentation (LIU *et al*. 2005).

Thus it was defined the values of each level to make a Placket-Burman matrix (PB-16), based on the combination of two known mineral resources (MARÓSTICA; PASTORE, 2007; VISHNIAC; SANTER, 1957) commonly used at Bioaromas Laboratory, to check the influence of variables on biomass production. For the experiment design, the variables and responses is presented in Table 4, during a period of 24 to 120 hours of fermentation. Values were expressed as absorbance value at 600 nm.

Table 4 - Plackett–Burman screening design matrix (PB-16) and the absorbance (600 nm) after 24, 48, 72, 96 and 120 h of fermentation

	A	B	C	D	E	F	G	H	I	AG	TE	GLY	Absorbance (600 ηm)				
													24 h	*48 h*	*72 h*	*96 h*	*120 h*
1	1	-1	-1	-1	1	-1	-1	1	1	-1	1	-1	0,2712	0,3907	0,5814	0,6104	0,6652
2	1	1	-1	-1	-1	1	-1	-1	1	1	-1	1	0,5321	0,6610	0,9142	1,5820	1,6692
3	1	1	1	-1	-1	-1	1	-1	-1	1	1	-1	0,4961	0,6022	0,7286	0,9862	1,1232
4	1	1	1	1	-1	-1	-1	1	-1	-1	1	1	0,5242	0,6276	1,2542	1,5631	1,6842
5	-1	1	1	1	1	-1	-1	-1	1	-1	-1	1	0,3720	0,7299	0,8981	1,2736	1,3960
6	1	-1	1	1	1	1	-1	-1	-1	1	-1	-1	0,3867	0,5142	0,5017	0,6121	0,6242
7	-1	1	-1	1	1	1	1	-1	-1	-1	1	-1	0,2711	0,2677	0,5256	0,7561	0,8001
8	1	-1	1	-1	1	1	1	1	-1	-1	-1	1	0,5642	0,6517	0,9225	1,2216	1,2997
9	1	1	-1	1	-1	1	1	1	1	-1	-1	-1	0,1734	0,2306	0,4821	0,5431	0,5421
10	-1	1	1	-1	1	-1	1	1	1	1	-1	-1	0,4037	0,5613	0,7003	0,8003	0,8321
11	-1	-1	1	1	-1	1	-1	1	1	1	1	-1	0,4821	0,5859	0,7992	0,9985	1,1042
12	1	-1	-1	1	1	-1	1	-1	1	1	1	1	0,5385	0,6796	1,2745	1,7521	1,7935
13	-1	1	-1	-1	1	1	-1	1	-1	1	1	1	0,5432	0,6273	1,1258	1,6972	1,7568
14	-1	-1	1	-1	-1	1	1	-1	1	-1	1	1	0,4912	0,5776	0,6334	1,3112	1,3524
15	-1	-1	-1	1	-1	-1	1	1	-1	1	-1	1	0,5074	0,7469	0,8562	1,2431	1,3175
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0,1524	0,2688	0,2955	0,3821	0,4057
17	0	0	0	0	0	0	0	0	0	0	0	0	0,3193	0,4721	0,7639	0,9312	0,9912
18	0	0	0	0	0	0	0	0	0	0	0	0	0,3059	0,4969	0,7559	0,9523	1,0223
19	0	0	0	0	0	0	0	0	0	0	0	0	0,3455	0,4753	0,7423	0,9442	1,0025
20	0	0	0	0	0	0	0	0	0	0	0	0	0,3182	0,4762	0,7781	0,9711	1,0632
21	0	0	0	0	0	0	0	0	0	0	0	0	0,3232	0,4852	0,7624	0,9572	1,0072

A K_2HPO_4, *B* NaH_2PO_4, *C* NH_4Cl, *D* $MgCl_2$, *E* $(NH_4)_2SO_4$, *F* $MgSO_4$, *G* NaCl, *H* KCl, *I* $NaNO_3$, *AG* Agitation, *TE* Temperature, *GLY* Glycerol and absorbance after 24, 48, 72, 96 and 120 hours. The levels of each variable are described in Table 1.

The center points for the screening design were chosen based on the conditions usually applied in the fermentation parameters (data not shown). Table 4 illustrates that, with few exceptions, the absorbance value increased during the course of the reaction, reaching a maximum after 72-120 h. However, the increase observed between this time might not be big enough to justify an extra 24 h of process. For this reason only these three periods were considered in the statistical analysis and for the central composite design.

The statistical evaluation of the results is shown in Table 5. In this case, a P value of 0,1 is currently recommended, since it is more conservative and lowers the risk of false excluding statistically significant parameters (RODRIGUES; IEMMA, 2005).

Table 5 - Estimates of the effects of the parameters analyzed after 72, 96 and 120 h of fermentation

Factor	Time (h)	Effect	SE	t(8)	*P* value
Mean	**72**	**0,775995**	**0,020437**	**37,97092**	**0,000001**
	96	**1,051843**	**0,026063**	**40,35774**	**0,000001**
	120	**1,116786**	**0,028099**	**39,74400**	**0,000001**
K_2HPO_4	**72**	**0,103138**	**0,046826**	**2,20257**	**0,058758**
	96	0,051062	0,059718	0,85506	0,417387
	120	0,054563	0,064384	0,84745	0,421370
NaH_2PO_4	**72**	**0,095563**	**0,046826**	**2,04080**	**0,075580**
	96	**0,133813**	**0,059718**	**2,24075**	**0,055361**
	120	**0,155163**	**0,064384**	**2,40995**	**0,042511**
NH_4Cl	72	0,047838	0,046826	1,02160	0,336867
	96	0,025062	0,059718	0,41968	0,685761
	120	0,058238	0,064384	0,90453	0,392137
$MgCl_2.6H_2O$	72	0,086237	0,046826	1,84166	0,102784
	96	0,018838	0,059718	0,31544	0,760498
	120	0,019688	0,064384	0,30578	0,767576
$(NH_4)_2SO_4$	72	0,070813	0,046826	1,51225	0,168924
	96	0,014263	0,059718	0,23883	0,817241
	120	-0,003862	0,064384	-0,05999	0,953634
$MgSO_4.7H_2O$	72	-0,085538	0,046826	-1,82671	0,105166
	96	0,013863	0,059718	0,23213	0,822262
	120	-0,008587	0,064384	-0,13338	0,897187
NaCl	72	-0,030863	0,046826	-0,65909	0,528350
	96	-0,013163	0,059718	-0,22041	0,831071
	120	-0,030613	0,064384	-0,47547	0,647165
KCl	**72**	**0,118762**	**0,046826**	**2,53625**	**0,034914**
	96	0,002737	0,059718	0,04584	0,964561
	120	0,004688	0,064384	0,07281	0,943748
$NaNO_3$	72	0,009137	0,046826	0,19514	0,850150
	96	0,051213	0,059718	0,85758	0,416077
	120	0,042913	0,064384	0,66651	0,523838
Agitation	**72**	**0,163462**	**0,046826**	**3,49084**	**0,008188**
	96	**0,251288**	**0,059718**	**4,20792**	**0,002964**
	120	**0,259412**	**0,064384**	**4,02915**	**0,003793**
Temperature	**72**	**0,169012**	**0,046826**	**3,60937**	**0,006888**
	96	**0,252112**	**0,059718**	**4,22173**	**0,002909**
	120	**0,274137**	**0,064384**	**4,25785**	**0,002769**
Glycerol	**72**	**0,408062**	**0,046826**	**8,71443**	**0,000023**
	96	**0,744387**	**0,059718**	**12,46509**	**0,000002**
	120	**0,771563**	**0,064384**	**11,98376**	**0,000002**

SE Standard error, Parameters in bold are statistically significant for the response ($P < 0.1$), considering the residual SS.

Table 5 presents the effects of variables on the response, in addition to their *t*- and *p*-values. Parameters in bold are statistically significant for the absorbance at 600 nm ($P < 0,1$). Shortly, although it could be observed that the effects of factors on biomass

production were not quite impactful, allowed to determine which parameters of the culture medium and cultivation conditions were significant in the proposed process.

Effect of the medium composition

Considering that the medium composition displays an important role in biotransformation processes, hence the effects of different sources of nitrogen and carbon on biomass production were investigated (ADAMS *et al*., 2003).

In this experiment, it could be observed that the minority of the compounds tested presented statistical effects on the response after 72, 96 and 120 hours of fermentation, considering the ranges tested. Thus, these variables were of no significant interest (at $P < 0.1$) to this process. The theory that they may have been masked by the variables most influential is not dismissed (RODRIGUES; IEMMA, 2005).

Therefore, according to the results show in Table 5, the best minerals in the medium for this process seemed to be K_2HPO_4 and NaH_2PO_4 and KCl, whose were chosen for further studies. Firstly, it was decided to keep them in the culture medium and even that its effects were significantly positive, were kept in intermediate and fixed concentrations, related to the level (-1) in the screening experiments and they were not included in the following Central Composite Design experiments. This alternative was adopted to minimize the costs of the application of these compounds in the medium, but at the same time makes them accessible to the microorganisms.

Glycerol, used as a carbon source, proved to be important throughout the period tested and remarkably a statistically significant parameter. This result is completely comprehensible since in fermentation processes (BICAS *et al*., 2008a), the substrate is usually the sole carbon source, and mineral mediums (saline solutions) or buffers are typically applied as the culture medium (MARÓSTICA; PASTORE, 2007b; SPEELMANS *et al*., 1998; TAN *et al*., 1998), even though some authors have proposed there is a correlation between best fungal growth and best bioconversion yield, suggesting mediums with other carbon sources (ADAMS *et al*., 2003).

Medium optimization has been studied for many authors and for many different applications (PUNDLE; SIVARAMAN, 2005; MACEDO et al., 2007; LIU et al., 2005; BICAS et al., 2008a). For example, Souza and co-workers (2006) observed that $FeSO_4.7H_2O$, $MgSO_4.7H_2O$ and Na_2HPO_4 were the best mineral sources for MTGase production, with activity reaching 0.246, 0.242 and 0.235 U/mL, respectively, after 8 days of cultivation. However, MTGase production with the addition of NaCl, NH_4Cl,

$ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$ and $NaNO_3$ was found to be low when compared with not supplemented medium.

Effect of temperature and agitation

It is known that the medium temperature directly influences biological reactions. Moreover, medium agitation promotes development of the microorganism and cell-substrate interaction. However, the use of high temperatures and agitation speeds, in addition to increasing the process energy costs, might enhance the loss of substrate and product and the occurrence of side reactions. Thus, an ideal balance must be searched for in order to achieve the best results (BICAS et al, 2008a).

At 72, 96 and 120 hours of fermentation both of parameters tested were statistically significant (at $P < 0.1$) on the levels tested. The temperature showed a positive effect on the fermentation process (Table 5) to biomass production, so it was decided to increase the range of study in the Central Composite Design. The positive effect also observed for agitation was probably related to the increase in cell-substrate contact. Hence, the optimal agitation may be situated at values above the maximum value tested in the screening design (250 rpm) and an ampler range is suggested (BICAS et al, 2008a). To relate the need for agitation with the process economics, a repetition with no agitation was run to ensure that the achievement of biomass was significantly minimized (data not shown).

Optimization using a central composite design

According to the variables screened, the significant factors ($P < 0.1$) for the process under study, considering the levels tested, which were worth considering in the further optimization design were only temperature, agitation speed and substrate (glycerol) concentration. The minerals (K_2HPO_4 and NaH_2PO_4 and KCl) only appeared to present an effect around 72 hours of fermentation, for this reason were not included in the following experiments and it was added in fixed concentrations, as discussed elsewhere.

The three variables (temperature, agitation and glycerol concentration) which were analyzed at the levels described in Table 2, were optimized using a 2^3 central composite design with six center points and six axial points resulting in 20 experiments,

as shown in Table 6, which also contains the absorbance values observed experimentally.

In Table 2, it can be seen that the levels chosen to study and optimize each variable were virtually the same as those used previously, but there was an alteration in the amplitude of the ranges studied, for all selected variables.

Table 6 - 2^3 Central composite design matrix and the absorbance value after 72, 96 and 120 h incubation

	G	T	A	Absorbance (600 nm)		
				72 h	96 h	120 h
1	-1	-1	-1	1,6200	1,8243	1,8432
2	+1	-1	-1	1,8276	2,5683	2,4763
3	-1	+1	-1	1,9240	2,7784	2,8684
4	+1	+1	-1	1,9432	3,0874	2,9457
5	-1	-1	+1	1,7824	2,9842	2,9874
6	+1	-1	+1	1,8005	2,9466	2,9185
7	-1	+1	+1	1,8643	3,0046	2,9985
8	+1	+1	+1	1,8946	2,7657	2,7985
9	-1,682	0	0	1,903	2,6573	2,7195
10	+1,682	0	0	1,8524	2,5732	2,5852
11	0	-1,682	0	1,7245	2,7745	2,7176
12	0	+1,682	0	1,8934	2,9746	2,9984
13	0	0	-1,682	1,8523	2,9037	2,8535
14	0	0	+1,682	1,6234	2,7353	2,7579
15	0	0	0	1,9445	2,9274	2,9047
16	0	0	0	1,9763	2,9594	2,9365
17	0	0	0	1,9111	2,9425	2,9476
18	0	0	0	1,9354	2,9473	2,9113
19	0	0	0	1,8935	2,9389	2,9014
20	0	0	0	1,9032	2,9413	2,9235

The levels of each variable are described in Table 2. G Glycerol, T temperature, A agitation

In summary, depending on the conditions applied in the fermentation process, the absorbance varied from 1,6 to 1,9 after 72 hours, 1,8 to 3,08 and 1,8 to 2,98, after 96 and 120 hours, respectively. Meanwhile, comparing the kinetic profile during the time analyzed it could be observed that the highest biomass production occurred around 96 hours where in some cases, as experiment 4, the absorbance was considerably higher. However, after this time, there was stagnation and a slight decreasing in the absorbance value, what could be explained by the accumulation of biomass and absence of substrate

or nutrient depletion of the medium. Additionally, it can be verified that the central points (experiments 15-20) showed a small variation, indicating good repeatability of the process, especially when dealing with a biological assay.

In 96 hours of fermentation, it was observed that the maximum values obtained correspond to the experiments 4, 5 and 7, and also for the tests carried out under the central point conditions. Meanwhile lower values and minimum variation in absorbance during the 120 hours, was collected in the Experiment 1, when the process was submitted to the minimum conditions (Level -1) for all variables. It is essential to point out that the absorbance (at 600 nm) presented a small variation in the fermentation process, where the highest and minimum values were very close.

All the experiments carried out at temperatures and agitation equal or inferior to 24 °C and 100 rpm are comprised in group with absorbance values below the overall average, demonstrating that lower temperatures clearly reduced the fermentation activity and retarded the process.

As a result, a 120 h-reaction period presented no practical advantage over 96 h, which was considered to be the optimum time for the statistical evaluation. This data (96 h-fermentation), were treated by the software STATISTICA® v. 7.0, which generated the regression coefficients and respective statistical analysis of the parameters considered (Table 7).

Table 7 - The least-squares and significances of the regression coefficients of the model parameters after 96h of fermentation

	RC	**SE**	**t(8)**	***p*-value**
Média	**2,944840**	**0,091643**	**32,13394**	**0,000001**
Glycerol (L)	0,046501	0,049670	0,93621	0,376561
Glycerol (Q)	**-0,119369**	**0,051610**	**-2,31290**	**0,049464**
Agitation (L)	**0,120762**	**0,049670**	**2,43130**	**0,041119**
Agitation (Q)	-0,027693	0,051610	-0,53658	0,606153
Temperature (L)	0,084901	0,049670	1,70932	0,125765
Temperature (Q)	-0,047156	0,051610	-0,91370	0,387584
G x A	-0,079537	0,064896	-1,22561	0,255201
G x T	**-0,166187**	**0,064896**	**-2,56081**	**0,033605**
A x G	**-0,204212**	**0,064896**	**-3,14674**	**0,013665**

RC Regression coefficient, SE standard error, T temperature, A agitation, L linear, *Q* quadratic.

Parameters in bold are statistically significant for the model (P < 0.05)

The significance of each coefficient was determined by t-*Student* e *p*-value. The greater the magnitude of the t-value and lower the p-value, then more significant is the corresponding coefficient. This implies that the linear and quadratic effects for the interactions between Glycerol x Temperature and Agitation x Temperature were significant at 5% of significance. This was also valid for glycerol (quadratic) and for agitation (linear), which were statistically significant, considering a *p* value of 0.05. These results are presented in Table 7.

After the measurement of the regression coefficients it was possible to assemble the model including only significant terms, with a significance level of 0.05, represented in the equation below:

Equation (1) – Predicted and adjusted model for the encoded values

$$\hat{Y} = 2{,}944840 - 0{,}119369\, x_1^2 + 0{,}120762\, x_2 - 0{,}166187\, x_1 x_3 - 0{,}204212\, x_2 x_3$$

In order to verify the validity of the model, an analysis of variance (ANOVA) was performed considering only the statistically significant ($P < 0.05$) variables (parameters in bold in Table 7) for the analysis.

Table 8 - ANOVA of the quadratic model for biomass production

Variation source	SS	*df*	SM	*F* value	*P* value
Regression	1,12	9	0,13	4,3	0,025
Residues	0,27	8	0,03		
Total	1,39	17			
$R^2 = 0{,}81$				$F_{0.95(9,8)} = 3{,}39$	

SS Sum of squares, *df* degrees of freedom, *SM* mean square

The ANOVA table (Table 8) demonstrated that the quadratic model adjusted for the process responses was satisfactory. The calculated F value was higher than the respective listed value, while the *P* value of the model was lower than 0,05. Although it is not ideal, a value for $R^2 = 0.81$ is a perfectly acceptable value for biological systems (RODRIGUES; IEMMA, 2005). Consequently, it is possible to define a statistically valid model ($P < 0.05$), given by Eq. 1.

Figure 14 displays the relationship between the predicted values by the model and the experimentally observed.

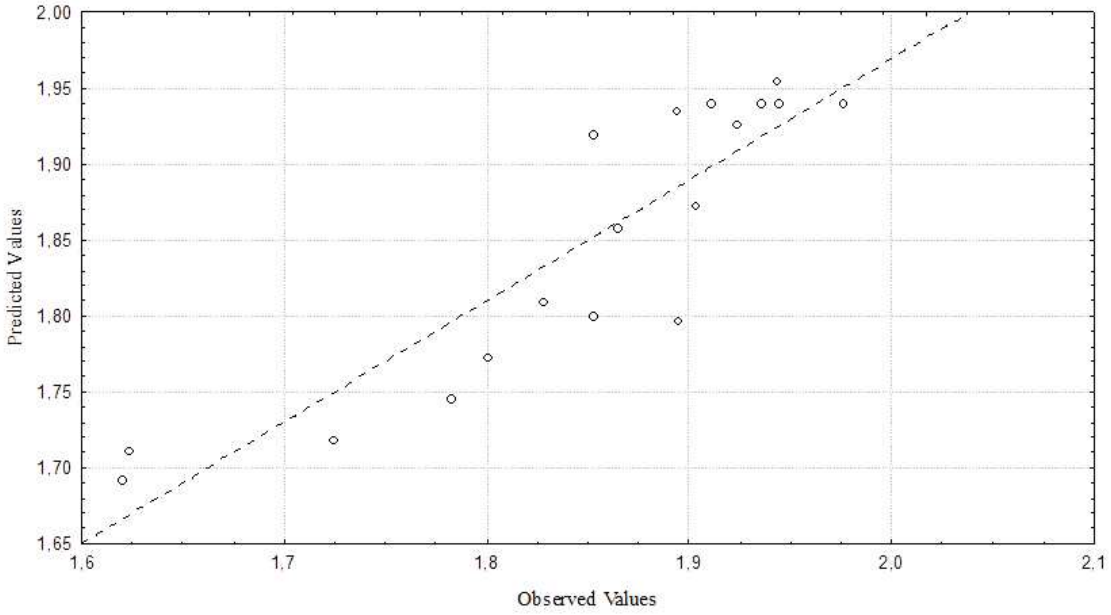

Figure 14 – Predicted and observed values for biomass production.

It is observed that some tests, especially 1 and 13, did not properly fit the model, due the conditions applied in each case. However, from this graphical view it was confirmed that there was a good distribution of values over the line and the collected data were well explained by the model. To confirm this fit to data collected it was built the Table 9, containing the absolute and relative error between predicted by the model and values observed experimentally, calculated as follows in equation 2 and 3.

Equation (2) – Calcule of absolute error

$$\mathbf{AE} = Y_{96h\text{-absorbance}} - \hat{Y}_{predicted}$$

Equation (3) – Calcule of relative error

$$\mathbf{RE} = \frac{Y96h\text{-absorbance} - \hat{Y}predicted}{Y_{96h\text{-absorbance}}} \times 100$$

Table 9 - Absorbance values provided by the model and differences from obtained experimentally after 96h-fermentation

Experiment	Predicted	AE	RE
1	2,3343	-0,51	-27,95
2	2,6666	-0,09	-3,83
3	2,9842	-0,20	-7,40
4	3,3166	-0,22	-7,42
5	3,0751	-0,09	-3,04
6	2,7427	0,21	6,91
7	2,9082	0,09	3,20
8	2,5758	0,18	6,86
9	2,6072	0,05	1,88
10	2,6072	-0,03	-1,32
11	2,7417	0,03	1,18
12	3,1479	-0,17	-5,82
13	2,9448	-0,04	-1,41
14	2,9448	-0,20	-7,66
15	2,9448	-0,01	-0,59
16	2,9448	0,01	0,49
17	2,9448	-0,01	-0,07
18	2,9448	0,01	0,08

AE Absolute error; *RE* Relative error

As may seen on Table 9, there was a deviation about 1-7% of the responses provided in relation to experimental, which represents a low value considering that the process involved microorganisms. The biggest deviations were found at the periphery of the area covering the points studied, which is supported by the fact that the models precisely suffers from largest deviations at the extremes points.

Thus, the largest variations could be found from the experimental responses, as noted in the exception Experiment 1, which reached a deviation of 27% and it was clearly higher than the others data collected.

As the model obtained was significant it was built the response surfaces to illustrate the behavior of each variable into the model. Figures 15, 16 and 17 comprise the surfaces responses and contour plots found in this study.

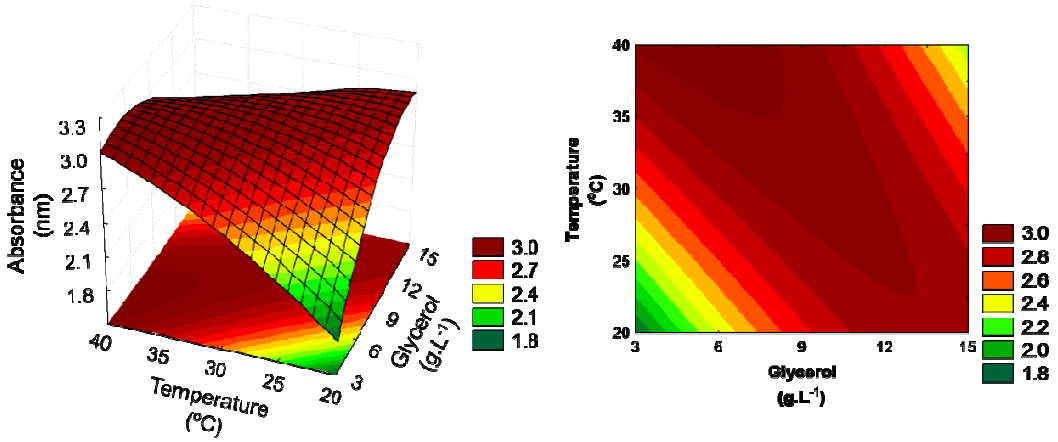

Figure 15 – Surface response and contour plot of the absorbance at 600 nm for biomass production after 96 h of fermentation as a function of temperature and glycerol. Agitation was fixed at central point value, 200 rpm.

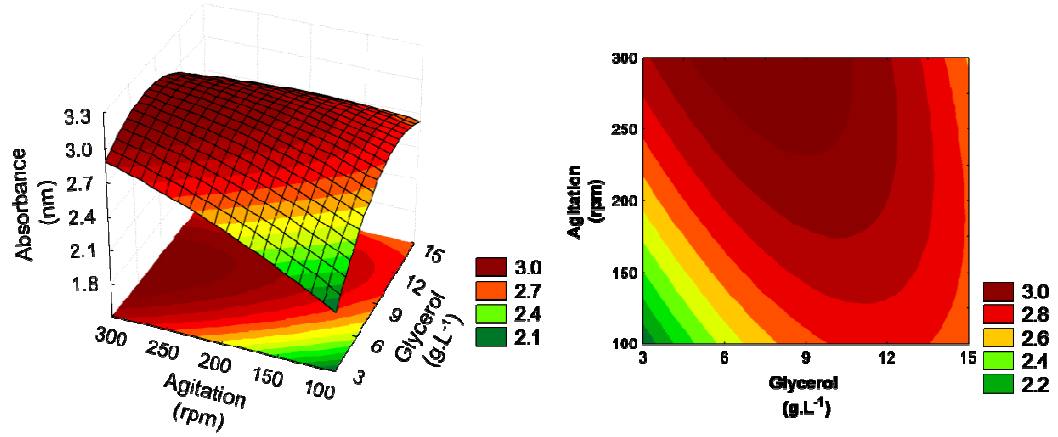

Figure 16 – Surface response and contour plot of the absorbance at 600 nm for biomass production after 96 h of fermentation as a function of agitation and glycerol. Temperature was fixed at central point value, 30°C.

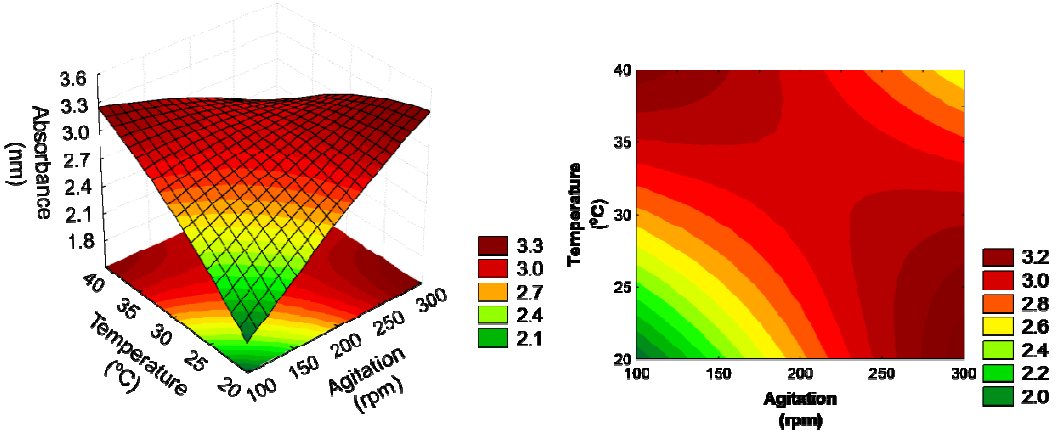

Figure 17 - Surface response and contour plot of the absorbance at 600 nm for biomass production after 96 h of fermentation as a function of temperature and agitation. Glycerol concentration was fixed at central point value, 9 g/L.

The profile of the response surfaces obtained was close to the ideal, since the Figure 15 and Figure 16 presented the predicted optimal regions, comprised inside the levels studied. It can be seen that a strict control of the biotransformation conditions was not necessary, which simplifies the process even more.

Figure 15 shows the effect of interaction between the concentration of glycerol and temperature on the absorbance, where it was clearly indicated which would be the proper combination to be used in fermentation parameters. An increase in temperature favors the increase in absorbance value and this phenomena can be explained by the higher dissolution of substrate in the medium, favored the higher temperatures. Besides that, when glycerol is dissolved in water, disrupts the hydrogen bonding between water molecules and that mixture cannot form a stable crystal structure unless the temperature is significantly lowered (SILVA et al., 2009).

The suitable temperature range for the process is in accordance with scientific references, as Pachauri and He (2006) who applied with temperatures between 33-37 °C in their process. Thus in the present case, temperatures between 30-40 °C should be considered, in order to obtain the best performance. On the other hand, it is know that very high temperatures can negatively affect biological systems, as discussed by Bicas and co-workers (2008a), possibly explained by inhibition of microbial growth and enzyme denaturation at temperatures close to 40 °C.

Meanwhile an increase in the glycerol concentration affects gradually and negatively the production of biomass, so it can be observed that the optimum range of glycerol concentration would be around 9 $g.L^{-1}$. High concentrations of this organic solvent in the médium may cause inhibition due to its cytotoxicity, evaluated by *log P* which glycerol value is around –3.0 (LAANE et al., 1987). *Log P* is the logarithm of the octanol:water partition coefficient of the solvent. Stability reaches a minimum for *log P* values between 0 to 2 for enzymes, and bctween 2 and 4 for microorganisms. Above these ranges increasing *log P* of the solvent (or for that matter substrate) results in increased biocatalyst stability. The transition point from cytotoxicity to non-toxicity for solvents typically occurs between a log P between 3-5 (TAN; DAY, 1998b).

Regarding Figure 16 between agitation and glycerol, it is possible to observe that a stirring among 250-300 rpm is able to provide higher yields of biomass, observed by an increase in the value of absorbance.

An analysis of Figure 17 showed a particular profile, where the maximum value of each variable, or temperature or agitation, offers an increase in the final value of absorbance. The temperature and agitation could oscillate from 37 to 40 °C and from 250 to 300 rpm, respectively, maintaining the absorbance value close to the maximal, when the glycerol concentration remained fixed at 9 $g.L^{-1}$.

The optimal conditions could be determined using mathematical methods (derivation of model´s equation). However the surface response and contour curves allows to verify the optimal conditions graphically and define the best conditions to each variable applied in the experiment. Due to practical reasons, it was decided to use 30 °C, 250 rpm and 9 $g.L^{-1}$ of substrate.

Table 10 - Experimental validation under optimized conditions after 96 h of fermentation

Variables	Optimal values	Absorbance (600nm)	Dry mass ($g.L^{-1}$)	α-Terpineol production($mg.L^{-1}$ – 72h)
Glycerol ($g.L^{-1}$)	9			
Temperature (°C)	35	3,0142*[A]	3,075*[B]	27*[C]
Agitation (rpm)	250			

**Average of experimental value; [A.B.C]Standard error: A: 0,2721; B: 0,13; C:1,58*

Finally, it was conducted seven experiments in the optimum conditions for the experimental validation of the model, as presented in Table 10, with the results of absorbance and dry mass collected. It was found that in the optimum conditions defined for utilization of glycerol as alternative medium for biomass growth, the average of absorbance value was 3,0142, a value very close to the maximum obtained during the sequential optimization process and the central composite design (CCD). Under these conditions, it was obtained 0,375 g of dry mass which was enough to conduct the experiments of biotransformation, achieving yields of 27 $mg.L^{-1}$ of α-terpineol.

It was also performed a comparison between the proposed cultivation media (containing glycerol) against the media usually applied for microorganisms growth (yeast and malt medium, YM), displayed graphically in Figure 18.

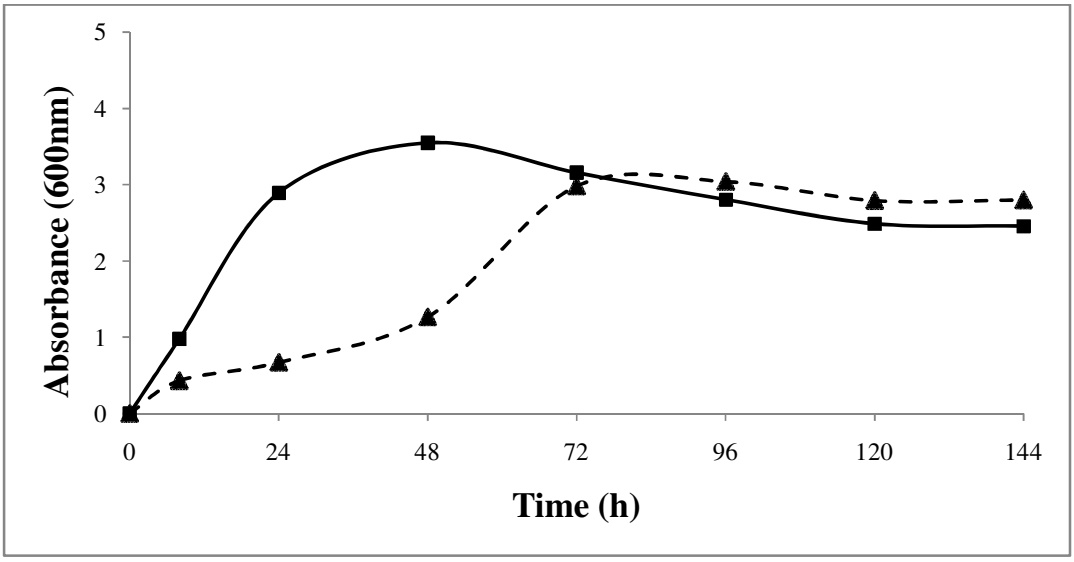

Figure 18 – Differences between growth under condition optimized in alternative glycerol medium (--▲--)and Yeast and Malt medium (-■-).

The growth of the strain in glycerol medium was low, showing a *Lag phase* considerably larger than when compared with the YM medium. Moreover, with YM medium it was reached higher values of absorbance and mainly dry biomass.

Although lower than predicted and lower than the synthetic media, the initial purpose of using glycerol as carbon source was achieved in the proposed experiment. Some studies have shown interesting results when glycerol was used on the system. Imandi and co-workers (2007) optimized the production of citric acid by the strain *Yarrowia lipolytica* NCIM3589 using glycerol as carbon source. Through the response surface methodology, they evaluated that the concentration of yeast extract, glycerol and saline solution were important factors to increase the production of citric acid. Optimum concentrations obtained were 0.2682 gL^{-1} of yeast extract, 54.4081 $g.L^{-1}$ of glycerol and 13.69% of saline solution.

The microorganism's growth using YM medium presents a great absorbance around 24-48 hours, while with the alternative medium containing glycerol next to 96 hours. This time may become unviable for large-scale production and possible use of an industrial process due to the long fermentation time. However, the use of renewable waste substrates is an environmental-friendly choice that also contributes to the reduction of waste treatment costs and increases the economic value of by-products.

CONCLUSION

An extensive screening of bacteria able to transform limonene into aroma compounds was performed. In addition to the lack of effective biotransformation systems for the production of limonene intermediates, terpene transformations in general suffer from the volatility of the substrate and from the toxicity of terpenes towards microorganisms.

The results in the screening showed that among 70 strains tested, five strains were able to convert *R*-(+)-limonene in aroma compounds, such as α-terpineol and carvone. It was observed that the most recurrent metabolites for the biotransformation of limonene were α-terpineol, obtained by three strains. It is worth noticing that it was obtained the highest concentration of α-terpineol derived from the bio-oxidation of limonene, when compared with other products. Strain LB285JLB was responsible for the best yields reached, approximately 29 $mg.L^{-1}$.

Although the yields obtained after bioconversion are not very high, they can be increased by further method optimization and study of the different culture conditions involved in order to produce these terpenoids on a large-scale.

The strain LB285JLB was used to evaluate an alternative culture medium, using glycerol as carbon and energy source for biomass. According to the contour plots and surface responses provided from the CCD experiment, the best conditions for the microorganism growth measured by its absorbance recovery were: 96 h-reaction in, temperature between 35-40 °C, agitation of 230 to 300 rpm and glycerol concentration of 6-9 $g.L^{-1}$.

These optimization techniques were very useful for a full understanding of the process and allowed a complete visualization of the robustness of the process, providing keys-information about its control.

The use of renewable waste substrates is an environmental-friendly choice that also contributes to the reduction of waste treatment costs and increases the economic value of by-products.

Considering the results obtained in the present study, the research described in this paper is an initial step for the exploration of aroma compounds production *via* biotransformation of *R*-(+)-limonene, a non-expensive by-product of citrus industry. Therefore, experiments involving other aspects of production (optimization of temperature and agitation conditions) and selection of new strains are being carried out.

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CAPÍTULO 3

PROSPECTION OF THE ENDOPHYTES ISOLATED FROM BARU (*Dipteryx alata* Vog.)

ABSTRACT

Background: Microorganisms associated with plants have been a promising source of natural products with biological activities. Considering that the Brazilian Cerrado present a huge biodiversity with unexplored microbial flora, especially endophytes, the bioprospection of endophytic microorganisms isolated from baru (*Dipteryx alata* Vog.) was evaluated in this paper.

Results: All the 26 microbial strains isolated from baru were identified as 16 bacteria and 11 filamentous fungus. Screening of the antimicrobial activity of the extracts of endophytics revealed a considerable activity against bacteria and yeasts tested. The most significant activity was observed for microorganisms labeled as LBBR1, LBBR15 and LBBR17 which inhibited the growth of all the pathogenic tested. Moreover, 81.48% displayed amylase activity, 93% displayed lipase activity and 63% displayed protease activity. Finally, the biotransformation of α-pinene resulted in verbenol as the most recurrent product.

Conclusion: The use of endophytic microorganisms isolated from the brazilian Cerrado biome demonstrates a partial use of these microorganisms in biotechnological processes. Thus, this paper represents the first of many works that are being developed in the laboratory of Bioaromas (FEA/Unicamp-Brazil) and represented a screening of the potential uses of these microorganisms.

Key-words: Endophytes, *Dipteryx alata* Vog., Biological activity, Biotransformation of terpenes.

1. INTRODUCTION

The term “endophytes” includes a suite of microorganisms that grow intra and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live (LI *et al*., 2008). Mutualism interaction between endophytes and plants may result in fitness benefits for both partners (KOGEL *et al*., 2006), providing protection and survival conditions to their host plant by producing a plethora of substances (TAN; ZOU, 2001). Once isolated and characterized, these substances may also have potential for use in industry, agriculture and medicine (STROBEL *et al*., 2004; STROBEL; DAISY, 2003).

Approximately 300,000 plant species on the earth growing in unexplored area is host to one or more endophytes (STROBEL; DAISY, 2003). Considering that a wide-spectrum of vegetal and microbial species present in the Brazilian Cerrado, a savanna-like vegetation, is still unknown (Ministério do Meio Ambiente, 2002), the opportunity to find new compounds from endophytes with potential to flavor and pharmaceutical industries is a promising strategy. Brazilian Cerrado is the second largest biome in South America after the Amazon rainforest, accounting about 160,000 species of plants, animals and microbes, many of which are endemic to this biome (QUIRINO *et al.*, 2009). Despite the rich biodiversity, the Cerrado is in rapid decline because of the expansion of modern agriculture (BRANNSTROM *et al.*, 2008; OLIVEIRA; MARQUIS, 2002) and its annual degradation rate is greater than in the Amazon (DRUMMOND, 2008). Thus, the preservation of this Biome is essential for the maintenance of world biodiversity (CAVALCANTI, 2002; COSTA *et al.*, 2002; RODRIGUES; CARVALHO, 2001). In this way, studies on the screening and characterization endophytes from Cerrado biome are important to investigate molecules with biological activities and potential applications in industry.

Microorganisms associated with plants require a number of hydrolytic enzymes to help degrade the cell wall. Endophytes have been the subject of several investigations for the production of enzymes of interest, such as amylases (STAMGORD *et al*., 2001; STAMFORD *et al*., 2002; MARLIDA *et al.*, 2000; SCHULZ *et al*., 2002), protease and lipase (SCHULZ *et al*., 2002). In addition to enzymatic activity, other biological functions from endophytic microorganisms extracts have also been described such as antibacterial, antifungal and antiviral properties (FIRÁKOVÁ *et al.*, 2007; TELES *et*

al., 2005; TELES *et al.,* 2006; SILVA *et al.*, 2006; WIYAKRUTTA *et al.*, 2004; PITTAYAKHAJONWUT *et al.*, 2005).

Baru (*Dipteryx alata* Vog.), also known as tonka beans (SANO *et al.*, 2004), is a native specie from Brazilian Cerrado with highly energetic and nutritive pulp and seeds, and rich in sugar and minerals, especially potassium, magnesium and calcium (CARVALHO, 1994). Biological activities have been demonstrated from baru nuts, such as antioxidant properties (SIQUEIRA *et al.*, 2009) and α-amylase inhibitors (BONAVIDES *et al.*, 2007). However, studies on the biotechnological potential of endophytic microorganisms isolated from baru have not been reported yet.

Compounds with biological activities can be obtained by either isolation from microbial extracts or biotransformation process, and be also labeled as natural (DE CARVALHO; DA FONSECA, 2006). 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). The biotransformation of terpenes, specially the α-pinene ($C_{10}H_{16}$), (SCHRADER; BERGER, 2001; YOO *et al.*, 2001; YOO; DAY, 2002; BICAS *et al.*, 2009) has been extensively employed for the production of volatile compounds with great industrial interest, since it allows the production of enantiomerically pure flavors and fragrances under mild reaction conditions (KRINGS *et al.*, 2006; BICAS *et al.*, 2008; BICAS *et al.*, 2009). The use of endophytes as biological systems to promote biotransformation reactions have received great attention in the last few years since they are able to modify chemical structures and produce novel metabolites of interest. Nevertheless studies on the potential of endophytes for biotransformation procedures are still waiting to be explored specially on biotransformation of terpenes.

The aim of the present work was to investigate the biotechnological potential of endophytes isolated from baru (*Dipteryx alata* Vog.). Accordingly, the antimicrobial activity, the enzymatic profile and the biotransformation of α-pinene were evaluated. This is the first report of isolation and investigation of endophytes from baru regarding their biotechnological applications, and is an important preliminary study that could help further researches on the identification of novel compounds with promising activity applicable in different fields of science.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemical standard used in this study was (*1S*)-(-)-α-pinene (~98%, Aldrich, Milwaukee, WI, USA Aldrich). All the chemicals and solvents were of the best available commercial grade.

2.2. Isolation of endophytic microorganisms

The microorganisms used in this study were isolated from baru fruits (*Dipteryx alata* Vog.), a native species from Cerrado, Brazil. The surface of fruit samples was sterilized by using ethanol 70% for 30 seconds and then treated with sodium hypochloride (1.5%) for 4 minutes. Samples were exhaustively rinsed with sterile water, and then parts of baru fruits including the seeds, pulp and internal part of peel were evenly spaced in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 30 °C and monitored every day to check the growth of endophytic microorganisms' colonies from the fruit segments.

The strains were cultivated and conserved by periodic replications (once a week) on Yeast-Malt agar (YM: bacteriological peptone 0.5 %, glucose 1.0 %, malt extract 0.3 %, yeast extract 0.3 %, and agar 2.0 %).

2.3. Industrial Applications of Endophytic Microorganisms

2.3.1. Antimicrobial compounds

Endophytic cultures were grown in Petri dishes containing solid YM medium and, in the case of bacteria, one full loop of the culture (24 hours old) was transferred to a 50 mL Erlenmeyer flask containing 10 mL of liquid YM medium, which was placed on the shaker at 30 °C and 150 rpm for 8 days. The cultures were then centrifuged at 7.100 g for 10 min at 5 °C using centrifugal Allegra ™ X-22R (Beckman Coulter). To obtain the extract of the endophytic microorganism, the supernatant obtained was further filtered with membrane 0.22 μm (Millex ® - Millipore).

In the case of fungi, a piece of agar (~1 cm^2) with a pre-grown culture of the fungal strains (72 hours old) was transferred to a 250 mL Erlenmeyer 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. After 8 days of incubation at 30 °C and 150 rpm, the biomass was recovered by

vacuum filtration using a Buchner funnel with Whatman nº 1 and then filtered again with membrane 0.22 µm (Millex ® - Millipore) to obtain the fungal extract.

The pathogenic microorganisms *Escherichia coli* CAT 0547, *Pseudomonas aeruginosa* ATCC 13388, *Staphylococcus aureus* CTC 2740, *Salmonella choleraesuis* CCT 4296 and *Candida albicans* ATCC 10231, obtained from the Culture Collection of the Laboratory of Microbiology CPQBA (Unicamp, Brazil), were inoculated in Petri dishes containing solid BHI (Brain Heart Infusion) medium and incubated at 30 °C. After 24 hours, one full loop of each organism was transferred to 50 mL Erlenmeyer flasks containing 10 mL of liquid BHI medium, and then was placed on the shaker at 30 °C and 150 rpm. After 24 hours, the absorbance of pathogenic cultures was adjusted with pure liquid BHI between 0.080 and 0.100 (625 nm). Then, 100 µL of each microorganism was spread in Petri dishes containing solid BHI, using a sterile Drigalsky spatula.

Thereafter, three filter paper discs (5 mm diameter) were placed on each BHI Petri dish previously inoculated with pathogenic microorganisms. It was deposited 10 µL of the extracts of endophytic cultures on each disc. After incubation at 30 ºC for 24 hours, the presence of inhibition zones around the discs was analyzed (adapted from Rabanal *et al.*, 2002 and Karaman *et al.*, 2003). Chlorine was assayed as positive antimicrobial reference, and dimethyl sulfoxide (DMSO) as negative control.

2.3.2. Screening of extracellular enzymes

The isolated strains were tested for their ability to produce extracellular enzymes that degrade culture medium containing starch, protein and olive oil, for a screening of enzymes amylase, protease and lipase production, respectively.

The production of all enzymes was determined in solid cultivation media and by an estimate based on the intensity of color, or on the diameter of halos formed.

2.3.2.1. Amylase

The endophytic microorganisms were inoculated on Petri dishes containing YM medium and incubated at 30 °C for 24 hours. The production of amylase was determined using agar starch medium with 0.5 % soluble starch (BASTOS, 2005). After 24 hours at 30 °C for bacteria and 48 hours for fungi, 10 mL of iodine solution (30 % iodine) was applied in each plate, and the halos around the colonies were measured. All assays were performed in triplicate.

2.3.2.2. Lipase

The medium contained the following per liter: peptone, 3.0 $g.L^{-1}$; K_2HPO_4, 2 $g.L^{-1}$; $MgSO_4$, 1 $g.L^{-1}$, rhodamine B, 0.01 $g.L^{-1}$; yeast extract, 2 $g.L^{-1}$; agar, 18 $g.L^{-1}$ and 20 $g.L^{-1}$ olive oil (LIN *et al.*, 1995). Inoculated plates were incubated for 72 hours at 30 °C for bacteria and 48 hours for fungal strains, in order to obtain colonial growth. The presence of orange fluorescent halos around colonies under UV rays observation indicated the presence of positive lipase-production. All assays were performed in triplicate.

2.3.2.3. Protease

The endophytic microorganisms were inoculated on Petri dishes containing YM medium and incubated at 30 °C for 24 hours. Protease activity were determined in a culture medium containing triptone, 46.43 $g.L^{-1}$; yeast extract, 2.79 $g.L^{-1}$; skim milk powder, 23.21 $g.L^{-1}$ and agar, 18.57 $g.L^{-1}$ (adapted from TANG *et al.*, 2008). After 72 hours at 30 °C for bacteria and 48 hours for fungi, the halos around the colonies were measured. All assays were performed in triplicate.

2.3.3. Biotransformation procedure

2.3.3.1. Inoculum for the biotransformation assays

Fungal strains

A piece of agar (~1 cm^2) with a pre-grown culture of the fungal strains (72 hours old) was transferred to a 250 mL Erlenmeyer 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. After 72 hours incubation at 30 °C and 150 rpm, the biomass was recovered by vacuum filtration using a Buchner funnel with Whatman n° 1 filter paper (BICAS *et al.*, 2008).

Bacterial strains

The microorganisms isolated from baru were used in the biotransformation process. A 24 hours culture grown on agar in a Petri dish was transferred to 250 mL Erlenmeyer flasks each containing 50 mL of YM medium. After 24 hours incubation at 30 ºC/150 rpm, the cultures were centrifuged at 2600 g/5 ºC during 10 min.

2.3.3.2. Biotransformation procedure

Fungal strains

The biomass obtained was distributed to a 250 mL Erlenmeyer flask filled with 50 mL of mineral medium (MM). Subsequently, 0.5 % (v/v) α-pinene was added. The flasks were incubated at 30 °C and 150 rpm and at fixed intervals (0, 24, 48, 72 and 96 hours after the addition of the terpene) 1 mL samples were extracted with the same volume of ethyl acetate. Chemical blanks (without biomass) were performed to ensure the absence of chemical and spontaneous transformations. Samples were analyzed directly by gas chromatography equipped with flame ionization detector (GC/FID), using decane as internal standard.

Bacterial strains

The whole cell mass was resuspended with 20 mL of sterile mineral medium and transferred to a 100 mL glass bottle with screw cap. It was added 0.5 % (v/v) of α-pinene and the inoculum was incubated at 30 °C and 150 rpm. 1 mL samples were extracted with the same volume of ethyl acetate at 0, 24, 48, 72 and 96 hours for analysis by gas chromatography (GC) of the possible products formed.

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

GC analysis of the biotransformation products were performed using a HP 6890 gas chromatograph (Agilent Technologies, USA) equipped with a split/splitless injector, FID detector and a fused silica capillary column. The stationary phase was DB-5 (Supelco) capillary column (20 m lenght; 0.100 mm i.d.; coating thickness of 0.10 μm). Working conditions were: injector 250 °C, detector 250 °C and helium was the carrier gas. Oven temperature was: initial temperature of 80 ºC for 2 min, rising at 20 °C.min^{-1} until 220 ºC, then held for 6 min.

GC/MS analyses were carried out in a GC-MS system, with a HP 7890 gas chromatograph coupled with a HP 5975 mass selective detector, under the following instrumental conditions: column: HP-5 MS fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm) from J&W Scientific (EUA), injector: split 1:10, temperature: 250 °C; helium flow rate: 0,8 mL.min^{-1}, oven: 80 °C for 3 min and 20 °C.min^{-1} until 200 °C, for 4 min; transfer line temperature: 250 °C, energy of impact: +70 eV and mass range of 35-350 m/z. The tentative identification of components was made by

comparing spectra with NIST 2005 mass spectral database libraries, with similarities higher than 85 % and supported by retention index data.

3. RESULTS AND DISCUSSION

3.1. Isolation of microorganisms and evaluation of antimicrobial compounds

In the continuous search for new products with pharmaceutical and agricultural applications for industries, natural selection has been considered superior to combinatorial chemistry regarding the discovery of novel substances with some potential for development of new industrial products (SCHULZ *et al*., 2002). Added to the attempt to show the wealth of Brazilian chemical and biological diversity, the bioprospection of endophytes isolated from baru (*Dipteryx alata* Vog.) was performed to find biologically active compounds that may result in promising lead applications. The baru is known to have some medicinal importance, for instance the peel of baru trunk is used to cure backache and the baru oil is used as anti-rheumatic and menstrual cycle regulator (DRUMMOND, 2008; SILVA *et al.*, 2001). Natural compounds such as triterpenes (lupeol, lupen-3 and betuline) isolated from the peel of trunk (DE ALMEIDA *et al.*, 1998; SANO *et al.*, 2004) and the betafarnese from the baru fruit (MATOS *et al.*, 1988) have been reported.

Considering the medicinal potential of baru, the investigation of biological properties of endophytic microorganisms isolated from this fruit has received our attention. Accordingly, a microbial screening was realized to provide a wide-spectrum of these endophytes, which were evaluated regarding their potential to produce antimicrobial compounds and enzymes and application in biotransformation assays.

The microbial strains were isolated and agreed in two principal classes (bacteria and filamentous fungi), after morphological analysis in optical microscope. The identification of bacteria strains was carried out based on the morphology of the colony and their characteristics after Gram coloration. Among 27 microbial strains, 16 bacteria and 11 filamentous fungus were identified in the present work. The majority of the bacteria isolated were Gram negative cocci (6 strains), followed by Gram positive cocci (4 strains), Gram negative bacilli (4 strains) and finally a lower number of Gram positive bacilli (2 strains).

Initially, to determine the spectrum of antimicrobial activity of the endophytic microorganisms, the disk diffusion method was used. This test is accepted by the FDA (Food and Drug Administration) and it is established as standard by NCCLS (National Committee for Clinical Laboratory Standards) (BARRY; THORNSBERRY, 1991). The diameters of the inhibition zones obtained with the extracts are presented in Table 1.

Table 1 - Antimicrobial results for the baru endophytes that inhibited the pathogenic microorganisms tested. The value is equal to the average of the 3 halos diameter in mm, without discounting the disc diameter (5 mm).

Strain	***C. albicans***	***E. coli***	***P. aeruginosa***	***S.choleraesuis***	***S. aureus***
LBBR01	8.0	5.0	13.3	9.0	7.0
LBBR02	9.7	0.0	9.0	7.0	10.0
LBBR03	10.3	0.0	11.0	10.7	12.7
LBBR06	0.0	9.3	0.0	0.0	0.0
LBBR07	8.0	8.0	0.0	0.0	8.0
LBBR08	5.3	6.7	7.3	0.0	7.0
LBBR12	9.7	0.0	10.3	0.0	11.7
LBBR14	9.3	0.0	10.7	10.7	11.7
LBBR15	10.3	5.0	8.7	8.0	7.7
LBBR16	0.0	0.0	0.0	8.3	7.0
LBBR17	8.7	5.5	7.7	7.7	8.7
LBBR18	10.0	0.0	9.3	7.7	8.3
LBBR20	0.0	6.3	0.0	0.0	0.0
LBBR21	7.5	7.0	0.0	6.5	0.0
LBBR23	7.0	7.3	6.5	0.0	6.3
LBBR25	7.0	0.0	0.0	0.0	0.0
DMSO	0.0	0.0	0.0	0.0	0.0
Chlorine	20.0	20.0	20.0	20.0	20.0

Screening of the antimicrobial activity of the extracts of endophytics revealed a considerable activity against bacteria and yeasts tested. Most of the extracts inhibited the growth of the *Candida albicans* and *Sthaphylococcus aureus*. The most significant activity was observed for the LBBR1, LBBR15 and LBBR17 which inhibited the growth of all the pathogenic tested. Identification of the secondary metabolites and studies involving other solvents to extraction and the MIC (minimal inhibitory concentration) should be performed in future studies.

Teles and co-workers (2006) studied the aromatic compounds produced by *Periconia atropurpurea*, an endophytic fungus associated with *Xylopia aromatica.* The biological analyses demonstrated that several compounds tested presented a potent cytotoxic activity against some cell lines, and one of these compounds showed a potent antifungal activity when was used a phytopathogenic fungi *Cladosporium sphaerospermum* and *C. cladosporioides*. In another study, the same research group evaluated the endophytic *Curvularia* sp., an isolate from the leaves of *Ocotea corymbosa*, a native plant of the Brazilian Cerrado. The biological evaluation of all the benzopyran compounds isolated were performed using antifungal activity against the same microorganisms cited before. Only two benzopyrans showed weak antifungal activity and, when the compounds were tested using *in vitro* antitumoral tests, one compound demonstrated a promising result (TELES *et al.*, 2005). Finally, five cadinane sesquiterpenes derivatives were isolated by bioassay-guided fractionation from *Phomopis cassiae*, an endophytic fungus isolated from *Cassia spectabilis*. One compound presented a potential antifungal activity of the isolates, evaluated against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides*. The activity against *Candida albicans* was reported in isolated compounds isolated from endophytes species (SILVA *et al*., 2006).

Studies evaluating the inhibition of the extracts of endophytes against bacteria and virus were reported by Wiyakrutta and co-workers (2004). From Thai medicinal plant species were obtained endophytes that could inhibit *Mycobacterium tuberculosis* and *Plasmodium falciparum*. Strong anti-viral activity against Herpes simplex virus type 1 was observed in some isolates. Compounds isolated by Pittayakhajonwut and co-workers (2005) demonstrated a beneficial effect against the same virus.

The results of the antimicrobial assays represent a promising alternative of the potential use of these microorganisms in biotechnological processes. In addition, an evaluation to their ability to produce some enzymes was investigated, considering that the endophytes usually produce the enzymes necessary for the colonization of plant tissues. The importance of the microorganisms in enzyme production is due its high production capability, low cost and susceptibility to genetic manipulation. The endophytic microorganisms occupy a relatively unexplored site in microorganism isolation so they can represent a new source in obtaining more enzymes with different potentialities (CARRIM *et al.*, 2006).

3.2. Screening of extracellular enzymes

Among 26 strains isolated from Baru, 81% displayed amylase activity, 93% displayed lipase activity and 63% displayed protease activity. Table 2 and 3 comprise the results of Enzymatic Index (EI) of the isolates tested.

Table 2. Enzymatic Index of fungal strains from baru.

	Halo diameter (mm)		
Fungal Strains	**Amylase**	**Lipase**	**Protease**
LBBR01	22.0	20.0	0.0
LBBR02	27.0	25.0	0.0
LBBR03	24.0	24.0	0.0
LBBR04	20.0	19.0	0.0
LBBR05	8.0	10.0	0.0
LBBR12	2.0	4.0	0.0
LBBR13	0.0	4.0	0.0
LBBR14	0.0	6.0	0.0
LBBR27	0.0	0.0	0.0

Table 3. Enzymatic Index of bacterial strains from baru.

	Halo diameter (mm)		
Bacterial Strains	**Amylase**	**Lipase**	**Protease**
LBBR06	6.0	9.0	12.0
LBBR07	9.0	38.0	11.0
LBBR08	8.0	14.0	9.0
LBBR09	4.0	15.0	24.0
LBBR10	4.0	12.0	18.0
LBBR11	10.0	10.0	16.0
LBBR15	7.0	13.0	15.0
LBBR16	11.0	16.0	13.0
LBBR17	8.0	11.0	15.0
LBBR18	8.0	13.0	19.0
LBBR19	6.0	25.0	13.0
LBBR20	3.0	22.0	17.0
LBBR21	6.0	17.0	13.0
LBBR23	9.0	10.0	19.0
LBBR24	7.0	11.0	23.0
LBBR25	7.0	8.0	24.0
LBBR26	0.0	18.0	24.0

Figure 1 show an example of negative (1-A) and positive results, as observed by microorganisms LBBR1 and LBBR2, that were able to grow on the medium and degrade the substrate.

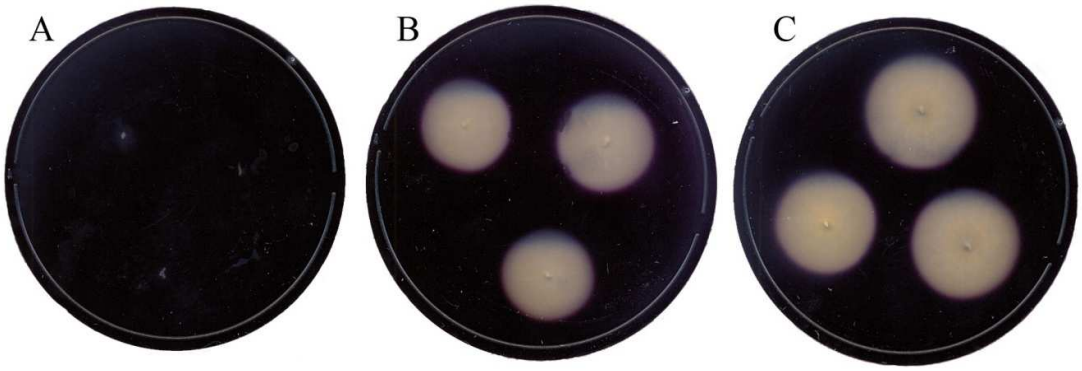

Figure 1 – Strain LBBR13 (A) as negative result and strains LBBR1 (B) and LBBR2 (C) showed amylase activity.

Furthermore, it was observed that the vast majority of bacteria showed a high level protease activity, and a small difference was observed between their behavior. However, Figure 2-A represents an example of negative result, observed by the strain LBBR27. The production of protease enzymes was detected for all bacteria isolates (Table 3) and can be assigned as a characteristic of the species, markedly different to the behavior of fungi, where all results collected were negative (Table 2).

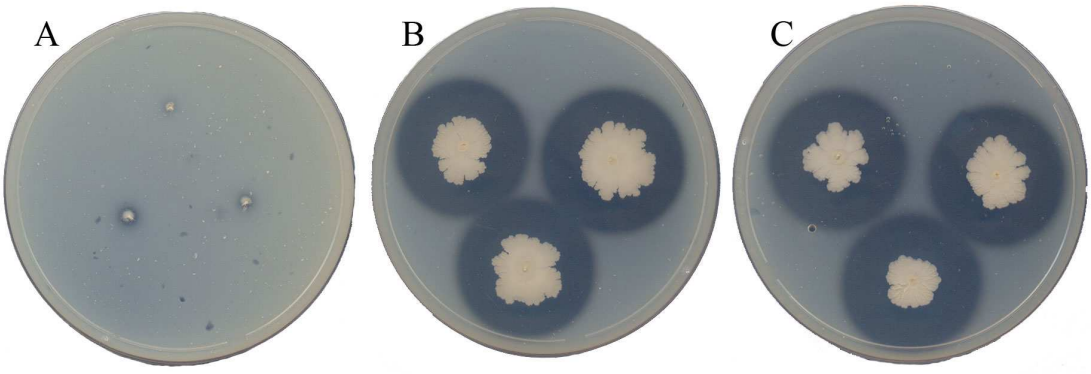

Figure 2 – Strain LBBR27 (A) as negative result and strains LBBR19 (B) and LBBR21 (C) showed protease activity.

The lipase activity although at varying levels, it was detected in most all isolates and showed the largest halos diameters collected in the present research.

The results obtained suggest that the microorganisms have the ability to use starch, proteins and lipids as energy sources. Also, it is assumed that during the incubation period, bacteria and fungi tested released enzymes (amylases, proteases and oxidases) that actively degraded components present in the culture medium. Comparative analysis of the production of extracellular enzymes detected variability among isolates of Baru, which can be very useful information to the identification of isolates.

To date references containing the screening of enzymes from endophytic microorganisms in petri dishes are still scarce. Some authors focuses on the mensurement of ligninolytic enzyme production, as realized by Urairuj and co-workers (2003), whose isolated endophytic *Xylariaceae* strains from healthy tropical native plants of northern Thailand. An activity of 195 U/L was achieved after cultivation at the optimum condition for 6 days and the enzyme activity was enhanced to 292 U/L when the media was supplemented with veratryl alcohol. In another study, authors made an evaluation of fungal endophytes for lignocellulolytic enzyme production, and concluded that in agar solid media the *Basidiomycetes* displayed positive reaction to phenoloxidase (PO) and cellulose (OSES *et al*., 2006).

Many endophytic fungi and bacteria strains isolated from endemic plants, collected in Hokkaido (Japan) produced useful extracellular enzymes that degrade xylan and mannan, common constituents of plant cells, into xylooligosaccharides and manno-oligosaccharides (TOMITA, 2003).

Enzymes have applications in many fields, including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. The application of enzymes to organic synthesis is currently attracting more and more attention. The discovery of new microbial enzymes through extensive and persistent screening will open new, simple routes for synthetic processes and, consequently, new ways to solve environmental problems.

This clearly evidences that the screening of endophytic enzymes is of great importance, according to the proposed purpose for specific application. It also should be highlighted the importance of the enzymatic activity measurement, and this stage of the research are under way with the microorganisms that exhibited positive preliminary results.

3.3. Biotransformation procedure

Finally, the endophytes were evaluated in biotransformation procedure using α-pinene as substrate. One of the most studied terpene in biotransformation process is the α-pinene ($C_{10}H_{16}$), a hydrophobic organic volatile compound emitted from the forest products industry (*e.g.*, wood products, pulp and paper industries). Because of their economic advantage, pinenes represent an ideal substrate for biotechnological processes and have been extensively employed in microbial conversion experiments (SCHRADER; BERGER, 2001; YOO *et al.*, 2001; YOO; DAY, 2002; BICAS *et al.*, 2008).

The biotechnological conversion of pinenes, abundant constituents of essential oils and versatile starters for the chemical conversion to flavours and fragrances (SCHRADER; BERGER, 2001), is not only hampered by the above mentioned substrate cytotoxicity, but also by low substrate solubility and by product degradation; however, many laboratory-scale attempts were published (SHUKLA *et al.*, 1968, GIBBON *et al.*, 1972, DHAVLIKAR *et al.*, 1974; NARUSHIMA *et al.*, 1982).

In our study, all the strains isolated from Baru were tested for their capacity to transform α-pinene, included bacteria and filamentous fungi. Interestingly, this appears to be one of the first works where a fruit of the Cerrado region is evaluated as the source of endophytic microorganisms for use in biotransformation processes. The preliminary results showed that the fungal strains LBBR01, LBBR02, LBBR04, LBBR05, LBBR14 and the gram negative cocci LBBR09 bioconverted the α-pinene into verbenol (85% similarity in MS results), after 24 hours of contact with the terpene. The bioconvertion occurred based on the biochemical reaction of hydroxylation of α-pinene (see Figure 1), and this reaction was reported in some articles.

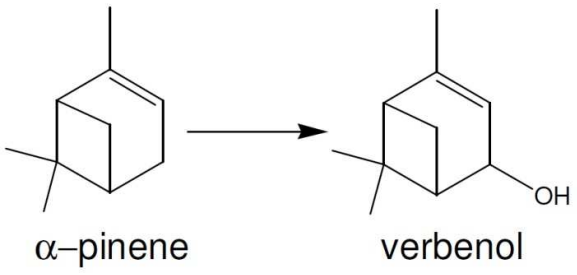

Figure 3 – Biochemical reaction of hydroxylation of α-pinene to verbenol.

Maróstica and co-workers (2007) showed the biotransformation of α-pinene from the turpentine oil, using fungal strains, and a microextraction in solid phase was used for extraction of the aroma compounds. The production of 50 mg/L of verbenol by *Mucor* sp. 2276 and 70 mg/L of verbenone form α- and β-pinenes was obtained. The production of verbenol/verbenone from α-pinene by *Aspergillus* sp. and *Penicillium* sp. strains was also reported by Agrawal, Deepika & Joseph (1999).

On the other hand, the potential to biotransform α-pinene using a yeast strain of *Hormonema* sp. was reported and *trans*-verbenol and verbenone have been produced in concentrations of 0.4 and 0.3 $g.L^{-1}$ after 96 hours (Van Dyk, van Rensburg & Moleleki 1998). Lindmark-Henriksson (2003) studied the biotransformation of α-pinene using cells of *P. abies* and the majoritary compounds produced were *trans*-pinocarveol, *cis*-verbenol, mirtenol and α-terpineol.

Tests are being conducted to identify the products of biotransformation of α-pinene. Further studies should be performed to quantify the products obtained and optimize the process parameters to increase the production of these compounds.

4. CONCLUSIONS AND FUTURE PROSPECTS

The use of endophytic microorganisms isolated in the Cerrado Brazilian biome demonstrates a partial use of these microorganisms in biotechnological processes. Thus, this paper represents the first of many works that are being developed in the laboratory of Bioaromas (FEA/Unicamp-Brazil) and showed a screening of the potential uses of these microorganisms. Future works should aim to identify such microorganisms, quantificate the enzyme activity and apply other terpenes as substrate in the biotransformation assays.

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CONCLUSÃO GERAL

Esta dissertação apresentou os resultados atingidos empregando processos biotecnológicos para a obtenção de bioaromas, especialmente dos derivados da biotransformação de monoterpenos, visando a prospecção de processos de interesse industrial. A literatura científica comprova o elevado interesse industrial por compostos de aroma naturais, especialmente aqueles obtidos por micro-organismos. No entanto, a seleção de linhagens com elevada produção tem se mostrado essencial para o desenvolvimento de processos economicamente viáveis. Nesse estudo, a utilização de 70 linhagens provenientes de fontes cítricas em ensaios de biotransformação mostrou que a grande maioria foi incapaz de acumular metabólitos de interesse, o que pode ser devido à completa utilização do substrato para crescimento e produção de energia. Entretanto, 5 micro-organismos foram capazes de biotransformar o limoneno em compostos de aroma, como α-terpineol, carveol e carvona. Apesar das concentrações não serem expressivas para uma possível otimização e aplicação industrial, destaca-se a importância deste estudo na tentativa de propror métodos biotecnológicos para a produção de aromas naturais. Além disso, o trabalho contemplou a produção de biomassa da linhagem LB285JLB utilizando glicerol como fonte de carbono e energia, a fim de utilizar fontes acessíveis para baratear o processo geral de desenvolvimento de biomassa e de biotransformação. Por fim, avaliou-se o potencial de micro-organismos endofíticos isolados do Baru (*Dipteryx alata* Vog.). A prospecção destas linhagens quanto o potencial de biotransformação de α-pineno, produção de enzimas e potencial antimicrobiano frente a micro-organismos patogênicos abre precedentes para que novas pesquisas sejam consideradas a fim de determinar o potencial deste nicho de micro-organismos.

SUGESTÕES PARA TRABALHOS FUTUROS

Considerando-se os resultados aqui apresentados sugere-se:

- Realizar a otimização da produção de α-terpineol pela linhagem LB285JLB, considerando as condições de cultivo e composição do meio;
- Quantificar os derivados obtidos a partir da biotransformação de α-pineno;
- Quantificar a atividade enzimática dos micro-organismos endofíticos;
- Utilizar novos substratos terpênicos para os ensaios de biotransformação;
- Utilização de resíduos industriais como fonte de terpenos para os ensaios de biotransformação;
- Aprofundar os estudos na atividade antimicrobiana e antitumoral dos compostos de aromas derivados de terpenos, assim como avaliar sua capacidade antioxidante.