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PRODUÇÃO E ESTUDO BIOLÓGICO DE COMPOSTOS DE AROMAS OBTIDOS A PARTIR DA BIOTRANSFORMAÇÃO DE CAROTENÓIDES

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RESUMO GERAL

A biotransformação de carotenóides é um campo de crescente interesse para a indústria de aromas, uma vez que podem ser obtidos compostos considerados como "naturais", de grande aplicabilidade industrial e que podem apresentar, em muitos casos, propriedades biológicas interessantes. Desta forma, o objetivo do presente trabalho foi efetuar um estudo de biotransformação de carotenóides seguida de uma otimização do processo, avaliar o uso de sub-produtos industriais para barateamento do processo, realizar a biotransformação de outros substratos terpênicos para formação de compostos voláteis e por fim, estudar as propriedades biológicas dos produtos voláteis derivados de carotenóides. O **capítulo 1** apresenta uma revisão bilbiográfica sobre bio-oxidação de terpenos, focando especialmente no uso monoterpenos e β-caroteno como substrato. O **capítulo 2** trata do isolamento de micro-organismos a partir de fontes naturais ricas em β-caroteno. Das mais de 400 linhagens avaliadas, cerca de 60 apresentaram um potencial para biotransformação de carotenóides, evidenciado por um halo de degradação característico formado ao redor da colônia inoculada em uma placa contendo β-caroteno. Em seguida, a técnica de microextração em fase sólida (SPME) foi usada para extração e concentração dos compostos voláteis formados na biotransformação pelas linhagens selecionadas, e os compostos foram identificados por CG-DIC e CG-EM. O estudo das variáveis envolvidas no processo de biotransformação de β-caroteno pela linhagem LB398APD é reportada no **Capítulo 3**. Para isso, um Placket-Burman (PB16) foi realizado inicialmente para seleção de variáveis, e seguindo o processo de planejamento experimental, um fatorial 2^2 com os parâmetros significativos do PB foi realizado. Em posse dos resultados da otimização, o

Capítulo 4 retrata o uso de meios alternativos para o desenvolvimento de biomassa pela linhagem LB398APD. O micro-organismo possuiu um desenvolvimento em meio manipueira superior ao meio convencional, com condições ótimas do sistema alcançadas usando 60 % de manipueira, 30 °C e 300 rpm (produção de biomassa em 24h = 4,6g.L^{-1}). Entretanto, quando glicerol foi usado como fonte de carbono, o desenvolvimento de biomassa não foi obtido com sucesso. Além dos ensaios usando β-caroteno como substrato, outros terpenos também foram avaliados com algumas linhagens fúngicas isoladas, e essa biotransformação é reportada no **Capítulo 5**. Carvona e carveol foram obtidos quando o limoneno foi usado como única fonte de carbono, e o uso de *Paecilomyces* sp. parece ser a primeira vez onde esse micro-organismo é reportado na literatura para ensaios e biotransformação. Por fim, o **Capítulo 6** trata de alguns estudos "in vitro" com compostos de aromas derivados de carotenóides. Os resultados alcançados geram a possibilidade para esses compostos seres utilizados industrialmente como "aromas funcionais", pois apresentaram resultados extremamente interessantes quando esses terpenos foram usados em 12 células tumorais, representando resultados muitas vezes superiores ao controle positivo (doxorrubicina).

ABSTRACT

The biotransformation of carotenoids is a field of growing interest to the flavor industry, once the compounds obtained can be labeled as "natural", with high industrial applicability and that may present, in many cases, interesting biological properties. Chapter 1 reviews a variety of techniques developed till the present days for the oxidation of terpenes, especially monoterpenes and β-carotene, to volatile compounds. In sequence, Chapter 2 deals with the screening of microorganisms from natural sources rich in β-carotene. Among more than 400 strains evaluated, 60 exhibited a potential for biotransformation of carotenoids, as evidenced by a characteristic degradation halo formed around the colonies inoculated on a plate containing β-carotene. Afterwards, the solid phase microextraction technique (SPME) was used to extrain and concontrate the aroma compounds produced by the selected strains, and the analysis were conduced by GC-FID and GC-MS. The study of the variables involved in the biotransformation of β-carotene by the LB398APD strain is reported in Chapter 3. For this, a Placket-Burman (PB16) was initially performed for the screening of the variables, and following the experimental design process, a 2^2 factor with the significant parameters selected in the PB was performed. In sequence, Chapter 4 shows the use of alternative culture media for the biomass development by the LB398APD strain. The microorganism demonstrated a development in the cassava wastewater media higher than in the conventional media, and the optimum system conditions were achieved by using 60 % of cassava wastewater, 30 °C and 300 rpm (biomass production in 24 hours = 4.6 gL^{-1}). In addition to the tests using β-carotene as substrate, other terpenes were also tested with some fungal strains, and this biotransformation is reported in Chapter 5. Carvone and carveol were obtained when limonene was used as the sole carbon source. The use

of *Paecilomyces* sp. seems to be the first report in literature for biotransformation assays. Finally, Chapter 6 deals with some in vitro studies with carotenoids derived aroma compounds. The promising results create the possibility for such compounds to be used as "functional flavors".

INTRODUÇÃO GERAL

TERPENOS

Hidrocarbonetos terpênicos e seus derivados oxifuncionalizados (terpenóides) representam uma vasta e diversa classe de compostos orgânicos derivados do isopreno (C_5H_{10}). São classificados quanto ao número de carbonos como hemiterpenos (cinco carbonos), monoterpenos (dez carbonos), sesquiterpenos (quinze carbonos), diterpenos (vinte carbonos), triterpenos (trinta carbonos) e tetraterpenos ou carotenos (quarenta carbonos) (Teisseire, 1994).

Os terpenos são metabólitos secundários de plantas, produzidos, em parte, para defesa contra micro-organismos e insetos. Devido a suas propriedades organolépticas diferenciadas, certos monoterpenos são utilizados em fragrâncias e como ingredientes em alimentos. Os terpenos e terpenóides mais simples (mono e sesquiterpenos/terpenóides) são amplamente utilizados na indústria de aromas e são os principais constituintes dos óleos essenciais (Bicas; Dionísio; Pastore, 2009; Bicas *et al.*, 2010). Di e triterpenos são menos voláteis e podem ser obtidos a partir de gomas e resinas vegetais (Trudgill, 1986). Os carotenos (tetraterpenos), por sua vez, são corantes naturais responsáveis pelas cores amarelas, laranja e vermelho de certos alimentos e flores, e apresentam um amplo espectro de uso nas indústrias alimentícia, farmacêutica, de cosméticos e ração (Valduga *et al*., 2009).

Carotenóides

Carotenóides são tetraterpenóides de 40 carbonos unidos por unidades opostas no centro da molécula (Fraser; Bramley, 2004; Rodriguez-Amaya, 1999). Ciclização,

hidrogenação, desidrogenação, migração de duplas ligações, encurtamento ou alongamento da cadeia, rearranjo, isomerização, introdução de funções com oxigênio ou a combinação destes processos resultam na diversidade de estruturas dos carotenóides (Rodriguez-Amaya, 1999). Pode-se distinguir entre hidrocarboneto carotenóides, chamados carotenos, composto somente de carbono e hidrogênio (por exemplo, o β-caroteno) e os carotenóides oxidados, denominados xantofilas, que apresentam grupos substituintes com oxigênio, como hidroxilas, grupo ceto e epóxi, como por exemplo a luteína.

Os carotenóides são pigmentos naturais com atividade biológica importante, destacando-se a inibição de doenças onde os radicais livres apresentam papel fundamental, como arteriosclerose, catarata, degeneração macular, esclerose múltipla, câncer, doenças degenerativas e cardiovasculares (Valduga *et al*., 2009). Comercialmente, são usados como corantes alimentícios e em suplementos nutricionais, principalmente pela sua ação pró-vitamínica A (Fraser; Bramley, 2004). Além das propriedades biológicas e de coloração destes compostos, os carotenóides apresentam destaque como precursores de compostos voláteis responsáveis pelo aroma de alguns alimentos e fragrâncias de algumas flores (Sanchez-Contreras *et al.*, 2000).

Há décadas, muitos compostos de aromas derivados de carotenóides têm atraído a atenção de químicos e aromistas, o que resultou no isolamento e elucidação da estrutura de uma extensa variedade de compostos de aroma derivados de carotenóides extraídos de plantas (Enzell, 1985; Winterhalter; Rouseff, 2002). Além de suas propriedades relativas a aromas e fragrâncias, alguns terpenos vêm sendo reconhecidos por suas propriedades funcionais, e diversos trabalhos relatam as propriedades preventivas de alguns monoterpenos, como álcool perílico, carvona e limoneno, contra

diversos tipos de câncer. Porém, poucas investigações biológicas revelam todas as potencialidades dos derivados dos carotenóides.

OBTENÇÃO DE COMPOSTOS DE AROMAS

Aroma é uma dos mais importantes atributos dos alimentos e bebidas, e uma vasta gama de compostos podem ser responsáveis pelos aromas destes produtos, como álcoois, aldeídos, ésteres, ácidos graxos livres de cadeia curta, metil cetonas, lactonas, compostos fenólicos e compostos de enxofre (Longo; Sanróman, 2006).

Atualmente, os compostos de aroma são obtidos por dois processos principais: extração de fontes naturais e síntese química. As sínteses químicas geralmente criam altos impactos ambientais por emitirem certa carga de resíduos não-biodegradáveis. Aromas extraídos diretamente de plantas estão sujeitos a instabilidades advindas da sazonalidade, dos efeitos geográficos e de eventual ataque de pragas. Essas desvantagens fazem com que os processos biotecnológicos sejam uma alternativa bastante atrativa (Pinheiro; Pastore, 2003) pelo fato de que, em geral, geram baixa carga de resíduos, utilizam condições brandas de processo e estão menos sujeitos a variações sazonais, oferecendo algumas vantagens sobre alguns dos processos existentes para a produção de compostos de aroma (Janssens *et al*., 1992). Além disso, os compostos gerados por *via* biotecnológica são denominados naturais sendo, portanto, mais valorizados no mercado que os aditivos químicos artificiais (Marques; Pastore, 1999).

A produção biotecnológica de compostos de aromas derivados de carotenóides podem ser provenientes de três métodos distintos: uso de enzimas, de micro-organismos e uso de tecidos vegetais ou culturas de células. Com relação ao uso de micro-organismos, estudos são recentes e poucos trabalhos podem ser encontrados.

Desta forma, os principais objetivos da Tese são:

- Isolamento e seleção de micro-organismos potencialmente biotransformadores de carotenóides em produtos de interesse industrial/farmacêutico;

- Otimização da produção de compostos de aromas derivados de carotenóides;

- Estudo da biotransformação de outros terpenos por algumas linhagens fúngicas isoladas para biotransformação de carotenóides;

- Verificação da potencialidade do uso de alguns resíduos agroindustriais para o desenvolvimento de biomassa de micro-organismo, utilizada em ensaios de biotransformação;

- Estudo de algumas atividades biológicas *in vitro* de compostos de aromas derivados de carotenóides.

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CAPÍTULO 1

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

BIOTECHNOLOGICAL PRODUCTION OF BIOFLAVORS BY BIOTRANSFORMATION OF TERPENES

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SUMMARY

This paper reviews a variety of techniques developed till the present days for the oxidation of terpenes, especially monoterpenes and β-carotene, to volatile compounds. Particular attention is given to biotransformation processes since, in comparison with traditional chemical methods, they proceed under mild conditions, have an elevated regio and enantioselectivety, do not generate toxic wastes and the products obtained can be labeled as "natural". Some of the bioflavors produced using this technique have attracted great interest by the flavor and pharmaceutical industries, not only for their pleasant fragrances but also for their bioactivity against cancer cells.

Key-words: biotransformation, terpenes, flavor.

1. INTRODUCTION

The terpenes 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). In mammals, terpenes contribute to stabilizing cell membranes, participate in metabolic pathways and act as regulators in some enzymatic reactions (De Carvalho; Da Fonseca, 2006a). Members of this class of chemicals have carbon structures which can be decomposed into isoprene (C_5H_8) residues and are classified, based on the number of carbons in the molecule, as monoterpenoids (ten carbons), sesquiterpenoids (fifteen carbons), diterpenoids (twenty carbons), triterpenoids (thirty carbons) and tetraterpenoids or carotenoids (forty carbons) (Bicas *et al.*, 2010; Teisseire, 1994). The simpler terpenoids (mono and sesquiterpenes) are the major constituents of essential oils and are widely used in the perfumery industry, while di and triterpenoids are less volatile and are obtained from plant gums and resins (Trudgill, 1986). Carotenoids are synthesized by bacteria, algae, fungi and by green plants and comprise more than 600 known structures (Sandmann, 2001).

Terpenes are a good starting material for the synthesis of many fine chemicals due to their similar carbon skeleton. *R*-(+)-limonene, for example, is the most abundant monocyclic monoterpene in nature and it represents more than 90 % of the orange peel oil, thus it is an inexpensive precursor (Fenaroli, 1971; Bauer; Garbe; Surburg, 2001). The oxygenated derivatives of limonene *eg* carveol, carvone and menthol, are recognized for their pleasant fragrances (Ravindranath, 1983), and some of them also present bioactivity against certain types of tumor cells, not only preventing the formation or progression of

cancer, but also regressing existing malignant tumors (Crowell, 1999; Jun; Jeong; Ho, 2006). α- and β-pinene, in turn, are found in high concentrations in turpentine, a paper and pulp industry residue, and are therefore, also available in bulk at a low price. These bicyclic monoterpenes are precursors of important flavor compounds, such as terpineols, borneol, camphor, citronellol, geraniol, menthol, verbenol and verbenone (Fenaroli, 1971; Bauer; Garbe; Surburg, 2001). The tetraterpene β-carotene, an orange pigment found mainly in tropical vegetables, is a precursor of norisoprenoid ionones, molecules responsible for desirable fruity and floral flavors (Baldermann; Naim; Leichmann, 2005; Bauer; Garbe; Surburg, 2001). Volatile carotenoid breakdown products have been long known as important flavour compounds (Winterhalter; Rouseff, 2001).

Of the approximately 6,500 known flavors, only 300 are commonly used. At present 50-100 are produced by microbial fermentation, while the rest are mainly obtained by chemical synthesis (Scragg, 2007). The scientific literature contains many examples of reviews about the chemical reactions of terpenes to produce flavors (Swift, 2004), the biotransformation of terpenes (De Carvalho; Da Fonseca, 2006a; Trudgill, 1990; Van der Werf; de Bont; Leak, 1997) and natural flavor production via biocatalysis (Giri *et al.*, 2001; Hagedorn; Kaphammer, 1994; Janssens *et al.*, 1992; Lomascolo *et al.*, 1999; Schrader *et al.*, 2004; Serra; Fuganti; Brenna, 2005; Welsh; Murray; Williams, 1989). However, no reference was found of a paper that congregates all these subjects. This paper discusses the methods for terpene oxidation in the production of molecules that attract great interest by the flavor industry, especially the monoterpenoid and norisoprenoid natural flavor compounds produced *via* microbial biotransformation.

2. CHEMICAL TRANSFORMATIONS

One of the most extensively studied reactions involving olefins is nitrosochlorination (Pisanchyn *et al.*, 1976). The first description of the transformation of terpenes using gaseous nitrosyl chloride was about 130 years ago (Tilden; Lond, 1975; Tilden; Shenstone, 1877). In the early 1950s, Royals and Horne Jr. (1951) applied the nitrosyl chloride method to produce *R*-(–)-carvone as the sole product from *R*-(+)-limonene, with an overall yield of 56-60 %. Years later, a similar procedure was followed for the preparation of carvone from orange oil (Rothenberger; Krasnoff; Rollins, 1980). Some other terpene nitrosochlorination processes and their variants have also been studied and patented (Derfer; Kane; Young, 1966; Magalhães; Koketsu; Wilberg, 1983; Mulder; Van Helden, 1979; Nakai; Harada, Nishimura, 1978; Reitsema, 1957).

Other widely investigated mechanisms for the allylic oxidation of olefins consist of the use of selenium dioxide (Trachtenberg; Carver, 1970; Schaefer; Horvath; Klein, 1968; Trahtenberg; Nelson; Carver, 1970; Wiberg; Nielsen, 1964), and many such terpene oxidation methods have been described in past years. In the case of limonene, the reaction carried out in ethanol leads to the formation of limonen-4-ol as the main product as well as limonen-10-ol and carveol in minor amounts (Sakuda, 1969; Thomas; Bucher, 1970), while limonene-1,2-diol was favored in the SeO_2-H_2O_2 system (Wilson; Shaw, 1973; Sumimoto; Suzuki; Kondo, 1974). Mirtenal may be prepared from α-pinene using SeO_2-V_2O_5 with relatively high yields (Li, 2000a; Zheng; Lu, 1995), or using SeO_2 in ethanol (Lin; Li; Deng, 1990), and the kinetics of this oxidation has been studied for the selenium dioxide-vanadium system (Li, 2000b). Selenium dioxide was also applied to induce the oxidation of

β-pinene (Coxon; Dansted; Hartshorn, 1970; Coxon; Dansted; Hartshorn, 1977; Joshel; Palkin, 1942; Stallcup; Hawkins, 1941; Stallcup; Hawkins, 1942), camphene (Hirsjarvi, 1956; Hirsjarvi; Hirsjarvi; Kaila, 1957; Hirsjarvi; Hirjarvi, 1965) and some sesquiterpenes (Sathe *et al.*, 1966). However, the possible formation of selenium and organoselenides in these kinds of reaction represent a problem to be considered, since selenium compounds are exceedingly toxic. This might be one of the reasons why such terpene oxidation methods are now in disuse.

The metal-catalyzed oxidation of terpenes has been extensively studied and might be an option for producing oxygenated derivatives. The palladium-catalyzed oxidation of limonene and α- and β-pinene occurs mainly at the allylic sites of the molecule, and the main products are generally carvyl derivatives (Gonçalves; Bueno; Gusevskaya, 2006; Gusevskaya; Golsalves, 1997; Gusevskaya; Robles-Dutenhefner; Ferreira, 1998; El Firdoussi *et al.*, 1998). In fact, the mechanism of such oxidations seems to be *via* the intermediate formation of π-allyl palladium complexes (Golçalves *et al.*, 2005).

Allal *et al.* (2003) evaluated the influence of the catalyst and the reaction conditions in the oxidation of α-pinene. The conclusion was that the system Cu/*t*-BuOOH/O2/70 °C promoted the formation of verbenone, while Pd/H_2O_2/70°C yielded verbenol. These systems were also studied in the oxidation of limonene, 3-carene and valencene, and the best results were obtained when using Cu/*t*-BuOOH/O_2, which, in the case of valencene, yielded nootkatone with 100 % conversion and 80 % selectivity. The oxidation of α-pinene catalyzed by other metal compounds, using H_2O_2 as the oxidizing agent has also been reported (Maksimchuk *et al.*, 2005, see also references cited).

The oxidation of monoterpenes using metal (Salen) complexes as catalysts has been widely described in recent years. These catalytic systems might be considered as cytochrome P450 analogs, since they involve oxometallic species (M=O) *via* a rebound mechanism such as the metalo-porphyrins (Gomes; Antunes, 1996a). When using relative catalyst concentrations of from 0.03-0.05 and iodosobenzene as the terminal oxidant, the conversion of limonene reached 50-60 %, and the selectivities observed for *cis*- and *trans*-l,2-epoxylimonene were 30 % and 16.7 %, respectively, 18.4 % for carvone and 10 % for the two diastereoisomers of 1-*p*-menthen-9-al (Gomes; Antunes, 1996b). Under the same conditions, a conversion of 50-60 % was observed for α-pinene, yielding, after 16h reaction, over 20 % of a mixture of the corresponding epoxides (55 % selectivities) and between 2 to 6 % of pinocamphone and myrtenol. For β-pinene the optimal conversion (55 %) was obtained after 4h reaction, with the maximum yield of myrtanal isomers and epoxide isomers varying from 6.5 to 23.2 % and 2 to 4 %, respectively (Gomes; Antunes, 1996a). In a recent study, Lima *et al.* (2006) evaluated the main reaction parameters that could affect the allylic oxidation or epoxidation of the metal(Salen)-catalyzed oxidation of limonene. The use of supercritical CO_2 instead of ordinary organic solvents was also investigated. In this system, the conversion was similar to that obtained in some organic solvents but the higher selectivities towards epoxide formation, as observed for organic solvents, only occurred after 4 h (Lima *et al.*, 2005).

Other cytochrome P450-biomimetic chemical systems (generally based on metaloporphyrins) capable of carrying out alkane hidroxylation and alkene epoxidation have also been reported (Groves; Subramanian, 1984; Moghadam *et al.*, 2004). In the specific case of terpenes, Skrobot *et al.* (2003) showed the production of epoxides from

these compounds. In an analogous work, the oxidation of monoterpenes by hydrogen peroxide catalyzed by porphyrins, was also described (Martins *et al.*, 1999; Martins *et al.*, 2001). Other authors have made use of a photoexitated porphyrin to oxidize limonene and produce a mixture of carvone and another unknown product (with a mass spectrum similar to that of verbenone) in concentrations of up to 3.4 $g.L^{-1}$ and 6.0 $g.L^{-1}$, respectively. However, the oxidative degradation instability of the metaloporphyrins and the difficulty of recovering this expensive catalyst limit their practical application. In this case one possible solution might be the immobilization of the metaloporphyrins on solid supports (Trytek *et al.*, 2005).

For more detailed information on the chemical transformations of terpenes focused on the flavor industry, the reading of Swift (2004) is recommended.

3. BIOTRANSFORMATION PROCESSES

During recent years there has been increasing pressure on the industries to adapt their processes and products to recent global tendencies. Environmental concern has forced the development of cleaner processes, according to the 3R rule (Reduce, Reuse and Recycle), while the "dietetic revolution" imposes a growing demand for natural and, more recently, functional products containing the so-called bioactive compounds. In this context, biotransformation emerges as an attractive alternative for terpene oxidation since, as compared to the traditional chemical methods, they proceed under mild conditions, have an elevated regio and enantioselectivity, do not generate toxic wastes, and the products

obtained can be labeled as “natural” (Giri *et al.*, 2001; Janssens *et al.*, 1992; Leuenberger, 1990; Serra; Fuganti; Brenna, 2005).

Biotransformations can be briefly described as chemical reactions catalyzed by microorganisms or enzyme systems (Kieslich, 1984) and are usually carried out with growing cultures, previously grown cells, immobilized cells, purified enzymes or multiphase systems (Leuenberger, 1990). According to De Carvalho and Da Fonseca (2006b), 7 % of the papers on terpene biotransformation published in the last ten years use purified enzymes as the biocatalyst, while plant cells, fungi, yeasts and bacteria account for 11 %, 33 %, 2 % and 41 %, respectively. In sequence, the (bio)oxidation of terpenes *via* biotransformation processes using isolated enzymes, whole plant cells and microorganisms will be considered in detail.

3.1. Use of purified enzymes

The use of purified enzymes in bioconversions may be advantageous or necessary in some cases, such as (i) when the membrane of the intact cell prevents appropriate substrate or product permeation, (ii) when there is posterior product degradation or undesirable side reactions involving other enzymatic systems, (iii) when the enzyme of interest is excreted by the cell and might be easily purified from the medium after biomass removal or (iv) when the enzyme of interest is commercially available. On the other hand, enzyme purification is often tedious, time consuming and expensive (Leuenberger, 1984).

The enzyme-generated reactive oxygen species process is a method that combines chemical oxidation and the enzymatic production of the oxidizing agent. This system can be applied to the bitechnological production of the aroma compound β-ionone from β-

carotene by the use of enzymes, *eg.* lipoxygenase (Waché; Ratuld; Belin, 2006; Wu *et al.*, 1999) and xanthine oxidase (Bosser; Belin, 1994). However, to be cleaved, the lipophilic substrate needs to be present in the aqueous phase where the enzymes work. An alternative to overcome this problem might be the use of micelles dispersed in water or solvent (Waché; Ratuld; Belin, 2006).

An enzymatic system developed by Trytek and Fiedurek (2002) is apparently less sensitive to substrate concentration and temperature variation when compared to the microbial transformation methods. In this study, the optimum medium conditions for the conversion of limonene to carvone (apart from other side products) using glucose oxidase and horseradish peroxidase were pH 7.0, 1.5 % substrate, 50 °C and a reaction time of 16 to 24 h. This work was of great scientific value, since it described an original method for biotransforming a monoterpene using cell-free enzymes. However, the yield obtained was too low (<10 $mg.L^{-1}$) for an industrial application, thus enzyme immobilization techniques might be considered for future similar studies.

Horseradish peroxidase was also studied in the enzymatic oxidation of citronellol. This reaction occurs predominantly after double C–C linkage epoxidation reactions, followed by epoxide solvolysis (Kaminska *et al.*, 1989). Another peroxidase, present in the mycelium-free culture supernatant of the edible fungus *Lepista irina*, was able to degrade β-carotene yielding flavor compounds. The degradation occurred most efficiently at 34 °C with a pH optimum between 3.5 and 4, and the main volatile breakdown products formed were β-ionone, β-cyclocitral, dihydroactinidiolide and 2-hydroxi-2,6,6-trimethylcyclohexanone (Zorn *et al.*, 2003b).

Alcohol dehydrogenase can be used in the production of food additives, especially flavoring agents. One example is the oxidation of geraniol to geranial using horse liver alcohol dehydrogenase in biphasic mediums (Legoy; Kim; Thomas, 1985). In the same paper, different organic solvents and three co-factor regenerating methods were studied. Other monoterpenoid oxidations catalyzed by alcohol dehydrogenase recovered from plants have been described elsewhere (Davies *et al.*, 1973; Hatanaka; Sekiya; Kajiwara, 1976).

The investigation of sesquiterpene biosynthesis in chicory led to the characterization of a cytochrome P450 hydroxylase which was shown to hydroxylate β-elemene (De Kraker *et al.*, 2001), and in a further study was shown to be able to hydroxylate a range of other sesquiterpenes exogenous to the plant, mainly yielding the respective isopropenyl or isopropylidene alcohols (De Kraker *et al.*, 2003).

Many other terpene enzymatic transformations in cell free systems have been described, although they were basically focused on the isolation and characterization of microbial enzymes (Cadwallader; Braddock; Parish, 1992) or plant enzymes for the elucidation of the biosynthetic pathways involving volatile terpenoid formation in vegetables, especially monoterpenoids in *Mentha* leaves (Kjonaas; Martinkus-Taylor; Croteau, 1982; Kjonaas; Venkatachalam; Croteau, 1985; Karp *et al.*, 1990) and norisoprenoids in quince (Fleischmann; Studer; Winterhalter, 2002), star fruit (Fleischmann; Watanabe; Winterhalter, 2003) or nectarines (Bladermann; Naim; Fleichmann, 2005).

3.2. Use of integer cells

According to Duetz, Van Beilen and Witholt (2001), there are four main reasons to use whole cells rather than purified enzymes: (i) apart from its simplicity and economy, the use of whole cells protects the enzyme from shear forces and might extend the enzyme activity half-life in a stirred bioreactor; (ii) the removal of an enzyme from a membrane environment often leads to complete or nearly complete loss of activity; (iii) cascades of enzymatic reactions may be too complicated to perform *in vitro* because of the number of enzymes, cofactors and substrates involved; (iv) the stoichiometric consumption of cofactors during the enzymatic reaction or chain of reactions may make the use of whole cells attractive. Besides, when using whole cells, the addition of cofactors is not required (Chatterjee, 2004).

3.3. Plant-cultured cells

Plant cell cultures exhibit a vast potential for the production of specific secondary metabolites and may be used to transform cheap and plentiful substances, such as industrial byproducts, into rare and expensive products (Giri *et al.*, 2001). Cytochrome P450 oxygenases from certain vegetable cells are known for their ability to oxidize monoterpenoids during their biosynthesis. Hence, the use of these cells in the biotransformation of terpenes has been investigated in recent years (Suga; Hirata, 1990).

The biotransformation capacity of culture suspensions of *Achillea millefolium* was investigated using different monoterpenes and a mixture of farnesol isomers. Except for geraniol, the other substrates tested (borneol, menthol, thymol and farnesols) yielded less than 1 $mg.L^{-1}$ of products. The authors concluded that part of the substrates added and the

biotransformation products were converted into and accumulated as the glycosylated forms (Figueiredo *et al.*, 1996). *Nicotiana tabacum* and *Catharanthus roseus* were investigated in the biotransformation of 3-carene and 2-pinene (Hirata *et al.*, 1994), and cell culture suspensions of the last specie were also tested in the conversion of (–)-piperitone, which was regioselectively hydroxylated at the 4- and 6-positions (Hamada *et al.*, 1994). Cultured cells of *Caragana chamlagu* were able to convert α- and β-ionone into 3-oxo-α-ionone and 5,6-epoxi-β-ionone with yields of 50 % and 87 %, respectively (Sakamaki *et al.*, 2004). Further terpenic and non-terpenic substrates were tested in biotransformation assays using a culture suspension of *Peganum harmala* (Zhu; Asghari; Lockwood, 2000).

Picea abies, from which the by-product turpentine is collected after the thermomechanical pulping process, has been widely studied in the biooxidation of terpenes using plant cell cultures. Their cell culture suspensions were tested in the biotransformation of α-pinene (Lindmark-Henriksson *et al.*, 2003), limonene and β-pinene (Lindmark-Henriksson *et al.*, 2004) and the main products obtained were, respectively, *trans*-verbenol, limonene-1,2-epoxide and *trans*-pinocarveol. α-Pinene proved to be the fastest reacting substrate, but immobilization of the *Picea abies* cells decreased the transformation rate without influencing the composition of the products or their absolute configuration (Vanêk *et al.*, 2005). Immobilized *Solanum aviculare* and *Dioscorea deltoidea* cells were also applied to oxidize (–)-limonene. However in this case the attack was preferentially at position 6, yielding mainly *cis*-carveol, *trans*-carveol and carvone (Vanêk *et al.*, 1999).

Despite great academic interest, the insufficient enzymatic activity and low yields obtained limit the application of plant cell cultures in industrial processes.

3.4. Fungi and yeasts

The use of microorganisms in monoterpene biotransformation is relatively recent, dating from the late 1950's and mid 1960's. In pioneer studies, a soil pseudomonad was used for the microbial degradation of camphor (Bradshaw, 1959; Bradshaw *et al.*, 1959; Conrad; Dubus; Gunsulus, 1961), limonene (Dhavalikar; Bhattacharyya, 1966; Dhavalikar; Rangachari; Bhattacharyya, 1966), α- and β-pinenes (Shukla; Bhattacharyya, 1968; Shukla; Moholay; Bhattacharyya, 1968), citronellol, farnesol and others (Seubert, 1960). The fungal-mediated oxidation of terpenes was described in the same period, after an *Aspergillus niger* capable of metabolizing α-pinene to oxygenated products was selected amongst different molds (Bhattacharyya *et al.*, 1960). Substrate concentrations of 0.6 % (v/v), an 8 h reaction time and a temperature range of 27-28 °C maximized the yields of the tree main metabolites (verbenol, verbenone and *trans*-sobrerol) (Prema; Bhattacharyya, 1962a). Subsequently, the same *A. niger* strain was investigated in the conversion of other mono and sesquiterpenes (Prema; Bhattacharyya, 1962b).

Currently *A. niger* is one of the most extensively studied fungal species involved in monoterpene biotransformation. Some of the parameters involved in the transformation of α-pinene to verbenone by an *A. niger* isolated from soil underneath citrus trees, were optimized one-at-a-time. The optimal conditions were obtained when the microorganism was incubated for 6h with 20 mg.100 mL^{-1} of substrate and 6 $g.L^{-1}$ of glucose in a sodium phosphate buffer at pH 7.0. Although the product formation increased, the yield remained low (32.8 mg.100 mL^{-1}) (Agrawal; Joseph, 2000a). *A. niger* ATCC 9462 was investigated for the conversion of (–)-α-pinene, (–)-β-pinene and (+)-limonene, but only the second compound was transformed by this strain. The best results, about 4 % conversion of (–)-β-

pinene to α-terpineol, were achieved when the substrate was supplemented in five subsequent additions as a 1:1 ethanol solution. Cell induction did not affect the reaction yield (Toniazzo *et al.*, 2005). Likewise, five monoterpene substrates, *i.e.* (+)- and (–)-limonene, α- and β-pinene and camphor, were used for the microbial production of aromas and fragrances by *A. niger* IOC-3913. The study was carried out in a liquid medium (with growing cells, pregrown cells and immobilized cells) or in a solid medium, with a substrate supply *via* the gas phase. (+)- and (–)-limonene were not metabolized by the strain tested, whilst verbenone and α-terpineol were the main products after the biotransformation of α- and β-pinene, respectively (Divyashree; George; Agrawal, 2006). Further papers have described the biotransformation of limonene to perillyl alcohol (Menéndez *et al.*, 2002) and the conversion of linalool to furanoid and pyranoid linalool oxides mediated by *A. niger* (Demyttenaere; Willemen, 1998).

Larger terpenoid molecules have also been used in biocatalytic studies with *A. niger*. Mikami *et al.* (1981) selected an *A. niger* strain capable of transforming β-ionone and β-metilionone into analogous tobacco-related aroma compounds. Another *A. niger* strain was found to be an efficient biocatalyst for a similar process, producing about 2.5 $g.L^{-1}$ hydroxyl and oxo derivatives from β-ionone after 230 h cultivation (Larroche; Creuly; Gros, 1995). Later, the same strain was immobilized in calcium alginate beads due to the low aqueous solubility of the precursor, and the reaction carried out in a two-phase liquid system. The best yield, 3.5 $g.L^{-1}$, was obtained after 400 h reaction (Grivel; Larroche, 2001). The physicochemical parameters of that system were analyzed elsewhere (Grivel; Larroche; Gros, 1999). In their paper, very interesting for its originality, Krings *et al.* (2006) reported the screening of submerged microbial cultures able to oxifunctionilize the

sesquiterpene α-farnesene. One culture, identified as *A. niger*, exhibited the most versatile and attractive flavour profile. The oxidation of the sesquiterpenes valencene and nootkatone could also be performed by a soil-isolated *A. niger* as well as by other fungal strains (Furusawa *et al.*, 2005).

In addition, *Aspergillus* sp., *i.e. A. cellulosae*, was capable of converting both enantiomers and the racemate of limonene into limonene-*trans*-1,2-diol as the main product (Noma; Yamasaki; Asakawa, 1992). In fact, some authors concluded that diols are common intermediates in the monoterpene metabolism of fungi (Mukherjee; Kraidman; Hill, 1973).

Penicillium sp. is another fungal genera well documented in terpene biocatalysis. It was observed that the biotransformation of limonene by *P. digitatum* occurred in the first instants of the log phase and that the bioconversion activity was expressively enhanced by the addition of substrate during the microbial (Tan; Day; Cadwallader, 1998). Other publications have described the biotransformation of limonene to α-terpineol using immobilized *P. digitatum* cells (Tan; Day, 1998b) and have studied the effects of cosolvents in this conversion (Adams; Demyttenaere; De Kimpe, 2003; Tan; Day, 1998a). According to Agrawal and Joseph (2000b), the culture conditions (age of the culture, pH, glucose concentration and nitrogen source), substrate concentration, the amount of biomass, the pH of the buffer, the temperature and the incubation time taken for biotransformation of α-pinene by a *Penicillium* sp., were found to be very critical for verbenone formation. A 15-fold increase in product recovery was observed under the optimized conditions: 100 mL of a 0.05 M phosphate buffer pH 7.0 incubated at 30 °C for 6 h with 20 mg of substrate and 200 mg of fungal biomass, which was harvested after 18h-growth at 30 °C and pH 5.75 in potato dextrose agar, supplemented with 1 % glucose and 0.025 % yeast extract. Curiously,

the biotransformation using spores of *P. italicum* (Demyttenaere; De Pooter, 1996) or *P. digitatum* ATCC 201167 (Demyttenaere; De Pooter, 1998) was feasible for, respectively, geraniol and nerol or citral and nerol, yielding 6-methyl-5-hepten-2-one. The pathway involved in this kind of transformation was subsequently studied (Wolken; Van der Werf, 2001).

Amongst various different mono and sesquiterpenoids transformations mediated by fungi, such as *Corynespora cassiicola* and *Diplodia gossypina*, Abraham *et al.* (1985) described a well distinguished process of recovering good yields of limonene-1,2-diols from limonene with continuous substrate feeding. The psychrotrophic *Mortierella minutissima* (Trytek; Fiedurek, 2005) was also studied for the fungal conversion of limonene into perillyl alcohol/perillyc acid and the best results, approximately 120 mg perillyl alcohol per liter, were obtained after 120 h at 15 °C and pH 6.0. The authors concluded that the use of lower temperatures might reduce volatilization of the substrate and product, favoring the biotransformation process. Apparently, this is the only terpene biotransformation process applying a psychrotrophic microorganism. In another manuscript, it was shown that *Cladosporium* sp. could transform limonene to α-terpineol (Kraidman; Mukherjee; Hill, 1969). The same transformation was feasible when an agroindustrial residue (cassava wastewater) was employed as an alternative culture medium for fungal cultivation, in this case a *Fusarium oxisporum* strain (Maróstica Jr; Pastore, 2007). Using a similar technique, Maróstica Jr. and Pastore (2006) noticed that *Penicillium* sp. was able to produce *cis* and *trans* rose oxides from citronellol, like *Cystoderma carcharias* (Onken; Berger, 1999). The use of agroindustrial residues in bioprocesses seems to be a rising trend to overcome high manufacturing costs (Pandey *et al.*, 2000a; Pandey *et*

al., 2000b; Pandey *et al.*, 2000c) (see also cited references), including the production of flavors.

An interesting alternative to generate flavor compounds was *via* the fungal conversion of larger terpene molecules to volatile breakdown products. In this context, Zorn *et al.* (2003a) described an original method for screening microorganisms able to cleave β-carotene to flavor compounds. From more than 50 filamentous fungi, ten bleached the zone surrounding the mycelium when grown in β-carotene-containing agar plates, suggesting the consumption of tetraterpene. Submerged cultures of four selected strains, *i.e.* *Ganoderma applanatum*, *Hypomyces odoratus*, *Kuehneromyces mutabilis*, and *Trametes suaveolens*, formed dihydroactinidiolide as the sole conversion product from β-carotene, while other carotenoid-derived volatile metabolites, mainly β-ionone, were detected in the mycelium-free culture supernatants from *Ischnoderma benzoinum*, *Marasmius scorodonius*, and *Trametes versicolor*. A mixed culture formed by *Bacillus* sp. and *Geotrichum* sp. produced tobacco aroma compounds from lutein after formation of the intermediate β-ionone. The second microorganism was responsible for the production of β-ionone, while the bacilli modified it to the aroma compounds 7,8-dihydro-β-ionone and 7,8-dihydro-β-ionol (Maldonado-Robledo *et al.*, 2003; Sanchez-Contreras; Jimenez; Sanchez, 2000).

As already reported for *A. niger*, the filamentous fungus *Lasiodiplodia theobromae* ATCC 28570 may also metabolize the flavor compound β-ionone to a complex mixture of metabolites reminding one of the tobacco flavor (Krasnobajew; Helmlinger, 1982). A similar hydroxilation of α- and β-ionone at positions 3 and 4, respectively, was performed by selected strains of the bacteria *Streptomyces*. It was demonstrated that the transformation of α-ionone proceeded with both high regio- and stereoselectivity (Lutz-Wahl *et al.*, 1998).

Other fungal species, such as *Armillariella mella* (Draczynska *et al.*, 1985) and *Botrytis cinerea* (Aleu; Collado, 2001; Farooq *et al.*, 2002) were able to biotransform, respectively, α-/β-pinenes and a great variety of other terpenes.

Novel fungal strains are continuously being selected based on their ability to biotransform terpenes, and a promising alternative for screening potential fungi is the solid phase microextraction (SPME) technique (Demyttenaere; Van Belleghem; De Kimpe, 2001; Demyttenaere; Vanoverschelde; De Kimpe, 2004).

Interestingly, as far as we know, there are only a few descriptions of yeast-mediated terpene biotransformation process. The yeast *Candida tropicalis* MTCC 230 has shown its capacity to oxidize α-pinene to α-terpineol with an overall yield of 77 % after 96 h/30 °C, when 0.5 $g.L^{-1}$ of substrate was used. The product concentration remained stable up to 120 h of reaction time (Chatterje; De; Bhattacharyya, 1999). In a recent manuscript, Pinheiro and Marsaioli (2007) described the use of whole *Trichosporum cutaneum* cells in batch reactions to prepare oxiderivates of *cis*-jasmine, *R*-(–)-carvone, α- and β-ionones and *R*-(+)-limonene. Other examples are the conversion of limonene, α-pinene, β-pinene and some monoterpenoids by yeast or yeast-like fungi (Van Dyk; Van Rensburg; Moleleki, 1998; Van Dyk *et al.*, 1998; Van Rensburg *et al.*, 1997), and the modification of hop aroma terpenoids by ale and lager yeasts (King; Dickinson, 2003). In this context, further descriptions of terpene biotransformations by yeasts would be of great scientific value.

3.5. Bacteria

Although the microorganism-mediated conversion of terpene seems to proceed *via* cytochrome P450 monooxygenases (Bernhardt, 2006; Duetz *et al.*, 2003; Unger; Sligar;

Gunsalus, 1986), there are indications that the cytochrome P450 oxygenases of *A. niger* are not involved in the transformation of limonene to perillyl alcohol (Menéndez *et al.*, 2002). Meanwhile it has also been observed that the bacteria *Rhodococcus erythropolis* DCL14 initiated the biotransformation of limonene with an epoxidation at position 1,2, catalyzed by a limonene monooxygenase whose activity was not dependent on cytochrome P450 (Van der Werf; De Bont, 1997).

Similar to the first studies, various pseudomonads have been applied to the biotransformation of terpenes (Trudgill, 1986). Members of this bacterial genus have shown good resistance to solvents (Inoue; Horikoshi, 1989), have the metabolic flexibility to grow in a wide range of organic compounds as the sole carbon source and possess a wide variety of oxygenases and related enzymes for the activation and cleavage of terpene molecules (Trudgill, 1986). Bicas *et al.* (2010) evaluated a bioprocess for the production of high concentrations of *R*-(+)-α-terpineol from *R*-(+)-limonene by two pseudomonads. Yoo, Day and Cadwallader (2001) isolated a soil pseudomonad that could metabolize both α- and β-pinenes, resisting concentrations of up to 10 % of these terpenes. The possible pathway for the degradation of α- and β-pinenes by this pseudomonad was later described (Yoo; Day, 2002). In earlier studies, some workers detected acid metabolites accumulated by *Pseudomonas* PX1 (Gibbon; Pirt, 1971) and *Pseudomonas putida* PIN11 (Tudroszen; Kelly; Millis, 1977) after the oxidation, followed by the ring cleavage of α-pinene, suggesting a different pathway from that determined in the above study. Years later, it was demonstrated that *P. putida* GS1 could convert limonene solely to perillyl acid, this remaining stable in the culture medium (Speelmans; Bijlsma; Eggink, 1998). In sequence, Mars *et al.* (2001) concluded, after analyzing two *P. putida* strains (GS1 e F1) and one recombinant *E. coli* strain, that the enzymes involved in this biocatalysis belonged to the *p*-

cymene degradation pathway. Another *P. putida* strain, MTCC 1072, has shown the ability to metabolize limonene producing perillyl alcohol and *p*-menth-1-en-6,8-ol, with yields of 36 % and 44 %, respectively (Chatterjee; Bhattacharyya, 2001).

Other members of this genus have also been applied in the oxidation of monoterpenes. The ability of *Pseudomonas gladioli* to utilize limonene as the sole carbon source was first described by Cadwallader *et al.* (1989). The microorganism attacked the molecule at positions 7 and 8 to form perillic acid and α-terpineol, respectively. A soil-isolated bacteria identified as *P. maltophilia*, was used to conduce the transformation of α-pinene using resting cells or culture broth in a 30L-fermentor. The main natural products were identified as limonene, borneol and camphor, while the acid fraction contained perillyl acid and 2-(4-metyl-3-cyclohexenylidene) propionic acid. Based on its O_2 uptake, it was demonstrated that this strain readily oxidized a diversity of monoterpenoids, *e.g.* β-pinene, limonene, α-phellandrene, 1,8-cineole and others (Narushima; Omori; Minoda, 1982).

Some *Pseudomonas* species were tested for the biodegradation of acyclic monoterpenoids, and special attention was given to the use of citronellol and geraniol by *P. citronellolis* (Cantwell *et al.*, 1978). One member of the last species, *P. citronellolis* DSM 50332, showed the ability to anaerobically degrade some monoterpenoids (Harder; Probian, 1995). This degradation can occur using a pathway earlier described for *P. citronellolis* (Seubert, 1960).

Rhodococcus opacus PWD4 cells, which can use toluene as their sole carbon source, hydroxylated *R*-(+)-limonene at position 6, forming enantiomerically pure *trans*-carveol. The maximal concentration of this product was obtained after 2.5 h and the final yield was 94-97 %. The posterior conversion of *trans*-carveol into (+)-carvone by *Rhodococcus globerus* PWD8 illustrates that this strain might have a potential application

in the industrial production of this ketone (Duetz *et al.*, 2001). Similarly, *Rhodococcus erythropolis* DCL14 was able to transform (–)-*trans*-carveol into (–)-carvone. In this case the use of a biphasic system improved the bioconversion rate (Tecelão; Van Keulen; da Fonseca, 2001). Some reaction parameters involved in the biotransformation of geraniol to geranic acid by *Rhodococcus* sp. strain GR3 were studied by Chatterjee (2004), who reported that the reaction occurred optimally at 30 °C and that the product concentration reached a maximum after 96 h and increased with increase in the geraniol concentration up to 1.0% (v/v). A patent application describes the preparation of hydroxylmethylated terpenes, more specifically perillyl alcohol, using the biotransformative capacity of a variety of bacteria, including members of the genus *Rhodococcus* sp. (Duetz; Witholt; Jourdat, 2004).

A *Xanthobacter* sp. isolated from river sediment, converted both enantiomers of limonene into its 8,9-epoxide using cyclohexane as its sole carbon source, with the suggested involvement of a P450-dependent monooxygenase. The best results, 0.8 $g.L^{-1}$ of epoxide, were achieved using 12 mM of substrate concentration (Van Der Werf; Keijzer; Van Der Schaft, 2000). The endobacterium *Serratia marcescens* has shown the capacity for two specific transformations of α-pinene. In one the main product was *trans*-verbenol together with minor amounts of verbenone and *trans*-sobrerol. Alterations in the culture conditions (use of another nitrogen source and the inclusion of glucose) changed the product profile, and in this case α-terpineol was the major product formed (Wright *et al.*, 1986).

Some papers involving the *Bacillus* sp. metabolism of monoterpenes have also been published. In one of them, a strain isolated from pine trees, identified as *Bacillus pallidus*

BR425, degraded α- and β-pinene, as well as limonene. In the first case, significant amounts of pinocarveol, pinocarvone, carveol, carvone and lesser amounts of myrtenol, myrtenal, limonene and β-pinene were recovered. Carveol was a common metabolite for all the monoterpenes tested, suggesting that this compound together with carvone, are central growth intermediates in BR425 pinene metabolism (Savithiry *et al.*, 1998). In the same research field, Chang and Oriel (1994) isolated a thermophilic *Bacillus stearothermophilus* strain from orange peel that could use limonene as the sole carbon source, converting it to perillyl alcohol as the main product, and α-terpineol and perillyl aldehyde as minor products. The same compounds were obtained, although not in the same proportions, when a 9.6 Kb chromosomal fragment was cloned and expressed in the recombinant *E. coli*, which could grow on limonene as its sole carbon source. However, the level of oxygenated monoterpenes recovered was considered insufficient for a possible industrial exploration of this process (Chang; Gage; Oriel, 1995). In a similar study, Savithiry, Cheong and Oriel (1997) noticed that growth on limonene could be conferred to *E. coli* after this microorganism received a 3.6 Kb DNA fragment from the wild strain. It was later demonstrated that the use of limonene as the sole carbon source by a recombinant *E. coli* resulted from the expression of a single gene, which codified a new monoterpene oxidative enzyme producing carveol, perillyl alcohol and subsequently carvone from limonene (Cheong; Oriel, 2000).

3.6. Use of unconventional biocatalysts

Although the greater part of the biooxidation processes described in the literature are performed by microorganims, different unusual biocatalysts have been tested for the

conversion of terpenes. Marine microorganisms, such as the cyanobacteria *Synechococcus* sp. PCC 7942, which could hydroxylate both *S*-(–)-limonene and its oxide (Hamada *et al.*, 2003), and the unicellular microalgae *Dunaliella tertiolecta*, which reduced aldehydes to the corresponding primary alcohols (Noma *et al.*, 1992), are interesting examples. However, the most curious biocatalysts applied to terpene conversions are the superior organisms and animals.

Suggesting that the larvae of the cutworm *Spodoptera litura* possesses a high level of enzymatic activity against terpenoids, some authors have tested their biotranformation potential for limonene (Miyazawa; Wada; Kameoka, 1998), α-terpinene (Miyazawa; Wada; Kameoka, 1996), β-myrcene (Miyazawa; Murata, 2000), terpinen-4-ol (Miyazawa; Kumagae, 2001), α-terpineol (Miyazawa; Ohsawa, 2002), menthol (Miyazawa; Kumagae; Kameoka, 1999), camphor (Miyazawa; Miyamoto, 2004), geraniol (Takechi; Miyazawa, 2006) and others. The terpenic substrate (1-10 $mg.g^{-1}$, depending on the terpene tested) was mixed into the larvae's artificial diet and the products analyzed (CG-MS) in their frass organic extract. In general, the unsaturated monoterpenes were hydroxylated at the allylic position. In this case, the terpene metabolism was similar to the terpene metabolism in mammals. Actually, there are several examples of terpenoid oxidations by mammals, although they have not received much attention (Ishida, 2005; Shimada; Shindo; Miyazawa, 2002).

As may be observed, there are interesting underexplored ways to biotransform terpenes, since every superior organism with a well developed enzymatic system, especially those involved in xeobiotic metabolism, have the potential to oxidize these compounds. Further research in this field could have great scientific value, especially if it discovers new

compounds, unknown metabolites with unique structures, potential biocatalysts or original biotechniques.

4. EMERGENT TECHNOLOGY AND FUTURE PROSPECTS

According to Leuenberger (1990), biotransformation might be a useful tool in organic chemistry, although some biotechnological developments are needed: optimization of the biocatalyst cultivation and biotransformation conditions (medium, temperature, agitation, pH *etc*.), strain improvement by classical methods or by genetic engineering, development of an appropriate production facility with an efficient product isolation procedure, process simplification to minimize the manufacturing costs and finally the scale up. Moreover, some techniques, which in combination with conventional methods could contribute to cost reductions and render further industrial biotransformation processes feasible and attractive, might be applied: the use of immobilized cells, improving biocatalyst stability and making a continuous production process possible; the use of biphasic media, increasing the solubility of the substrate and avoiding its toxic effect towards the microorganism; and the use of recombinant DNA and protein engineering to improve the yields (Leuenberger, 1990). In this context, many scientists are in search of genetically modified organisms for a more effective terpene oxidation process.

Unspecific genetic modifications through induced mutation (colchicine, ethyl methanesulphonate or ultraviolet radiation) of *Aspergillus* sp. and *Penicillium* sp., or protoplast fusion between members of these two genera were applied in order to improve verbenol yields in the biotransformation of α-pinene (Agrawal; Deepika; Joseph, 1999).

However, after the advent of DNA recombinant techniques, direct genetic approaches for increasing biotransformation rates and simplifying the process have been driving studies in this area.

Plant recombinant enzymes applied to hydroxylate *S*-limonene (Wüst *et al.*, 2001) and to cleave carotenoids producing apocarotenoid flavor compounds (Schwartz; Qin; Zeevaart, 2001) are already a reality. Additionally, some wild types and mutants of $P450_{cam}$ and $P450_{BM-3}$ have been investigated as a way to oxidize (+)-valencene to (+)-nootkatone. The latter presented higher activity although less selectivity when compared to $P450_{cam}$ (Sowden *et al.*, 2005). *Pseudomonas putida* $P450_{cam}$, which is known to convert (+)-camphor to 5-*exo*-hydroxycamphor, was remodeled by designed mutagenesis, greatly enhancing activity for the oxidation of α-pinene and *S*-limonene. The authors suggested that this technique could give rise to novel fragrances and flavorings or new biologically active compounds (Bell; Sowden; Wong, 2001).

Although the approach of cloning and the expression of terpene biotransforming genes have already been performed in *E. coli* (Chang; Gage; Oriel, 1995; Cheong; Oriel, 2000; Savithiry; Cheong; Oriel, 1997) there are still only a few descriptions of the bio-oxidation of terpenes as carried out by cloned microorganisms, and the viability of such processes seems to be distant. One exception is the production of perillyl alcohol from limonene by *Pseudomonas putida,* expressing an alkene hydroxylase purified from *Mycobacterium* sp. HXN-1500. This process was performed in a 2-L bioreactor with a biphasic medium. After 75 h, the perillyl alcohol accumulated in the organic phase reached 6.8 $g.L^{-1}$, equivalent to 2.3 $g.L^{-1}$ when calculated for the entire bioreactor contents (Van Beilen *et al.*, 2005).

However, it is worth noting that the success of innovative flavor biosynthesis does not depend exclusively on genetically improved biocatalysts, but also on process engineering, particularly when it comes to terpenoid flavor compounds (Schrader *et al.*, 2004).

5. CONCLUSION

The flavor and fragrance industries have grown constantly with growth in the world economy. In parallel, the chemical oxidation of terpenes for flavor synthesis is gradually becoming anachronous, and the rising quest for natural sources of aroma compounds is forcing an adaptation of the manufacturing methodology. Therefore, biotransformation processes, particularly those applying filamentous fungi or bacteria, have arisen as a promising alternative. However, the low transformation rates and high production costs are still obstructing their wide-scale adoption. To overcome these problems, the genetic engineering technique seems to be a suitable choice, although such investigations are still in an embryonic stage. Therefore, more studies are essential to ensure the economical adoption of biotechnology for the production of flavor and fragrances.

This novel frontier in the field of food ingredients not only aims to provide good and economic technological applicability, but is also part of the quest for functional ingredients and nutraceuticals that is directing scientific and technological development in this area. Hence, flavor terpenes produced *via* biotransformation are completely adapted to the new market demand since, despite their natural nature, many of them have been proven to play an important biological role against certain types of cancer in *in vivo* studies.

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CAPÍTULO 2

CAPÍTULO 2

ISOLAMENTO E SELEÇÃO DE MICRO-ORGANISMOS POTENCIALMENTE BIOTRANSFORMADORES DE CAROTENÓIDES

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RESUMO

Micro-organismos produzem diversos metabólitos secundários, incluindo aromas, compostos de fragância e de maior apelo funcional. A produção biotecnológica de aromas é um campo emergente pois, diferentemente da tradicional síntese química, reações de biotransformação podem produzir compostos classificados como naturais, o que representa uma poderosa vantagem de *marketing* sobre compostos sintetizados quimicamente. Os principais objetivos deste trabalho consistiram no isolamento e seleção de micro-organismos potencialmente biotransformadores de carotenóides em compostos de aromas. Micro-organismos foram isolados de frutas e vegetais ricos em carotenóides, usando o método de placa constituído de solução de β-caroteno e o potencial para formação de voláteis de interesse foi evidenciado com o uso de micro-extração em fase sólida (SPME-CG-DIC) como técnica de extração e concentração dos voláteis do meio reacional. A linhagem selecionada (LB398APD) foi avaliada quanto a produção de enzimas de interesse industrial, para uma possível co-produção aliada ao processo de biotransformação.

Palavras-chave: isolamento, seleção, biotransformação.

1. INTRODUÇÃO

Os carotenóides são tetraterpenos amplamente difundidos na natureza. São um dos responsáveis pelas cores do amarelo ao vermelho de frutas e flores, e podem ser sintetizados em plantas (Hirschberg, 2001), algas (Yuan *et al*. 2002) e micro-organismos (Maldonade; Rodriguez-Amaya; Scamparini, 2008). Comercialmente, os carotenóides são usados como corantes alimentícios e em suplementos nutricionais (Fraser; Braimley, 2004) e são considerados precursores de muitos compostos químicos importantes, responsáveis pelo aroma de alguns alimentos, fragrâncias de algumas flores (Sanchez-Contreras; Jiménez; Sánchez, 2000), coloração específica e fotoproteção (Marasco; Schimidt-Dannert, 2003).

Compostos voláteis naturais estruturalmente derivados de carotenóides, estão freqüentemente distribuídos na natureza. Devido ao seu baixo valor de *treshold*, muitos deles apresentam alto potencial aromático e por isso possuem grande interesse para a indústria de aromas e fragrâncias (Zorn *et al.*, 2003). Compostos como β-ionona, α-ionona, diidroactinidiolida, damascenol e β-ciclocitral são alguns dos voláteis derivados dos carotenóides que nas plantas, têm a função de anti-fungicida, de afastamento de herbívoros e até mesmo atrair agentes polinizadores (Weeks, 1986).

A produção de compostos de aromas pode ser realizada em diferentes processos, com destaque para produção através da síntese química e extração de fontes naturais. Porém, a síntese química geralmente apresentam um alto impacto ambiental e por sua vez, aromas extraídos diretamente de plantas apresentam problemas relacionados a instabilidade advindas da sazonalidade e efeitos geográficos. Neste contexto, a produção biotecnológica estão menos sujeitas a variações sazonais, emitem menor carga de resíduos e os produtos

advindos desse processo são considerados como "naturais", o que pode fornecer uma importante estratégia de mercado (Janssens *et al.*, 1992; De Carvalho; Da Fonseca, 2002; Marques; Pastore, 1999; Pinheiro; Pastore, 2003). Devido às suas vantagens inerentes, estudos na área de produção biotecnológica de aromas (bioaromas) estão, cada vez mais, ganhando espaço, principalmente quando terpenos são usados como substratos processos de biotransformação (Bicas; Dionísio; Pastore, 2009).

A micro-extração em fase sólida (MEFS ou SPME) é uma técnica de extração e concentração dos compostos voláteis de amostras sem utilização de solventes, minimizando a geração de resíduos e a exposição ocupacional, constituindo uma técnica simples, rápida e de fácil aplicação (Komatsu; Vaz, 2004). Esta técnica de extração vem sendo adotada em diversas pesquisas de biotransformação de terpenos (Demyttenaere; Belleghem; De Kimpe, 2001; Maróstica Junior *et al.*, 2007; Bicas *et al.*, 2008).

Neste trabalho, o isolamento e seleção de micro-organismos potencialmente biotransformadores de carotenóides é proposto, e o uso de micro-extração em fase sólida como técnica de extração dos produtos voláteis formados na biotransformação de carotenóides é reportado pela primeira vez na literatura. Aliado ao estudo do processo de biotransformação, uma prospecção de enzimas de interesse industrial pela linhagem selecionada foi conduzido, com a finalidade de avaliar uma possível co-produção entre compostos de interesse obtidos biotecnologicamente.

2. MATERIAIS E MÉTODOS

2.1. Reagentes

β-Ciclocitral (Sigma pureza ~ 90 %) e β-caroteno (Fluka pureza ~ 97 %) foram mantidos sob baixas temperaturas (-10 ºC). Tween 40 (emulsificante) foi adquirido da Sigma-Aldrich. Todos os demais reagentes foram grau analítico.

2.2. Isolamento de micro-organismos

Os micro-organismos utilizados nesse estudo foram isolados de vegetais ricos em β-caroteno comercializados na região de Campinas-SP.

O isolamento foi realizado em meio de cultura líquido *yeast malt* (em $g.L^{-1}$: extrato de malte = 3; extrato de levedura = 3, peptona = 5 e glicose = 10) onde amostras de cenoura (*Daucus carota* L.), rúcula (*Eruca sativa* L.), acerola (*Malpighia glabra* L.), pêssego (*Prunus persica*), manga (*Mangifera indica*), caqui (*Diospyros kaki* L.) e batata-doce (*Ipomoea batatas* (L.) Lam) foram inseridas no meio de cultura, e mantidas sob 30 ºC para crescimento em estufa bacteriológica. Após 72 horas, alíquotas do meio líquido foram transferidas para placas de Petri, contendo meio sólido PDA (*Potato Dextrose Agar*). Cada micro-organismo isolado foi transferido sucessivamente para outras placas de PDA, até o completo isolamento da colônia.

2.3. Seleção do potencial biotransformador de β-caroteno em placa

O potencial biotransformador de β-caroteno foi evidenciado por meio da metodologia proposta por Zorn *et al.* (2003), porém com algumas modificações, onde a presença de halos indica um potencial de metabolização dos carotenóides presentes na placa. Para preparação de placas de crescimento contendo β-caroteno, uma solução contendo 200 $mg.L^{-1}$ de β-caroteno, 2 $g.L^{-1}$ de tween 40 e 3.35 % de etanol, foi adicionada em meio PDA pré-esterilizado. O meio de cultura suplementado com o substrato para o processo de biotransformação foi vertido em placas estéreis, e após solidificação, foram inoculadas com uma "picada" contendo o micro-organismo já em crescimento (24 horas). A cor das placas foi examinada diariamente e a degradação do β-caroteno foi detectada visualmente pela formação de halo, após 3-14 dias.

2.4. Seleção de micro-organismos produtores de compostos de aromas por SPME (Solid Phase Micro Extration).

Os micro-organismos selecionados quanto à capacidade de biotransformação de carotenóides em placa foram avaliados em meio de cultura líquido, onde os compostos voláteis foram extraídos e analisados. Para isso, três alçadas de cada linhagem foram inoculadas em 50 mL de meio líquido YM e acondicionadas em *shaker* sob condições 150 rpm e 30 °C. Após 24h de crescimento, as amostras foram centrifugadas a 10.000 rpm, a 5 °C por 15 minutos, e a biomassa foi transferida para *vials* de 100 mL, contendo 20 mL de meio mineral (em $g.L^{-1}$: $MgSO_4$ =0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 e Fe_2SO_4 = 0.01). Em cada *vial* contendo a biomassa, 18 mL de meio mineral e 2 mL de solução de β-

caroteno, preparada conforme descrito anteriormente (item 2.3), foram adicionados. O inóculo foi incubado em *shaker*, a 150 rpm e 30 °C, por 72 horas.

2.5. Procedimento de extração

Para a extração dos compostos voláteis da amostra foi utilizada a técnica de microextração em fase sólida no espaço confinado (*headspace*) entre a amostra e o frasco vedado. Nesta técnica, a extração dos compostos voláteis é realizada por exposição da fibra dentro do frasco da amostra, que permanece sob agitação e em temperatura controlada (veja Figura 1). Foi empregado uma fibra de revestimento misto CARBOXEN-PDMS, relatada para uso em extração de compostos de aromas (Valente; Augusto, 2000), de 75µm de espessura de filme e 10 mm de comprimento (Supelco, Bellefonte, PA, USA). A fibra de SPME foi pré-condicionada a 280 °C por 60 minutos no injetor do cromatógrafo gasoso. No tempo 0, 24, 48 e 72 horas a fibra foi exposta, onde o frasco permaceu sob agitação, a 30°C, durante 30 minutos.

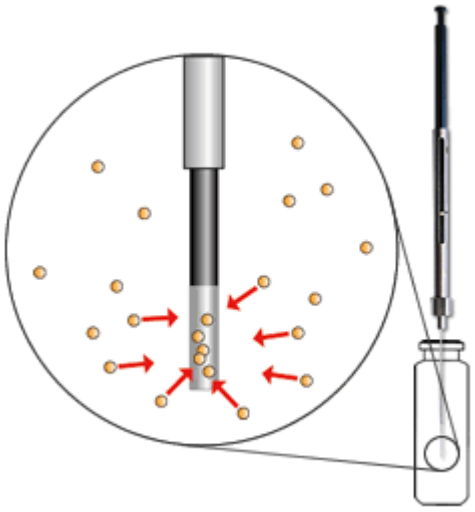

Figura 1 – Fibra exposta ao *headspace* da amostra, e os compostos sendo adsorvidos (Fonte: www.labhut.com).

2.6. Análises cromatográficas (CG-DIC)

Cromatógrafo gasoso (Agilent, GC 7890 A) acoplado a um detector de ionização em chama (CG-DIC) foi usado para detecção dos compostos voláteis formados na biotransformação do β-caroteno. A dessorção dos compostos voláteis da fibra de SPME foi realizada em um injetor do tipo split/splitless a temperatura de 250 °C, no modo splitless por 6 min. A fibra foi mantida no injetor por mais 4 minutos para eliminar o efeito memória. Uma coluna capilar de sílica fundida HP-5 (J&W Scientific, Folsom, Califórnia, USA) de 30 m x 0,25 mm de diâmetro interno e 0,5 μm de espessura de fase estacionária foi utilizada para separar os componentes voláteis. Gás Hélio foi utilizado como gás de arraste, a uma vazão constante de 1,0 $mL.min^{-1}$. A temperatura do detector de ionização em chama foi mantida a 250 °C. A programação de temperatura do forno do cromatógrafo gasoso foi iniciada a 40 °C, permanecendo nesta temperatura por 1 min, em seguida iniciou-se uma rampa de 5 °C min^{-1} até atingir 110 °C permanecendo nesta etapa por 5 minutos, após houve um incremento de 10 $°C.min^{-1}$ até 220 °C, permanecendo nesta temperatura por 5 min.

2.7. Estudo das enzimas extracelulares da linhagem LB398APD

2.7.1. Potencial de produção de lipase alcalina em placa

O meio de cultivo em placa de petri para avaliação da produção de lipase foi constituído de: (% m/v em água destilada) óleo de oliva = 2,0; peptona = 0,3; extrato de levedura = 0,2; K_2HPO_4 = 0,2; $MgSO_4.7H_2O$ = 0,1; Na_2CO_3 = 0,1; ágar = 2,0; rodamina B = 0,001 (Lin *et al.*, 1995). A linhagem LB398APD foi inoculada e incubada a 30 °C por 72

horas, e após esse período, o halo claro ao redor da colônia devido à hidrólise do óleo de oliva foi mensurado.

2.7.2. Potencial de produção de protease em placa

O meio de cultivo em placa de petri para avaliação da produção de protease foi constituído de: ($g.L^{-1}$) triptona = 46.43; extrato de levedura = 2.79; leite em pó = 23.21 e ágar = 18.57 (Tang *et al.*, 2008). A linhagem LB398APD foi inoculada e incubada a 30 °C por 72 horas, e após esse período, o halo claro ao redor da colônia foi mensurado.

2.7.3. Potencial de produção de amilase em placa

O meio de cultivo em placa de petri para avaliação da produção de amilase foi constituído de: ($g.L^{-1}$) extrato de carne = 3; peptona = 3 e amido solúvel = 0,5 % (Bastos, 2005). A linhagem LB398APD foi inoculada e incubada a 30 °C por 72 horas, e após esse período, adicionou-se à placa 10 mL de solução de iodo (30 %), onde halo claro ao redor da colônia foi mensurado.

3. RESULTADOS E DISCUSSÃO

3.1. Isolamento, seleção e ensaios de biotransformação

Os micro-organismos isolados no presente trabalho e as suas respectivas matrizes, encontram-se na tabela a seguir.

Tabela 1 – Matrizes para o isolamento de micro-organismos e respectivo número de isolados.

FONTE	TOTAL
RUCULA (*Eruca sativa* L.)	78
PESSEGO (*Prunus persica*)	38
ACEROLA (*Malpighia glabra* L.)	64
MANGA (*Mangifera indica*)	50
CAQUI (*Diospyros kaki* L.)	47
CENOURA (*Daucus carota* L.)	71
BATATA-DOCE (*Ipomoea batatas* (L.) Lam)	56
PESSEGO (*Prunus persica*) – fungo endofítico	1
OUTROS	3
TOTAL	**408**

As linhagens isoladas (Tabela 1) e três linhagens pertencentes a Coleção do Laboratório de Bioaromas, foram testadas quanto ao potencial de biotransformação de β-caroteno em placa. As três linhagens avaliadas (*Fusarium oxisporum*, *Bacillus subtilis* e *Scopulariopsis* sp.) são estudadas em processos de biotransformação (Bicas *et al.*, 2008) e produção de lipase alcalina (Prazeres; Pastore, 2006); produção de biossurfactantes (Barros *et al.*, 2008) e produção de galacto-oligossacarídeos (Santos *et al.*, 2009) no Laboratório de Bioaromas.

Por sua vez, um micro-organismo endofítico isolado da sementre de pêssego, também foi avaliado. Endofíticos incluem um grupo de micro-organismos, geralmente

fungos e bactérias, que vivem sistematicamente no interior das plantas, sem causar dano a seus hospedeiros (Li *et al.*, 2008; Tan; Zou, 2001). Diversos estudos têm demonstrado a capacidade destes micro-organismos na produção de compostos com atividade antimicrobiana (Pongcharoen *et al.*, 2008; Pittayakhajonwut *et al.*, 2008; Boonphong *et al.*, 2001), de ação anti-câncer (Firáková; Šturdíková; Múčková, 2007; Gangadevi; Muthumary, 2008) e antioxidante (Liu *et al.*, 2007; Huang *et al.*, 2007). Os compostos antimicrobianos isolados destes micro-organismos endofíticos representam diversas classes de compostos, como alcalóides, peptídeos, esteróides, terpenóides, fenóis, quinonas e flavonóides (Yu *et al.*, 2010). Desta forma, sua aplicação em ensaios de biotransformação é de grande interesse, especialmente pela capacidade natural que muitos destes micro-organismos possuem em metabolizar terpenos. No presente estudo, um micro-organismo endofítico foi isolado e avaliado quanto a capacidade de degradar o β-caroteno em placa; contudo, não apresentou a capacidade de biotransformação deste tetraterpeno, nas condições testadas.

Todos os micro-organismos isolados foram testados quanto à capacidade de biotransformação do β-caroteno em placa. Técnica semelhante de *screening* foi utilizada por Zorn *et al.* (2003), usando β-caroteno como substrato; e também por Sanchez-Contreras *et al.* (2000), onde a luteína foi usada como única fonte de carbono. Nesse caso, a luteína foi extraída da flor de marigold (*Tagetes erecta*) e adicionada ao meio de cultura sólido. O halo de degradação foi visualizado em 19 culturas e, quando testados em meio líquido, duas linhagens apresentarm a capacidade de produção de compostos voláteis. Posteriormente, essas linhagens foram identificadas como *Paenibacillus amylolyticus* e *Trichosporon asahii* (Rodríguez-Bustamante *et al.*, 2005). Com relação ao β-caroteno, Zorn e seus colaboradores (2003) realizaram a seleção de algumas cepas para a degradação deste

tetraterpeno, sendo que diidro-actinidiolida foi o único produto da bioconversão observado nas culturas submersas de *Ganoderma applanatum, Hypomyces odoratus, Kuehneromyces mutabilis*, e *Trametes suaveolens*. Compostos voláteis derivados de carotenóides foram detectados no meio de cultura de *Ischnoderma benzoinum, Marasmius scorodonius e Trametes versicolor*, sendo a β-ionona o metabólito principal para essas linhagens.

No presente estudo, um halo de degradação do tetraterpeno foi evidenciado ao redor da colônia em aproximadamente 60 linhagens, representando 14 % do total de micro-organismos testados. Porém, a degradação do β-caroteno em placa é apenas um indicador inicial da potencialidade destes micro-organismos para aplicação no processo de biotransformação para formação de compostos de aromas.

A degradação do β-caroteno podem produzir diversos compostos voláteis com 9 a 13 carbonos, dependendo do precursor e da posição de quebra (Enzell, 1985) (veja Figura 2), onde as enzimas lipoxigenases, xantine oxidases, fenoloxidases e peroxidades estão envolvidas (Bossser; Belin, 1994; Wu *et al.*, 1990; Waché *et al.*, 2002, Waché *et al*., 2006). Os compostos de aromas derivados de carotenóides são apreciados na indústria de aromas devido aos baixos valores de *threshold*, características das notas de aromas, seu histórico e significância comercial. Desta forma, após seleção em placa dos micro-organismos com potencialidades para metabolizar carotenóides, o uso da cromatografia gasosa (CG-DIC) tornou-se essencial para identificar os possíveis produtos formados na quebra do β-caroteno. A técnica de extração escolhida (SPME) foi fundamental para garantir uma técnica limpa e simples, sem uso de elevadas temperaturas, considerando que carotenóides são termossensíveis.

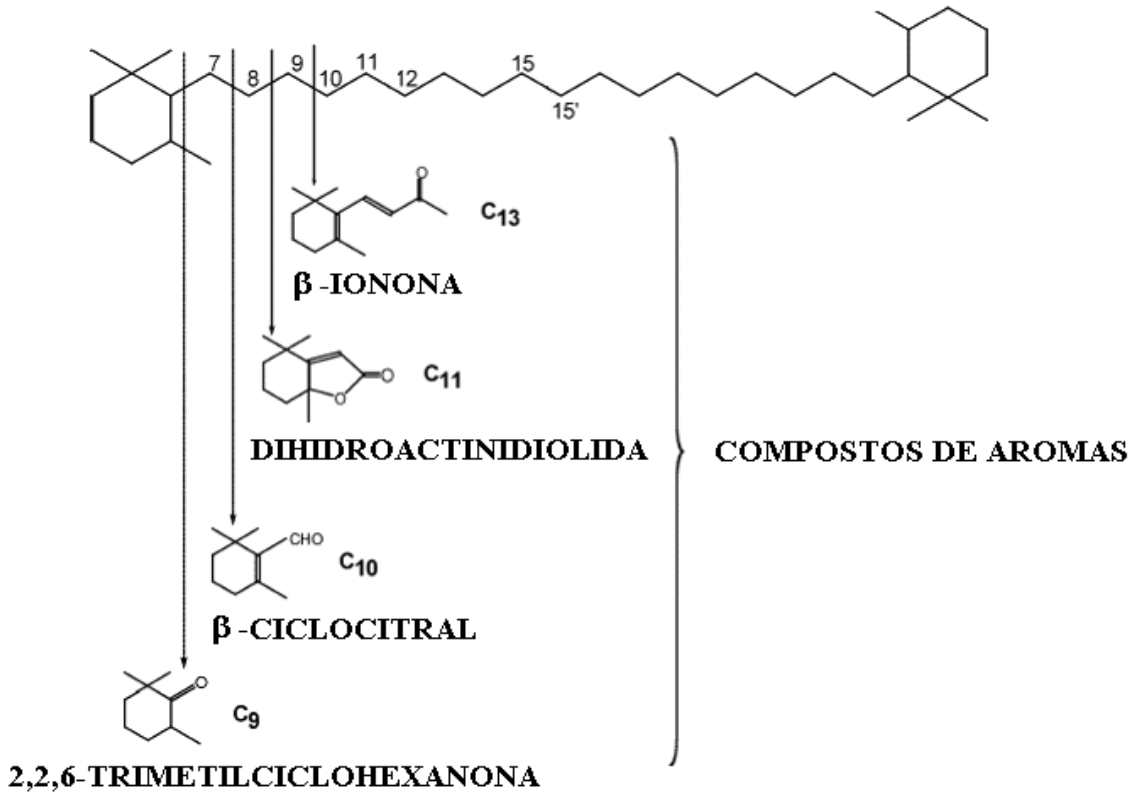

Figura 2 - Produtos da clivagem de carotenóides (Rodríguez-Bustamante; Sánchez, 2007).

Dos resultados encontrados, vinte linhagens apresentaram a capacidade de metabolizar o β-caroteno, e a bactéria epifítica Gram negativa LB398APD, isolada de cenoura, se destacou na produção dos compostos de aroma derivadas de carotenóides (Figura 3). Dentre os compostos produzidos pela linhagem, destaca-se a produção de β-ciclocitral, um composto volátil derivado dos carotenóides que nas plantas, têm a função anti-fungicida, de afastamento de herbívoros e até mesmo atrair agentes polinizadores (Weeks, 1986). Uma otimização da produção deste composto de aroma pela linhagem LB398APD foi realizada e reportada no próximo capítulo.

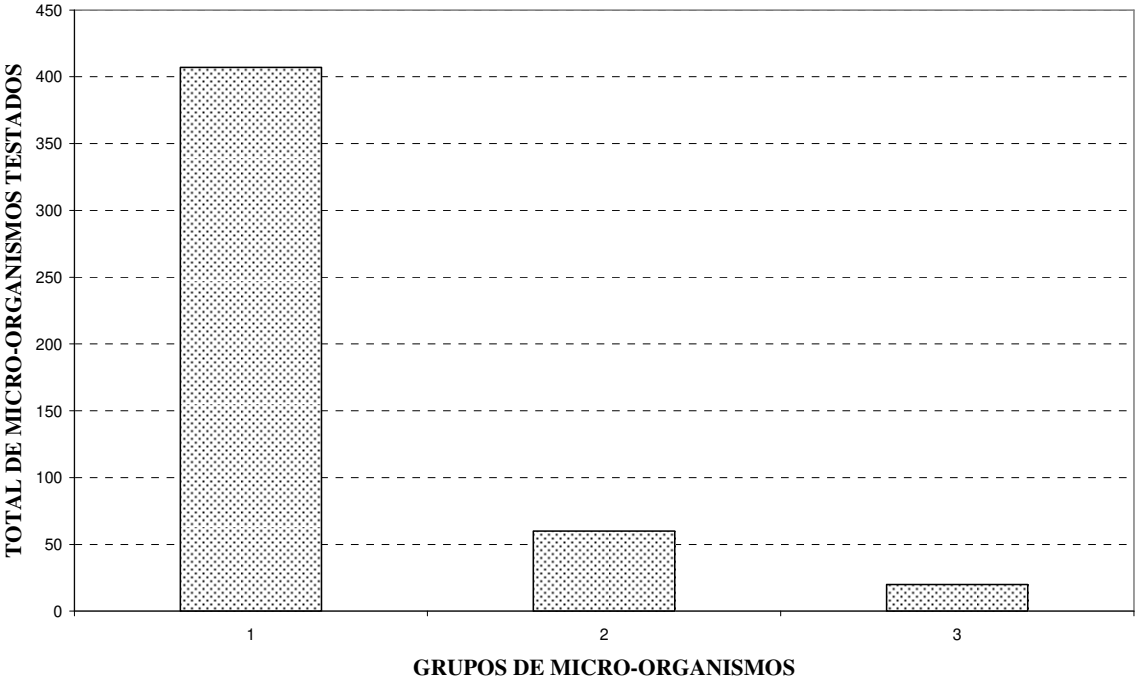

Figura 3 – Sendo: **(1)** micro-organismos isolados e testados quanto a capacidade de metabolizar β-caroteno em placa; **(2)** micro-organismos selecionados devido a formação de halo de degradação do β-caroteno em placa e **(3)** micro-organismos que produziram compostos voláteis pela biotransformação do β-caroteno em meio líquido.

3.2. Estudo das enzimas extracelulares da linhagem LB398APD

No presente estudo, o isolamento e a seleção de micro-organismos potencialmente biotransformadores de β-caroteno foi realizado, e a formação de β-ciclocitral foi evidenciada pela linhagem LB398APD. No processo de biotransformação, o crescimento da linhagem ocorre em meio de cultura rico em nutrientes (YM) e após 24 horas de crescimento, o sobrenadante é descartado e a biomassa é transferida para meio mineral contendo o substrato para o processo de biotransformação (veja tópico 2.4). No entanto, para a produção de enzimas extracelulares, a biomassa é descartada pois as enzimas

extracelulares estão contidas no sobrenadamente do meio de cultura. Desta forma, avaliar as enzimas extracelulares produzidas pela linhagem LB398APD é interessante para verificar uma possível co-produção de enzimas de interesse industrial aliado ao processo de biotransformação, tornando o processo biotecnológico mais factível, pelo fato de reduzir custos pela produção de dois compostos de interesse: aromas e enzimas. As aplicações das enzimas são variadas, podendo ser usadas na indústria de detergentes (proteases e lipases), de alimentos (amilases e proteases) e de couro (proteases e lipases) (Nielsen; Oxenboll, 1998).

Lipases são enzimas que catalisam a hidrólise de triglicerídeos para di e monoglicerídeos, glicerol e ácidos graxos; e em condições de baixa atividade de água, catalisam as reações inversas (Sharma; Chisti; Banerjee, 2001). As amilases formam o principal grupo de enzimas utilizado na indústria de alimentos, principalmente em panificação (Bon; Pereira, 1999). São responsáveis pela hidrólise do amido e são classificadas em várias formas, dependendo de como atuam sobre as moléculas de amido. As proteases, por sua vez, são usadas em processos industriais de tratamento de couro e peles, na formulação de detergentes, na indústria de alimentos, assim como na panificação, juntamente com as amilases, sendo responsáveis pela hidrólise do glúten e no processamento de carnes, conservas e peixes (Bon; Pereira, 1999). Incluem as proteinases, as quais catalisam a hidrólise da molécula de proteína em fragmentos grandes, e as peptidases, que hidrolisam estes fragmentos de polipeptídeos até chegarem a aminoácidos (Frazier; Westhoff, 1988).

Com este intuido, a produção de lipase alcalina, protease e amilase foi determinada através da mensuração do tamanho do halo característico ao redor do inóculo em meio apropriado para cada enzima. Metodologia semelhante é amplamente descrita na literatura

para seleção de micro-organismos produtores de lipases (Prazeres; Pastore, 2006), proteases (Souza; Oliveira; Andrade, 2008) e amilases (Lealem; Gashe, 1994; Souza; Oliveira; Andrade, 2008). Contudo, no presente experimento, a atividade enzimática não foi realizada devido a baixa produção destas enzimas extracelulares.

Os testes em placa são considerados ideias para uma seleção inicial de micro-organismos produtores de enzimas, onde o diâmetro do halo é mensurado e comparado ao tamanho da colônia. Desta forma, os testes conduzidos com a linhagem LB398APD demonstraram uma baixa produção de lipase alcalina extracelular e amilase, evidenciados pela formação de halo ao redor da colônia inferior a 0,2 cm, e crescimento mínimo da linhagem nesses meios de cultura. Lealem e Gashe (1994) recomendam um valor do índice de atividade enzimática $\geq 2,0$ para mostrar a habilidade do micro-organismo em degradar amido em meio sólido, onde o índice enzimático (IE) representa o diâmetro do halo de degradação/ diâmetro da colônia. Com relação a lipase, trabalhos realizados por Colen *et al.* (2005) e Golçalves (2007), consideram cepas que apresentam IE maior que 1,2 e 1,0, respectivamente, como promissoras para a produção de lipase.

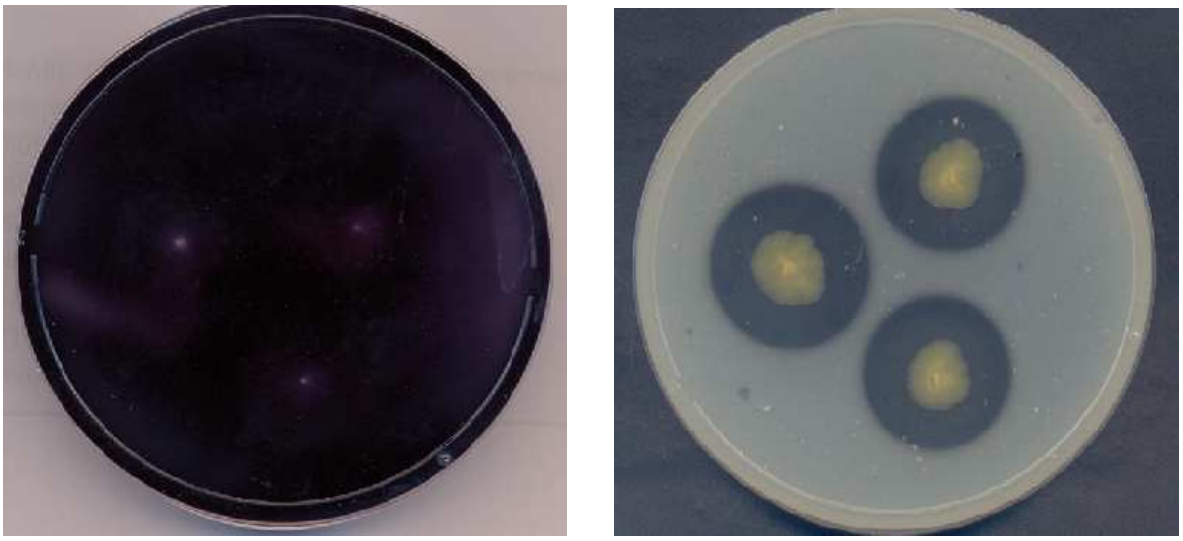

Figura 4 - Linhagem LB398APD para avaliação de enzimas amilolíticas (A) e enzimas proteolíticas (B).

A produção de protease pela linhagem LB398APD, por sua vez, demonstrou um halo significativamente maior em comparação as outras duas enzimas, com IE = 0,9 (Figura 4). O valor relativamente baixo desse índice impossibilita uma possível aplicação industrial desta enzima.

4. CONCLUSÕES

O isolamento e seleção de micro-organismos potencialmente biotransformadores de carotenóides em compostos voláteis foi realizada com sucesso neste trabalho. A extração e concentração dos compostos de aromas voláteis derivados de carotenóides foi realizado através do uso de micro-extração em fase sólida, e a formação de β-ciclocitral foi evenciado através de CG-DIC. O estudo de três enzimas (amilase, protease e lipase) para aliar uma co-produção entre compostos foi realizado para a linhagem selecionada (LB398APD), porém a mesma não apresentou capacidade para produção destas enzimas extracelulares em

quantidades que sejam interessantes para processo industrial. Por fim, a técnica de SPME é reportada pela primeira vez para ensaios de biotransformação de carotenóides.

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CAPÍTULO 3

CAPÍTULO 3

RESPONSE SURFACE METHODOLOGY TO EVALUATION THE PARAMETERS INVOLVED IN THE BIOTRANSFORMATION OF β-CAROTENE BY LB398APD STRAIN

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ABSTRACT

Natural volatile compounds structurally derived from carotenoids are considered interesting to flavor and fragance industries due to their often low flavor tresholds and its industrial significance. In this paper, the response surface methodology (RSM) to evaluation the parameters involved in the biotransformations of β-carotene by LB398APD strain was conduced. Moreover, the solid phase microextraction was used to extrain and concentrate the volatiles formed in the headspaces of the experiment.

Key words: biotransformation, tetraterpene, SPME.

1. INTRODUCTION

Isoprenoids, terpenes, or terpenoids are the most structural diverse and abundant compounds in nature, primarily constituting part of essential oils in plants (Rodriguez-Bustamante; Sanchez, 2007). Members of this class of chemicals have carbon structures which can be decomposed into isoprene (C_5H_8) residues and are classified, based on the number of carbons in the molecule, as monoterpenoids (ten carbons), sesquiterpenoids (fifteen carbons), diterpenoids (twenty carbons), triterpenoids (thirty carbons) and tetraterpenoids or carotenoids (forty carbons) (Teisseire, 1994).

Carotenoids are synthesized by bacteria, algae, fungi and green plants (Sandmann, 2001), and recently, the aroma compounds derived from this terpene has been studied in several areas due to their biological importance (Janakiram *et al.*, 2008; Duncan *et al.*, 2004; Tatman *et al.*, 2002; Lee *et al.*, 2003), low threshold values and characteristic aroma notes (Rodríguez-Bustamente; Sanchez, 2007).

Nowadays, flavours represent over a quarter of the world market for food additives and most of the flavouring compounds are produced *via* chemical synthesis or by extraction from natural materials. However, the disadvantages of both methods and the increasing interest in natural products have directed many investigations towards the search for other strategies to produce natural flavours (Longo; Sanromán, 2006). The biotransformation can be considered an advantageous alternative for chemical synthesis, since it proceeds under mild conditions, has an elevated regio and enantioselectivity, does not generate toxic wastes and the products obtained can be labeled as "natural" (Janssens *et al*., 1992; Giri *et al*., 2001; Serra *et al*., 2005; Leuenberger, 1990). On the other hand, the production of natural flavours by direct extraction from plants is also subject to various problems, including these

raw materials often contain low concentrations of the desired compounds, making the extraction expensive (Longo; Sanromán, 2006). In this context, studies envolving the enzymatic and biotransformative procedures to produce aroma compounds derived from carotenoids has been evaluated recently (Zorn *et al.*, 2003; Sanchez-Contreras *et al.*, 2000; Maldonado-Robledo *et al.*, 2003; Baldermann *et al*, 2005; Zorn *et al.*, 2004; Scheibner *et al.*, 2008).

Solid phase micro extraction (SPME) technique is a simple and effective desorption and adsorption technique that is used for concentrating volatile or non volatile compounds in liquid samples or head spaces, and does not require the use of solvents. It is also compatible with gas chromatography (Alpendurada, 2000). The other techniques of extracting liquid samples are different from SPME due to their use of solvents and their need of more time of analysis, which generates more waste and the sample is more exposed. Some studies using SPME in biotransformation processes can be found in the literature recently using citronelol (Demyttenaere; Vanoverschelde; De Kimpe, 2004) and limonene (Bicas *et al.*, 2008; Demyttenaere; Van Belleghem; De Kimpe, 2001) as substrate.

Although relatively little is known about the microbial conversion of carotenoids into aroma compounds, the aim of this paper was to evaluate the parameters envolved in the process of the biotransformation using the LB398APD straim. For this, a screening design methodology (PB-16) followed by factorial design was proposed to evaluate the parameters involved in the biotransformation procedure. Solid phase microextration (SPME), as technique for the extraction and concentration of the volatile compounds formed in the biotransformation assay, was used in this study.

2. MATERIALS AND METHODS

2.1. General

All of the solutions containing β-carotene were prepared freshly before use, to avoid carotenoids degradation by heat or light. Standard sterile techniques were used and all cultivations were conducted in the complete absence of light. The experiments took place in the Laboratory of Bioflavors at Unicamp (Campinas-SP, Brazil).

2.2. Microorganism

The microorganism LB398APD used in this study was isolated from carrot (*Daucus carota* L.) commercialized in Campinas-SP (Brazil). The strain was selected in experiments envolving the use of solid phase microextraction to extract and concentrate the volatiles compounds formed in the biotransformation (Dionísio *et al.*, 2009a).

2.3. Chemicals

β-Carotene (~97 %), β-cyclocitral (~90 %) and tween 40 were purchased from Aldrich Chemical Company. All of the chemicals and solvents were analytical grade.

2.4. Inoculum

The strain LB398APD was used to the biotransformation process. A 24 h culture grown on agar in a Petri dish was transferred to 250 mL Erlenmeyer flasks each containing 50 mL of Yeast Malt medium (YM). After 24 h incubation at 30 ºC/ 150 rpm, the cultures were centrifuged at 10,000 rpm/ 5 ºC during 15 min and the cell mass absorbance was adjusted (Abs~0.8 at 600 nm).

2.5. Optimization experiments

A study was carried out in order to define the effects of the substrate concentration, cultivation conditions, medium composition and inoculum on the area of β-cyclocitral peak in GC (response). The strategy used was to run a Plackett-Burman screening design (Rodrigues; Iemma, 2005) with 16 experiments (PB-16) and three center points to estimate the experimental error and select the main parameters (Table 1). The central points were the usual conditions used in the process of biotransformation (see chapter 2), where agitation = 150 rpm; temperature = 30 ºC; carotenoid solution ($g.L^{-1}$: 0.2 of β-carotene; 2 of tween 40 and 3.35 % of ethanol) and a mineral medium (in $g.L^{-1}$: $MgSO_4$ =0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 and Fe_2SO_4 = 0.01).

Table 1 - Variables and levels evaluated in the screening design.

Variables	Codified	Levels		
		-1	**0**	**+1**
Medium composition				
$MgSO_4$ ($g.L^{-1}$)	***a***	0	0.5	1.0
$NaNO_3$ ($g.L^{-1}$)	***b***	0	3.0	6.0
K_2HPO_4 ($g.L^{-1}$)	***c***	0	1.0	2.0
KCl ($g.L^{-1}$)	***d***	0	0.5	1.0
Fe_2SO_4 ($g.L^{-1}$)	***e***	0	0.01	0.02
Solution of β-carotene				
Alcohol (% $v.v^{-1}$)	***f***	2.6	3.35	4.1
Tween 40 ($g.L^{-1}$)	***g***	1.0	2.0	3.0
β-carotene ($g.L^{-1}$)	***h***	0.1	0.2	0.3
Cultivation conditions				
Temperature (ºC)	***i***	25	30	35
Agitation (rpm)	***j***	0	150	300
Inoculum				
Inoculum (Abs at λ=600 nm)	***k***	~0.6	~3.6	~6.6

To define the optimal process conditions, a central composite design was carried out using the parameters selected (Table 2). The second optimized the two statistically significant variables (agitation and concentration of substrate) in a 2^2 Central Composite Design with three central points, totalizing 11 experiments.

The variables were coded according to the following equation:

$$xi = (Xi - X_0) / \Delta X1 \quad \text{(Equation 1)}$$

Where: xi is the dimensionless value of an independent variable, Xi is the real value of an independent variable, X_0 is the real value at the central point and ΔX_1 is the difference between the real value at level +1 (X_1 which corresponds to x1 = +1) and the central point (X_0 which corresponds to $x_0 = 0$).

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

Variable	Levels				
	- 1.41	**- 1**	**0**	**+ 1**	**+ 1.41**
Agitation (rpm)	0	45	160	275	320
β-carotene (g. L^{-1})	0.5	0.65	1.0	1.35	1.5

2.6. Biotransformation procedure

The cell mass (inoculum) was aseptically transferred to 100 mL sterile screw-top flasks each containing 20 mL of minimal medium (the composition varied according to the experimental designs as shown in Tables 1 and 2). The solution containing β-carotene was then prepared, by homogenizing β-carotene in Tween-40 (2 $g.L^{-1}$), dissolving in alcohol (3.35 % $v.v^{-1}$), with the proportion of each component also varying as shown in Tables 1 and 2, and then completing with sterile distilled water to a volume of 1 mL. Each flask was incubated in a shaker under their respective conditions. In previous experiments, the

biotransformation was conduced up to 144 h. However, as the transformation rate reached its maximum in 110 h (data not shown), it was decided to carry out all the experiments only in this time.

2.7. GC-FID and GC-MS analysis

A SPME device (Supelco, Bellefonte, PA, USA) containing a fused-silica fiber (10 mm in length) coated with a 75 μm layer of Carboxen/Polydimethylsiloxane (CAR-PDMS) was used. The fiber was preconditioned in the GC injection port at 280 ºC for 60 min.

Analyses were performed using an Agilent 7890 A gas chromatograph (GC) equipped with a flame ionization detector (FID). Desorption proceeded in the injection port of the GC for 6 min at 250 °C in split less mode. After, the fiber remained a further 4 min in the injector port to eliminate the possibility of any carry-over of analyte between samples. The volatile compounds were separated in a 30 m x 0,25 mm HP-5 (J&W Scientific, Folsom, California, USA). The oven temperature program was: 40 °C for 1 min, and them increase to 110 °C at 5 °C min^{-1} where it was held for 5 min, and increase to 220 °C at 10 °C.min^{-1} where it was held for 5 min. Helium was the carrier gas at flow rate of 1,0 mL.min^{-1} and the detector temperature was 250 °C. The quantification was done using a β-cyclocitral standard.

GC/MS analyses were carried out in a GC/MS system (Shimadzu GC-17A/QP-5000 high performance quadrupole, Japan) under the following instrumental conditions: column: HP-5 MS (5 % phenyl/95 % dimethylpolysiloxane) fused silica capillary column (30m x 0.25mm i.d. x 0.25 μm) from J&W Scientific (EUA), injector: split 1:5, temperature: 250° C; purge time: 1.00 min., purge flow: 20 mL.min^{-1}; helium flow rate: 1.0 mL.min^{-1}, oven: 40 °C for 1 min, and them increase to 110 °C at 5 °C min^{-1} where it was held for 5 min, and

increase to 220 °C at 10 °C.min^{-1} where it was held for 5 min; transfer line temperature: 240 °C, energy of impact: +70 eV, 35-350 m/z). The tentative identification of components was made by comparing spectra with Adams and NIST 2005 mass spectral database libraries, with similarities higher than 85 %.

2.8. Data analysis

The results were analyzed in the software Statistica^{TM} version 7.0, considering a significance level of 5 % ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1. Screening of the variables (Plackett-Burman experiments)

In preliminary studies, more than 400 strains were tested for their capacity to degradate β-carotene contained in the culture medium (Dionísio *et al.*, 2007). In sequence, the strain LB398APD was selected to bioconvert the β-carotene into β-cyclocitral after one day of contact with the solution, and the highest concentration occurred after 110 h of transformation (data not shown). The volatile compound is formed when the enzymatic cleavage occurre in the 7-8 position of the tetraterpene. Figure 1 shows the structure of the compound reported in this investigation.

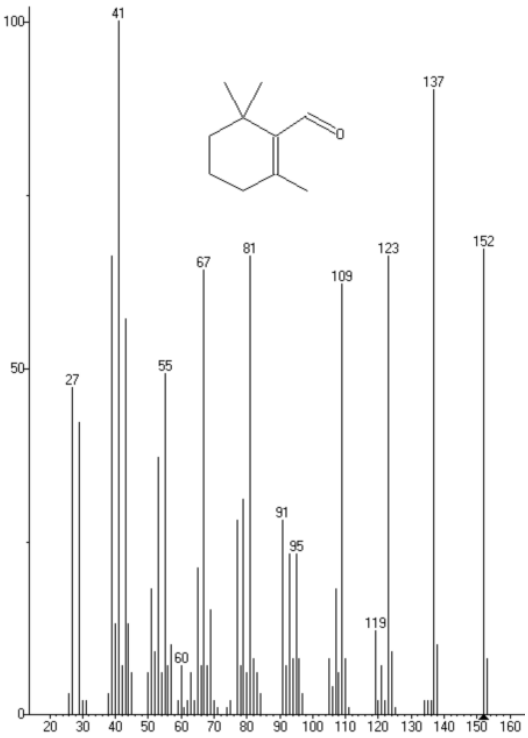

Figure 1 - Mass spectra of β-cyclocitral

In the present work, for the screening of the variables involved in the biotransformation of the β-carotene, a PB-16 was used. The center points for the screening design were chosen based on the conditions usually applied for this biotransformation: a mineral medium (in $g.L^{-1}$: $MgSO_4$ = 0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 and Fe_2SO_4 = 0.01) commonly used in assays of biotransformation (Dionisio *et al.*, 2009b; Maróstica; Pastore, 2007), a solution of carotenoid containing 0.2 $g.L^{-1}$ of β-carotene emulsified in 2 $g.L^{-1}$ of tween 40 and 3.35 % of ethanol (adapted by Zorn *et al.*, 2003), and the cultivation conditions usually applied in assays of biotransformation (Dionisio *et al.*, 2009a).

The levels of the variables tested in the screening design are described in Table 1. These codified values were applied in the PB-16 Plackett-Burman matrix (Table 3).

Table 3 - Plackett-Burman design matrix (PB-16) and the β-cyclocitral GC-FID area after 110 h of biotransformation.

	a	***b***	***c***	***d***	***e***	***f***	***g***	***h***	***i***	***j***	***k***	***Area***
1	+1	-1	-1	-1	+1	-1	-1	+1	+1	-1	-1	218.20
2	+1	+1	-1	-1	-1	+1	-1	-1	+1	+1	-1	315.53
3	+1	+1	+1	-1	-1	-1	+1	-1	-1	+1	+1	276.26
4	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	+1	307.11
5	-1	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	70.62
6	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	-1	147.88
7	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	126.62
8	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	213.72
9	+1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	167.47
10	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	-1	528.96
11	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	+1	287.95
12	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	82.84
13	-1	+1	-1	-1	+1	+1	-1	+1	-1	+1	+1	779.24
14	-1	-1	+1	-1	-1	+1	+1	-1	+1	-1	+1	52.08
15	-1	-1	-1	+1	-1	-1	+1	+1	-1	+1	-1	492.21
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	85.86
17	0	0	0	0	0	0	0	0	0	0	0	456.99
18	0	0	0	0	0	0	0	0	0	0	0	436.72
19	0	0	0	0	0	0	0	0	0	0	0	437.72

The identification and levels of each variable are described in Table 1. SPME fibers were exposed into the *vial* for 30 min at 30 °C

The results obtained in this experiment demonstrated that only the concentration of β-carotene and the agitation of the system were statistically significant for the response

($p<0.05$) (Table 4) and, for the next step, the others variables are not used in a central composite design experiment and remained fixed in the central points (temperature = 30 ºC; 2 $g.L^{-1}$ of tween 40 and 3.35 % of ethanol to prepared the β-carotene solution, and mineral medium compositium (in $g.L^{-1}$): $MgSO_4$ = 0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 and Fe_2SO_4 = 0.01).

Table 4 - Estimates the effects of the parameters analyzed after 110h of biotransformation.

Factor	Effect	SE	t(7)	*p* value
Mean	**288.6305**	**28.59381**	**10.09416**	**<0.0001**
a	-86.8162	62.31876	-1.39310	0.206229
b	123.8838	62.31876	1.98790	0.087160
c	-47.9237	62.31876	-0.76901	0.467036
d	-98.3937	62.31876	-1.57888	0.158373
e	22.9513	62.31876	0.36829	0.723544
f	3.5538	62.31876	0.05703	0.956119
g	-34.0288	62.31876	-0.54604	0.601996
h	**229.6463**	**62.31876**	**3.68503**	**0.007808**
i	-88.1563	62.31876	-1.41460	0.200091
j	**208.6488**	**62.31876**	**3.34809**	**0.012283**
k	13.5062	62.31876	0.21673	0.834601

Where (***SE***) Standard error, (***a***) $MgSO_4$ ($g.L^{-1}$), (***b***) $NaNO_3$ ($g.L^{-1}$), (***c***) K_2HPO_4 ($g.L^{-1}$), (***d***) KCl ($g.L^{-1}$), (***e***) Fe_2SO_4 ($g.L^{-1}$), (***f***)alcohol (% $v.v^{-1}$), (***g***) tween 40 ($mg.L^{-1}$), (***h***) β-carotene ($mg.L^{-1}$), (***i***) temperature (ºC), (***j***) agitation (rpm), (***k***) absorbance of inoculum. Parameters in bold are statistically significant for the response ($p<0.05$), considering the residual SS.

The medium composition was studied in the PB-experiments, because it usually displays an important role in biotransformation processes, being one of the main factors responsible for alterations in the yield (Adams *et al.*, 2003). In this paper, an usual culture medium for biotransformation was tested (Maróstica; Pastore, 2007), however all the components analyzed were not statistically significant.

The concentration of the inoculum is usually an important factor in the fermentation process. However, it had no statistical significant influence considering the levels tested. Bicas *et al.* (2008), using a similar sequential Plackett-Burman design, evaluated the parameters involved in the biotransformation of limonene into α-terpineol. The results showed that the inoculum size presented not significance in the levels tested.

It is known that the medium temperature influence biological reactions. Moreover, medium agitation promotes development of the microorganism and cell-substrate interactions. The temperature showed no effect and was not used in the central composite design, being fixed in 30 ºC in the DCCR experiments. On the other hand, the agitation presented a positive effect ($p<0.05$) in the β-cyclocitral area, probably related to the increase in cell-substrate contact.

Another parameter tested in the experiment is the solution containing the substrate for the biotransformation, composed of β-carotene, alcohol and the emulsifier tween-40. Basead in the PB results, the β-carotene concentration had a positive effect on the levels tested, and the optimal concentration may be situated at values above the maximum value tested in the screening design and an ampler range is suggested.

3.2. Optimization using a Central Composite Design (CCD)

According to the variables screened and considering the levels tested, the significant factors ($p<0.05$) for the biotransformation of β-carotene into β-cyclocitral, were *agitation* and *substrate concentration*. These two variables were worth considering in the further optimization design analyzed, and the levels are described in Table 2. The optimization occurred using a 2^2 central composite design with three center points (Table 5) and the central points of the PB-16 were used for the other variables no significants.

Table 5 - Central composite design matrix and the β-cyclocitral GC-FID area after 110h of biotransformation by the LB398APD stran.

Assays	Agitation (rpm)	β-carotene ($g.L^{-1}$)	Area
1	-1	-1	404.90
2	+1	-1	612.38
3	-1	+1	754.97
4	+1	+1	1282.91
5	-1.41	0	676.44
6	+1.41	0	926.32
7	0	-1.41	570.97
8	0	+1.41	1279.72
9	0	0	1048.03
10	0	0	1036.89
11	0	0	1106.61

The levels of each variable are described in Table 2. (***Area***) β-cyclocitral area peak.

Considering the results obtained in this study (Table 5), the importance of the agitation and concentration of β-carotene for the biotransformation procedure was confirmed. The area of the assay four (320 rpm and 1.35 $g.L^{-1}$ of β-carotene) is three times bigger than the area of the assay one (0 rpm and 0.5 $g.L^{-1}$ of β-carotene).

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 (Table 6). Considering the intervals tested a model for the two variables based on the following formula (Equation 2) was proposed.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \quad \textbf{(Equation 2)}$$

Where: Y = dependent variable, b_0 is the average, b_1 and b_2 are the linear regression coefficients for the variables x_1 and x_2, respectively, b_{11} and b_{22} are the quadratic regression coefficients for the variables x_1 and x_2, respectively, and b_{12}, are the regression coefficients for the interactions of the variables x_1 and x_2.

Table 6 - The least-squares and significances of the regression coefficients of the model parameters.

Parameter	RC	SE	t(5)	*P* value
Mean	1063.843	52.23065	20.36818	0.000005
Agitation	**136.100**	**31.98461**	**4.25519**	**0.008052**
Agitation^2	**-156.125**	**38.06930**	**-4.10107**	**0.009345**
Substrate	**252.865**	**31.98461**	**7.90585**	**0.000521**
Substrate^2	-94.142	38.06930	-2.47292	0.056328
1L x 2L	80.115	45.23307	1.77116	0.136743

Where (***RC***) Regression coefficient and (***SE***) standard error. Parameters in bold are statistically significant for the model ($p<0.05$).

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 3 – Predicted and adjusted model for the encoded values

$$\hat{Y} = 1063.84 + 136.1\ x_1 + 252.86\ x_2 - 156.12\ x_1^2$$

The ANOVA (Table 7) demonstrated that the quadratic model adjusted for the process responses was satisfactory. The calculated *F* value was four times higher than the respective listed value, while the P value of the model was lower than 0.001, and the R^2= 0.9242.

Table 7 - ANOVA of the quadratic model.

Variation source	SS	df	SM	F-value	*P* value
Regression	811736.8	4	202934.2	18.28	0.001
Residues	66594.2	6	11099.03		
Total	878331.0	10	87833.1		
R^2=0. 9242				$F_{4;\,6;\,0.05} = 4.53$	

(***SS***) Sun of squares, (***df***) degrees of freedom, (***SM***) mean square.

According to the contour plot (Figure 2), the best conditions for the highest β-cyclocitral recovery was: agitation of 200 rpm and β-carotene concentration of 1.35 $g.L^{-1}$, with the conditions fixed at 3.35 % of ethanol, 2 $g.L^{-1}$ of tween-40, temperature at 30 °C absorbance (λ=600nm) of inoculum ~ 3.6 (drymass ~ 2.62 $g.L^{-1}$) and 110 h-reaction in mineral medium standard.

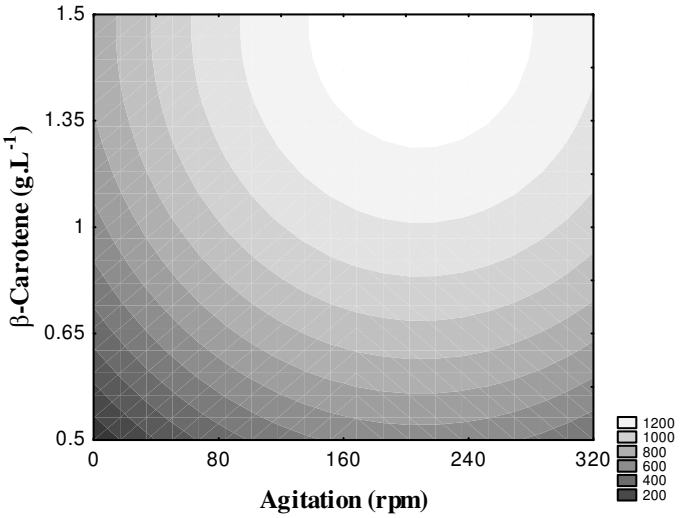

Figure 2 - Contour plot of the β-cyclocitral area after 110 h-biotransformation as a function of β-carotene concentration and agitation.

The quantification was done by calibration using a β-ciclocytral standard. However, the production of β-cyclocitral in the best condition were ~ 10 $mg.L^{-1}$, that were not enough for an industrial application. On the other hand, the biotransformation of β-carotene using a bacterial strain and an the use of the statistical analysis to improve the yields are reported by the first time. Moreover, further studies envolving validation process, β-carotene evaluation by liquid cromatography (HPLC) in the biotransformation experiment, and extraction of the volatiles compounds using a solvent should be performed.

4. CONCLUSION

This paper describes a sequence of experiments applying response surface methodology (RSM) to optimize the biotransformation of β-carotene using the LB398APD strain. A screening design methodology (PB-16) followed by factorial design was proposed to evaluate the parameters involved in the biotransformation procedure. In the fisrt step, a significant influence of substrate concentration and agitation were noted with Plackett-Burman and a central composite design (CCD) was subsequently employed for further optimization. To date, no studies have reported the use of statistical analysis for the biotransformation of β-carotene.

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CAPÍTULO 4

CAPÍTULO 4

FUNGAL BIOTRANSFORMATION OF TERPENES BY Aspergillus sp., Penicillium sp. AND Paecilomyces sp. STRAINS

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ABSTRACT

In this paper, fungal biotransformations of terpenes were investigated. *Aspergillus* sp., *Penicillium* sp. and *Paecilomyces* sp. were tested for their abilities to produce oxidized metabolites by degrading five different substrates (limonene, α-pinene, γ-terpinene, citronellol and α-farnesene). Biotransformation products were only evidenced for the experiments with (*R*)-(+)-limonene and γ-terpinene. Analysis using GC/MS confirmed the accumulation of mainly carvone and carveol, for the biotransformation of limonene. This is the first report on the biotransformation of terpenes using *Paecilomyces* sp.

Keywords: bioflavor, citronellol, α-farnesene, limonene, α-pinene, γ-terpinene.

1. INTRODUCTION

The oxidation of terpenes generates interesting derivatives which have best chemical stability and a stronger odor than their precursors (Krings *et al.*, 2006). These compounds are widely applied in industry, mainly as pharmaceuticals, chemicals, flavors, fragrances, pesticides and disinfectants (Krings *et al.*, 2009). The description of their activity as natural insecticides, antimicrobial agents and their roles in prevention and therapy of several diseases (Crowell *et al.*, 1999) has increased the interest in such compounds.

Biotransformation, carried out by microorganisms or their enzymatic systems, is a biotechnological tool that may be used to produce natural aroma compounds by the oxidation of terpenes (Bicas *et al.*, 2009). This technique is particularly interesting because, unlike traditional chemical processes, biotransformations occur under mild conditions, are enantioselective, do not generate toxic wastes and they may summarize in a single stage a series of steps of chemical synthesis (Ribbons, 1990; Krings; Berger, 1998). Therefore, considering the increasing demand for natural and environmentally friendly products, biotransformation of terpenes represents a very attractive alternative to chemical aromas.

Many examples of biotransformation of inexpensive terpene hydrocarbons, *e.g.* limonene and α-pinene, are found in the literature (Bicas *et al.*, 2008), being limonene the most studied (Duetz *et al.*, 2003; Maróstica Junior; Pastore, 2007; Bicas *et al.*, 2008). However, the biotransformation of some other terpenes, *e.g.* citronellol, *γ*-terpinene and farnesene, into aroma compounds has not been fully explored yet and only few studies are available. In this paper, nine newly isolated microorganisms from the genus *Aspergillus* sp. (LB403APD*,* LBPKAPD*,* LB155AP, LB160APD), *Penicillium* sp. (LB326AP, LB47APD,

LB99APD) and *Paecilomyces* sp. (LB91APD, LB116APD) were screened for their ability to biotransform limonene, α-pinene, γ-terpinene, citronellol and α-farnesene for the production of bioflavors.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemical standards used in this study were: (1*S*)-(-)-α-pinene (~98 %, Aldrich, Milwaukee, WI, USA Aldrich), (*R*)-(+)-limonene (~98 % purity, supplied Fluka, St. Gallen, SG, Switzerland), (*R*)-(+)-citronellol (~95 % purity, supplied by Aldrich, Milwaukee, WI, USA Aldrich), γ-terpinene (97 % purity, supplied by Aldrich, Milwaukee, WI, USA Aldrich) and α-farnesene (~97 % purity, supplied by Aldrich, Milwaukee, WI, USA Aldrich). All the chemicals and solvents were of the best available commercial grade.

2.2. Microorganisms

The strains *Aspergillus* sp. (LB403APD, LBPKAPD, LB155AP and LB160APD), *Penicillium* sp. (LB326AP, LB47APD, LB99APD) and *Paecilomyces* sp. (LB91APD and LB116APD) from the Bioflavours Laboratory Culture Collection, were cultivated and conserved by periodic replications (once a week) on Yeast-Malt agar ($g.L^{-1}$: bacteriological peptone = 0.5; glucose = 1.0; malt extract = 0.3; yeast extract = 0.3 and agar = 2.0). All the strains were isolated from Brazilian fruits and vegetables rich in terpenes (*Daucus carota*, *Citrus reticulate*, *Prunus persica*, *Mangifera indica*, *Ipomoea batatas* and *Eruca sativa*).

2.3. Inoculum for the biotransformation assays

A piece of agar (~2 cm^2) with a pre-grown culture of the fungal strains (72 h old) was transferred to a 250 mL conical flask filled with 50 mL of yeast and malt (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 h 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).

2.4. Biotransformation procedure

The biomass obtained was distributed to a 250 mL conical flask filled with 50 mL of mineral medium (MM) (in $g.L^{-1}$: $MgSO_4$ =0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 and Fe_2SO_4 = 0.01) (Maróstica Junior; Pastore, 2007). Subsequently, 0.5 % (v/v) of the substrate tested (α-pinene, limonene, citronellol, γ-terpinene or farnesene) was added. The flasks were incubated at 30 °C and 150 rpm and at fixed intervals (8, 24, 48, 72 and 96 h 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.

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

Samples were analyzed directly by gas chromatography equipped with flame ionization detector (GC/FID), using decane as internal standard. 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 (carrier gas He at 0.8 mL/min). 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 (Bicas *et al.*, 2008).

GC/MS analyses were carried out in a GC-MS system (Shimadzu GC-17A/QP-5000 high performance quadrupole, Japan) under the following instrumental conditions: column: HP-5 MS (5 % phenyl/95 % dimethylpolysiloxane) fused silica capillary column (30m x 0.25 mm i.d. x 0.25 μm) from J&W Scientific (EUA), injector: split 1:5, temperature: 250 °C; purge time: 1.00 min., purge flow: 20 $mL.min^{-1}$; helium flow rate: 1.0 $mL.min^{-1}$, oven: 80 °C to 3 min and 20 °C min^{-1} up to 200 °C, for 4min; transfer line temperature: 240 °C, energy of impact: +70 eV, 35-350 m/z). A mixture of aliphatic hydrocarbons (C5–C20) (PolyScience, Illinois, EUA) was injected under the above temperature program to calculate the retention index (I) of each compound. The tentative identification of components was made by comparing spectra with Adams (2007) and 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.

3. RESULTS AND DISCUSSION

3.1. Biotransformation of limonene

Table 1 show the biotransformation products in the liquid mediums after 72 hours after the addition of the substrate.

Table 1 - Main products accumulated after 72 h of biotransformation of limonene by the selected strains.

Strain	Microorganism	Source	Compounds*
LB155APD	*Aspergillus* sp.	*Prunus persica*	Carveol Carvone
LB116APD	*Paecilomyces* sp.	*Eruca sativa*	Carveol Limonene-1,2-diol
LBPKAPD	*Aspergillus* sp.	*Citrus reticulate*	Carveol Limonene-1,2-diol
LB160APD	*Aspergillus* sp.	*Prunus persica*	Carveol Limonene-1,2-diol
LB326APD	*Penicillim* sp.	*Mangifera indica*	Dihydrocarveol Dihydrocarvone Carveol Carvone

* Positive identification by GC/MS using standards. Extraction and chromatography conditions are described in the text.

The biotransformation of limonene occurred mainly in the first 72 h of reaction, as shown in Figure 1 for carvone. After this period, the concentration of all accumulated products remained stable or decreased. Therefore it was decided to show the main accumulated products at the time of 72 h (Table 1).

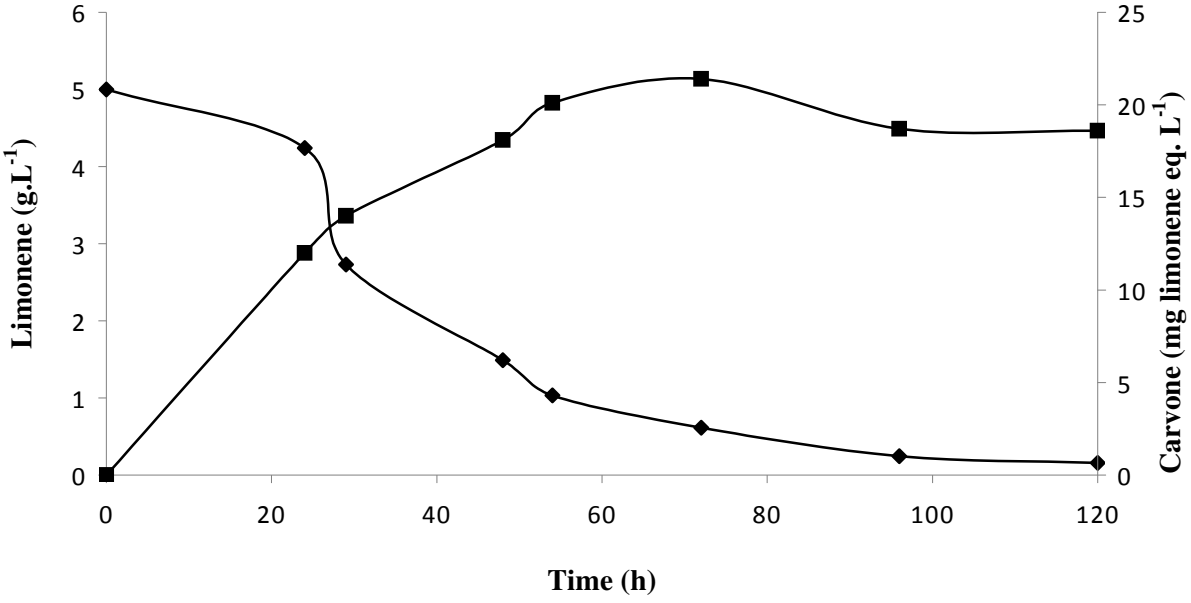

Figure 1 - Time course of carvone production (■) from limonene (◆) by LB326APD strain.

It was possible to observe that the most recurrent metabolites for the biotransformation of limonene were carveol and/or carvone (Table 1), indicating that the microorganisms *Aspergillus* sp. (strains LB155APD, LBPKAPD, LB160APD), *Paecilomyces* sp. (strains LB116APD) and *Penicillium* sp. (strain LB326APD) showed a similar pathway to that already described for the strains *Rhodococcus opacus* PWD4 (Duetz *et al.*, 2001), *Pleurotus sapidus* (Onken; Berger, 1999) and *Rhodococcus erythropolis* DCL14 (Tecelão *et al.*, 2001).

Figure 2 shows the results of GC-MS of carveol and carvone production by the strain LB155APD that seemed capable to oxidize limonene in C6. Another strain, LB326APD (*Penicillium* sp.), also accumulated trace amounts of dihydrocarvone, and intermediate of the same pathway (Bicas *et al.*, 2008). 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). Carvone is one of the most distinguished commercial flavor compounds, with a strong mint odor. Besides its use in the fragrance and flavor industry, it may also be applied as potato sprouting inhibitor, antimicrobial agent, building block, biochemical environmental indicator and also it has applications in the medical field, justifying the research aimed to increase the world production of this monoterpene (De Carvalho; Da Fonseca, 2006).

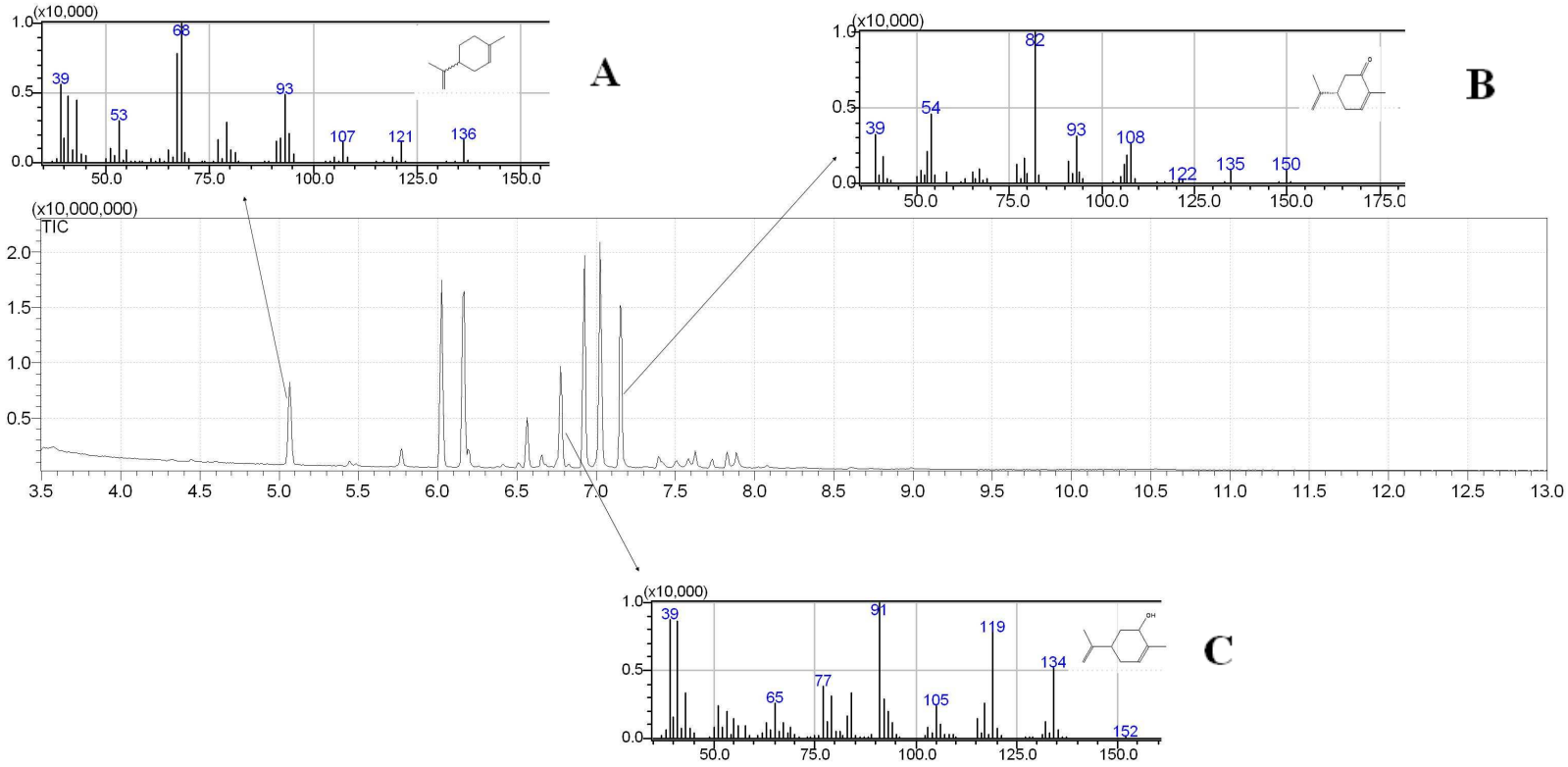

Figure 2 - Products observed in the biotransformation of limonene by the strain LB155APD, after 72 hours: (A) Limonene; (B) Carvone and (C) Carveol.

One of the most promising process for the production of carvone by microbial biotransformations were described for the strains *Rhodococcus opacus* PWD4 and *R. globerus* PWD8, which converted limonene to *trans*-carveol and subsequently to carvone, in an overall yield of 94-97 % (Duetz *et al.* 2001). Although the amount of carveol and carvone accumulated in our process (~12-22 mg limonene equiv. per liter) was much lower than the concentration expected for a viable industrial process, it was reported here, at the best of our knowledge, the first description of a biotransformation of limonene by a *Paecilomyces* strain.

Limonene-1,2-diol was another common product for three strains (LB116APD, LBPKAPD and LB160APD) (Table 1). This alcohol is an intermediate for a energy-producing pathway already observed for *Rhodococcus erythropolis* DCL14 (Van der Werf *et al.*, 1999) and *Pseudomonas fluorescens* (Bicas *et al.*, 2008), which involves the epoxidation of limonene as initial step. However, the others intermediates of this pathway have not been detected, even in trace amounts.

Low products concentrations in these cases could be justified due the toxicity and inhibitory effect of limonene on microorganisms, reported by several studies as one of the main problems commonly encountered in monoterpenes biotransformation experiments (Bowen, 1975; Chang; Oriel, 1994). In addition, some researchers have noticed that limonene inhibits the energy-producing process (oxidative phosphorylation) in cells, and causes membrane damage in microorganisms (Uribe; Pena, 1990).

3.2. Biotransformation of γ-terpinene, α-pinene, citronellol and α-farnesene

In these experiments, it was observed biotransformation products only for (*R*)-(+)-limonene as substrate, using the strains of *Aspergillus* sp., *Penicillium* sp. and *Paecilomyces* sp. None of the other substrates, *i.e.* γ-terpinene, α-pinene, citronellol and α-farnesene,

resulted in the accumulation of biotransformation products. However, it is possible to find in the literature some examples for the biotransformation of these products. Bicas and others (2008) describe and review some of the main α-pinene biotransformation processes for the production of flavor compounds. Citronellol can also generate a large amount of metabolites (Ornken; Berger, 1999) and one of the most interesting compounds is rose oxide (Maróstica Junior; Pastore, 2006). Finally, α-farnesene, a sesquiterpene present in many kinds of fruits, has been employed in biotransformation trials for the first time by Krings and co-workers (2006). Four major aroma compounds were formed: one of them, identified as 6-hydroxy-farnesene, with an impacting citrus smell and two other presenting sweet and herbaceous odors and the last one had no smell.

4. CONCLUSION

Interesting aroma compounds, as carveol and carvone could be detected in this study, although their concentrations were yet too low to attract the interest for industrial process. More biochemical engineering studies are needed to increase the yields and the production rates, what is already in course at our lab. The biotransformation of terpenes by a *Paecilomyces* strain described in this work were reported for the first time in literature.

5. ACKNOWLEDGEMENTS

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CAPÍTULO 5

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EVALUATION OF CASSAVA WASTEWATER AND GLYCEROL FOR BIOMASS PRODUCTION IN BIOTRANSFORMATION ASSAYS

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ABSTRACT

The biomass production by the LB398APD strain, which is used in carotenoid biotransformation assays, has been proposed using two agro-industrial by-products as culture media: cassava wastewater and glycerol. Cassava wastewater is a liquid waste from the cassava-processing industry, rich in carbon and nitrogen. Glycerol, in turn, is a residue from the production of biodiesel, which is gaining prominence worldwide. The Response Surface Methodology (RSM) was used in this experiment and the factors concentration of residue, temperature, agitation and concentration of peptone (only for the case of glycerol) were evaluated. The results obtained in this study showed that the cassava wastewater can be used for the development of biomass, with a very interesting production when compared to the usual medium (yeast and malt) tested. On the other hand, glycerol did not demonstrate promising results at the evaluated level.

Keywords: residue, biomass, biotransformation, cassava wastewater, glycerol.

1. INTRODUCTION

The biotechnological production of natural flavors has been driven by consumer demand for natural and healthier products. This interest has increased the number of researches on microbial production of the bioflavors (Demyttenaere; De Kimpe, 2001) and studies, particularly with terpenes, are gaining more space on the worldwide context in order to increase the production of these compounds (Bicas *et al.*, 2009; Bicas *et al.*, 2008;) and to evaluate their potential as functional ingredients (Dionisio *et al.*, 2009a; Maróstica *et al.*, 2009). Berger (2009) recently mentioned the use of unconventional culture media as one of the points to be considered for the biotechnological production of aroma compounds, considering the attractiveness of lower production costs of these ingredients. The use of industrial residue as culture medium is a feasible alternative, since the raw material represents 30 % of total costs in a biotechnological process (Cameotra; Makkar, 1998).

Cassava (*Manihot esculenta* Crantz) pulp is the solid waste generated as a consequence of starch production and its use can be observed in some studies, for example, to improve biomass utilization (Sriroth *et al.*, 2000) and to serve as substrate for microorganism growth in bioconversion processes (Pandey *et al.*, 2000). On the other hand, cassava wastewater is quantitatively the most important liquid residue of the cassava industry and its production is estimated at 30 % by weight of raw material in flour mills (Wosiacki *et al.*, 2000) and represents a substrate with a high content of carbon source.

This effluent is usually discharged into the environment, resulting in a major environmental problem, because it has a large load of organic materials dispersed and in solution. This high organic load causes the decrease of oxygen concentration in the water, with damage to the aerobic forms of life (Wosiaki *et al.*, 2000; Campos *et al.*, 2006). The use

of the cassava wastewater has been reported in many studies, especially for the production of surfactants (Barros *et al.*, 2010; Nitschke; Pastore, 2006; Costa, 2005; Wosiaki *et al.*, 2000).

Glycerol is the principal residue obtained during transesterification of vegetable and animal oils (Silva *et al.*, 2009) and is especially important as a result of the development of biodiesel production. Due to its ample occurrence of glycerol in nature, many known microorganisms can naturally utilize this alcohol as a sole carbon and energy source. These microorganisms have attracted attention because of their potential use in bioconversion of abundant glycerol produced from biodiesel (Solomon *et al.*, 1995; Barbirato; Bories, 1997; Menzel *et al.*, 1997). The use of this by-product adds significant value to the productivity of the biodiesel industry. Actually, a possible low-cost alternative is the use of glycerol as a carbon source in biotechnological processes (Tang *et al.*, 2009; Ciapina *et al.*, 2007; Imandi *et al.*, 2007; Maneerat *et al.*, 2005; Papanikolaou; Aggelis, 2002; Biebl, 2001).

In this context, the main objective of this paper was to evaluate cassava wastewater and glycerol as carbon source for production of biomass for biotransformation assays, specifically for the LB398APD strain, already reported as potential for the biotransformation of β-carotene (Dionisio *et al.*, 2009b).

2. MATERIAL AND METHODS

2.1. Cassava wastewater: Preliminary studies

2.1.1. Substrate preparation

Cassava wastewater was collected in an industry of cassava flour and transported to the processing location at room temperature where it was homogenized, boiled, cooled, centrifuged and stored in a freezer (Barros *et al.*, 2010).

2.1.2. Determination of the cassava medium composition

2.1.2.1. Determination of the reducing sugar contents

Total reducing sugars were quantified according to Somogy (1944) and Nelson (1945) using glucose as the standard solution.

2.1.2.2. Determination of the nitrogen content

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

2.1.2.3. Determination of the mineral content

Cassava medium (1 mL) was incinerated for 3 hours at 500 °C, after which 5.0 mL of 6M HCl was added and evaporated to dryness. The residue was transferred to a 50 mL volumetric flask, water was added to the volume, and it was then shaken and filtered. The minerals were determined using an atomic emission spectrophotometer (Maróstica; Pastore, 2007).

2.1.3. Inoculum

The inoculum was prepared from a 24 hours-old culture of the LB398APD strain. One full loop was transferred to a 250 mL conical flask containing 50 mL of Yeast Malt medium (in $g.L^{-1}$: malt extract = 3; yeast extract = 3; peptone = 5 and glucose = 10) and incubated at 30 °C for 24 hours in a rotary shaker (150 rpm). After the incubation period, the culture was homogenized and the inoculum was standardized by checking the optical density (λ = 600 nm). After, 1 mL was transferred to the conical flasks (250 mL) containing 50 mL

of cassava wastewater (100 %) or YM, and incubated at 30 °C in *shaker* (150 rpm) for up to 144 hours.

2.1.4. Biomass and pH

After 0, 8, 24, 48, 72, 96, 120 and 144 hours (in duplicates) 1 mL of the culture medium was collected, and spectrophotometric analysis was done. Biomass was determined by measuring the optical density of the samples at 600 nm ($O.D._{600}$) and the cell concentration was determined as a function of the optical density, where C ($g.L^{-1}$) = $1,4532OD_{600}$ - 0,2182. After, the culture medium was collected, the pH measurement was performed and the samples were frozen (-10 °C). At the moment of analysis, were centrifuged (10,000 rpm for 15 minutes, 5 °C) and the biomass was transferred to Petri dishes for 24 hours at 80 °C, to verify the dry mass.

2.2. Optimization experiments

2.2.1. Cassava wastewater

After the first results obtained from the study of the cassava wastewater, using 150 rpm, 30 °C and 100 % of substrate, a complete factorial design (2^3), including six axial points and three replications at the central point was performed for the production of biomass by the microorganism. The parameters used were: concentration of residue (%), agitation (rpm) and temperature (°C). Table 1 shows the variables and levels evaluated in the central composite design.

Table 1 - Variables and levels evaluated in the central composite design.

Variables		Levels				
		-1.68	**-1**	**0**	**+1**	**+1.68**
Cassava wastewater (%)	x1	20	36	60	84	100
Temperature (°C)	x2	20	24	30	36	40
Agitation (rpm)	x3	0	60	150	240	300

2.2.2. Glycerol

Due to the large variation of results found in the literature, in the beginning of this study, a 2^{4-1} factorial design with three replicates at the center point was performed, varying the factors: glycerol concentration (%), peptone concentration (%), agitation (rpm) and temperature (°C). Table 2 shows the variables and levels evaluated in the screening design. In this case, it is more interesting to explore all of the variables in the fractionated design to analyze the effects and only then proceed with a complete design with a smaller number of factors.

Table 2 - Variables and levels evaluated in the screening design.

Variables		Levels		
		-1	**0**	**+1**
Glycerol (%)	x1	5	15	25
Peptone (%)	x2	0	5	10
Agitation (rpm)	x3	0	150	300
Temperature (°C)	x4	20	30	40

2.3. Data analysis

The results of the optimization experiments were analyzed using the software Statistica™ version 7.0, considering the *p*-value of 0.05 for the complete design and *p*-value of 0.10 for the fractional.

2.4.Biotransformation procedure using cassava wastewater as culture medium

2.4.1. Microorganism

The strain LB398APD from the Bioflavours Laboratory Culture Collection, were cultivated and conserved by periodic replications (once a week) on Yeast-Malt agar (in $g.L^{-1}$: bacteriological peptone = 0.5, glucose = 1.0; malt extract = 0.3; yeast extract = 0.3 and agar = 2.0).

2.4.2. Biotransformation assay

The inoculum was prepared from a 24 hours-old culture of the strain. One full loop was transferred to a 250 mL conical flask containing 50 mL of cassava wastewater (60 %) and incubated at 30 °C for 24 hours in a rotary shaker (300 rpm). After the incubation period, the cell mass (inoculum) was aseptically transferred to 100 mL sterile screw-top flasks each containing 20 mL of mineral medium (in $g.L^{-1}$: $MgSO_4$ = 0.5; $NaNO_3$ = 3; K_2HPO_4 = 1; KCl = 0.5 and Fe_2SO_4 = 0.01) and the substrate (solution of β-carotene) was added (Dionisio *et al.*, 2009a). The samples were incubated at 30 °C and 150 rpm for 144 hours. Chemical blanks (without biomass) were performed to ensure the absence of chemical and spontaneous transformations. The SPME fibers were then exposed to the headspace every 24 hours, and the temperature/time was 30 °C and 30 minutes.

2.4.3. SPME analysis (GC-FID)

A SPME device (Supelco, Bellefonte, PA, USA) containing a fused-silica fiber (10 mm in length) coated with a 75 μm layer of CARBOXEN-PDMS was used. The fiber was preconditioned in the injection port of the chromatograph at 280 ºC for 60 min.

Analyses were performed using an Agilent 7890 A gas chromatograph (GC) equipped with a flame ionization detector (FID). Desorption proceeded in the injection port of the GC for 6 min at 250 °C with the purge valve off (split less mode). The volatile compounds were separated in a 30 m x 0,25 mm i.d. HP-5 (J&W Scientific, Folsom, California, USA). The temperature program was: 40 °C for 1 min, and them rose to 110 °C at 5 °C min^{-1} where it was held for 5 min, and rose to 220 °C at 10 °C.min^{-1} where it was held for 5 min. Helium was the carrier gas at flow rate of 1,0 mL.min^{-1} and the detector temperature was 250 °C. After desorption, the fiber remained a further 10 min in the injector port to eliminate the possibility of any carry-over of analyte between samples. The quantification was done using a β-cyclocitral standard.

3. RESULTS AND DISCUSSION

3.1.Cassava wastewater

Cassava wastewater is rich in sugars, nitrogen and mineral salts. The presence of C-source turns this residue into a suitable medium for the cultivation of microorganisms, contributing to make this a feasible industrial biotechnological process (Cameotra; Makkar 1998). On the other hand, this medium also contains high levels of substances, such as cyanide, that could limit growth or inhibit biotransformation (Maróstica; Pastore, 2007). Table 3 presents the composition of the cassava wastewater used in this study.

Table 3 - Physicochemical composition of cassava wastewater.

Components	Concentration	
Umidity	93.1	%(m/m)
Organic carbon	329.0	$g.kg^{-1}$
Reducing sugars	36.6	$g.L^{-1}$
Total nitrogen	26.3	$g.kg^{-1}$
Phosphorous	4.0	$g.kg^{-1}$
Potassium	41.4	$g.kg^{-1}$
Calcium	2.2	$g.kg^{-1}$
Magnesium	7.6	$g.kg^{-1}$
Sulfur	2.0	$g.kg^{-1}$
Iron	46.4	$mg.kg^{-1}$
Zinc	12.8	$mg.kg^{-1}$
Manganese	19.8	$mg.kg^{-1}$
Copper	7.4	$mg.kg^{-1}$
pH	5.7	

Preliminary results using cassava wastewater as culture medium showed that this residue presented higher results of dry mass and absorbance than the Yeast Malt, a conventional medium used for biomass development (Figure 1).

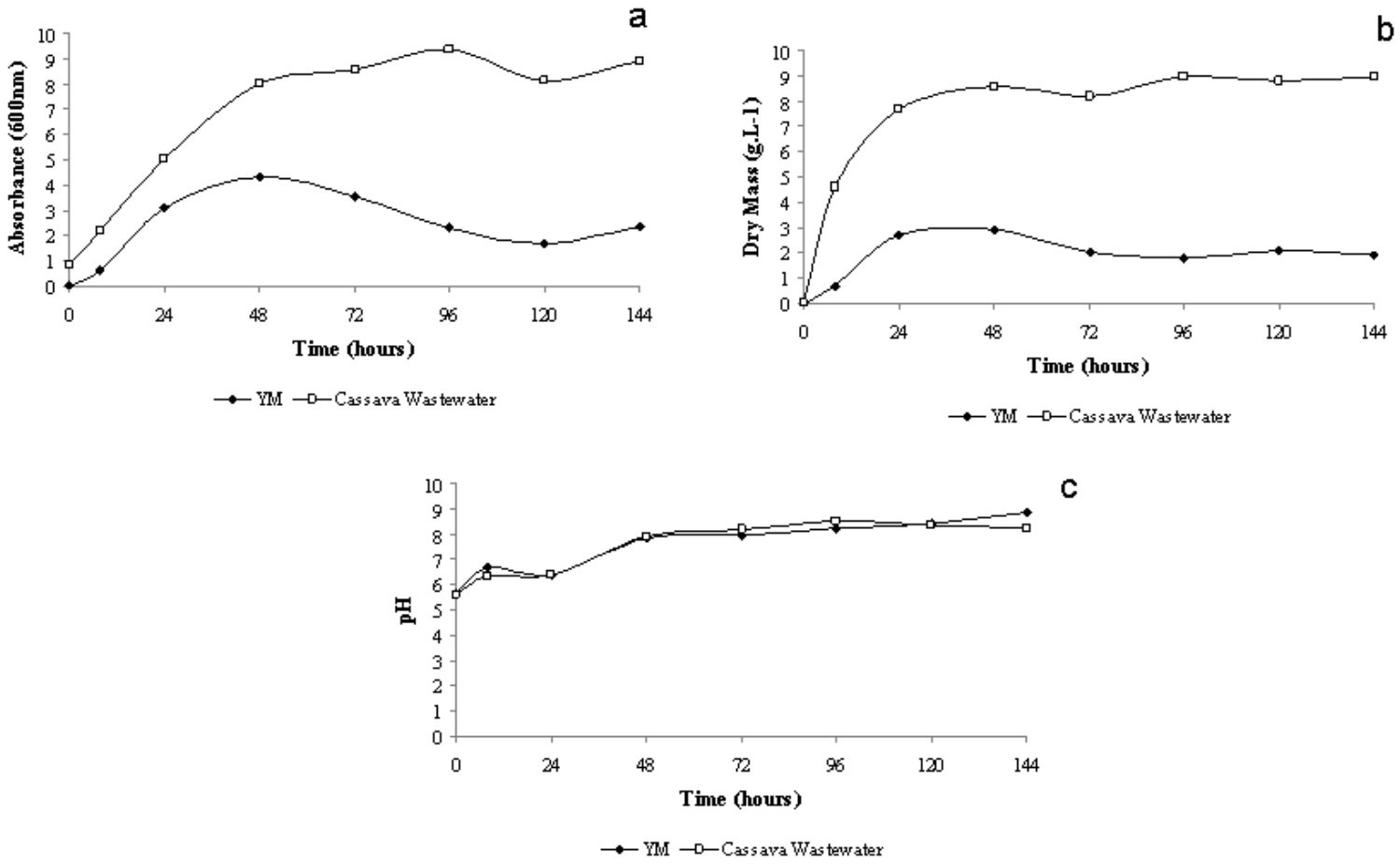

Figure 1 - Absorbance (a), dry mass (b) and pH values (c) of the LB398APD strain in YM culture medium and cassava wastewater culture medium.

Some studies suggest the use of cassava wastewater for biomass growth using *Trichosporon* sp. (Wosiacki *et al.*, 2000), for the production of citric acid by *Aspergillus niger* (Leonel; Cereda, 1995), for the production of fruity aroma compounds by *Geotrichum fragrans* (Damasceno *et al.*, 2003), for biotransformation assays of *R*-(+)-limonene to *R*-(+)-α-terpineol (Maróstica; Pastore, 2007) and production of biosurfactants (Nitschke; Pastore, 2006; Costa, 2005).

An optimization of the key parameters involved in the process was carried out using the preliminary results of this experiment. A central composite design (CCD) with three variables (2^3) and three central points (total of seventeen experiments) was proposed (Myers *et al.*, 2009, Rodrigues; Iemma, 2005). The results shown in figure 2 demonstrated the absorbance values during the fermentation process and the table 4, 5 and 6 show the results of the experiment.

Table 4 - Central Composite Design (CCD) for the biomass production by the LB398APD strain using cassava wastewater (Absorbance values).

	T	C	A	0h	8h	24h	48h	72h	96h	120h	144h
1	-1	-1	-1	0.000	0.354	**0.796**	2.798	2.914	2.934	3.224	3.482
2	1	-1	-1	0.000	0.756	**2.226**	3.006	3.044	2.826	2.960	2.764
3	-1	1	-1	0.000	0.643	**3.006**	3.337	2.858	3.296	3.720	3.576
4	1	1	-1	0.000	1.443	**3.245**	3.908	3.865	3.910	3.900	3.118
5	-1	-1	1	0.000	0.184	**0.165**	2.174	2.368	4.233	4.850	5.317
6	1	-1	1	0.000	0.066	**2.259**	4.766	6.446	6.400	6.281	4.231
7	-1	1	1	0.000	0.004	**3.474**	5.700	6.433	7.008	7.135	7.357
8	1	1	1	0.000	0.161	**2.544**	4.041	4.918	5.329	3.683	3.720
9	-1,68	0	0	0.000	0.000	**3.132**	2.892	3.480	5.074	5.452	5.720
10	1,68	0	0	0.000	0.081	**2.717**	2.614	2.840	3.358	3.804	3.567
11	0	-1,68	0	0.000	0.109	**2.484**	3.566	3.812	3.900	4.212	3.253
12	0	1,68	0	0.000	1.896	**5.465**	6.356	6.452	6.339	6.272	5.993
13	0	0	-1,68	0.000	1.825	**1.965**	2.137	2.293	2.192	2.297	2.158
14	0	0	1,68	0.000	0.113	**2.112**	4.046	4.861	5.590	5.462	5.666
15	0	0	0	0.000	1.246	**4.496**	5.475	5.687	6.586	6.669	6.911
16	0	0	0	0.000	1.069	**4.316**	5.390	6.293	7.111	6.873	5.144
17	0	0	0	0.000	1.050	**4.233**	4.983	5.598	5.901	6.160	5.938

Where: (T) temperature in °C; (A) agitation in rpm and (C), cassava wastewater in %

Table 5 - Dry mass values ($g.L^{-1}$) of the LB398APD strain using cassava wastewater.

	T	C	A	0h	8h	24h	48h	72h	96h	120h	144h
1	-1	-1	-1	0	3.4	**4.2**	4.8	4.8	5.2	5.8	6.0
2	1	-1	-1	0	0.8	**1.6**	2.2	2	2	2.2	2.0
3	-1	1	-1	0	0.8	**2.0**	1.4	2.4	2.2	2.8	2.6
4	1	1	-1	0	1	**2.0**	2.6	1.8	2.8	2.4	2.6
5	-1	-1	1	0	4.4	**3.6**	5.4	6.2	8	10.4	9.2
6	1	-1	1	0	0.6	**2.6**	4.6	4.8	4.4	4.6	4.0
7	-1	1	1	0	1.2	**4.2**	5.6	6.2	6.4	6.0	6.0
8	1	1	1	0	1.8	**4.4**	4.4	5.2	7.0	4.2	4.0
9	-1.68	0	0	0	0	**2.8**	4.4	3.8	4.6	4.6	4.4
10	1.68	0	0	0	1.2	**2.8**	2.8	0.4	2.4	3.0	3.0
11	0	-1.68	0	0	0.6	**1.8**	3.6	3.6	2.8	3.8	3.2
12	0	1.68	0	0	2.6	**4.6**	5.4	5.6	5.0	4.8	5.0
13	0	0	-1.68	0	1.2	**2.4**	1.2	1.2	2.2	1.6	2.0
14	0	0	1.68	0	1.6	**3.0**	5.4	6.6	6.2	5.4	6.2
15	0	0	0	0	1.8	**3.6**	4.6	4.6	4.8	4.8	4.2
16	0	0	0	0	0.6	**3.8**	4.8	4.6	5.6	4.2	4.0
17	0	0	0	0	1.6	**3.4**	4.6	4.6	5.6	5.4	4.8

Where: (T) temperature in °C; (A) agitation in rpm and (C), cassava wastewater in %.

Table 6 - pH values of the LB398APD strain using cassava wastewater.

	T	C	A	0h	8h	24h	48h	72h	96h	120h	144h
1	-1	-1	-1	5.77	6.67	**7.5**	7.89	8.1	7.99	8.52	8.55
2	1	-1	-1	5.72	7.7	**8.36**	8.87	8.91	9.03	9.15	9.18
3	-1	1	-1	5.63	6.95	**8.32**	8.55	8.62	8.69	8.89	8.93
4	1	1	-1	5.79	7.43	**8.32**	8.75	8.81	8.94	8.99	9.12
5	-1	-1	1	5.65	5.70	**6.01**	6.32	7.65	8.33	8.49	8.54
6	1	-1	1	5.68	5.81	**7.82**	8.76	9.01	9.22	9.36	9.25
7	-1	1	1	5.56	5.71	**7.92**	8.63	9.16	8.81	8.89	8.94
8	1	1	1	5.78	6.02	**7.53**	7.81	9.14	9.16	9.18	0.00
9	-1.68	0	0	5.64	6.29	**7.71**	7.96	7.99	8.28	8.33	8.67
10	1.68	0	0	5.61	5.73	**7.76**	7.88	6.79	9.14	9.23	9.51
11	0	-1.68	0	5.62	5.77	**7.46**	8.14	8.21	8.32	8.39	8.49
12	0	1.68	0	5.71	7.57	**8.22**	8.54	8.69	8.78	8.82	8.73
13	0	0	-1.68	6.03	8.00	**8.17**	8.22	8.24	8.41	8.52	8.61
14	0	0	1.68	5.57	5.63	**5.92**	6.71	8.33	8.35	8.34	8.87
15	0	0	0	5.64	7.09	**7.88**	8.21	8.28	8.55	8.82	8.15
16	0	0	0	5.61	7.06	**7.95**	8.15	8.32	8.58	8.88	8.13
17	0	0	0	6.26	7.72	**8.36**	8.52	8.84	8.89	8.94	9.06

Where: (T) temperature in °C; (A) agitation in rpm and (C), cassava wastewater in %.

The biomass growth was evaluated by the values of absorbance and dry mass, and the pH was monitored throughout the fermentation process. The results show that the biomass values ranged from 0 to ~ 7.35 for absorbance, and 0 to ~ 10.4 $g.L^{-1}$ for dry mass throughout the whole period. However, an efficient process to obtain biomass must occur in a short period of time in order to reduce the costs of the biotechnological process. For this reason, only the results at 24 hours were used for the statistical analysis.

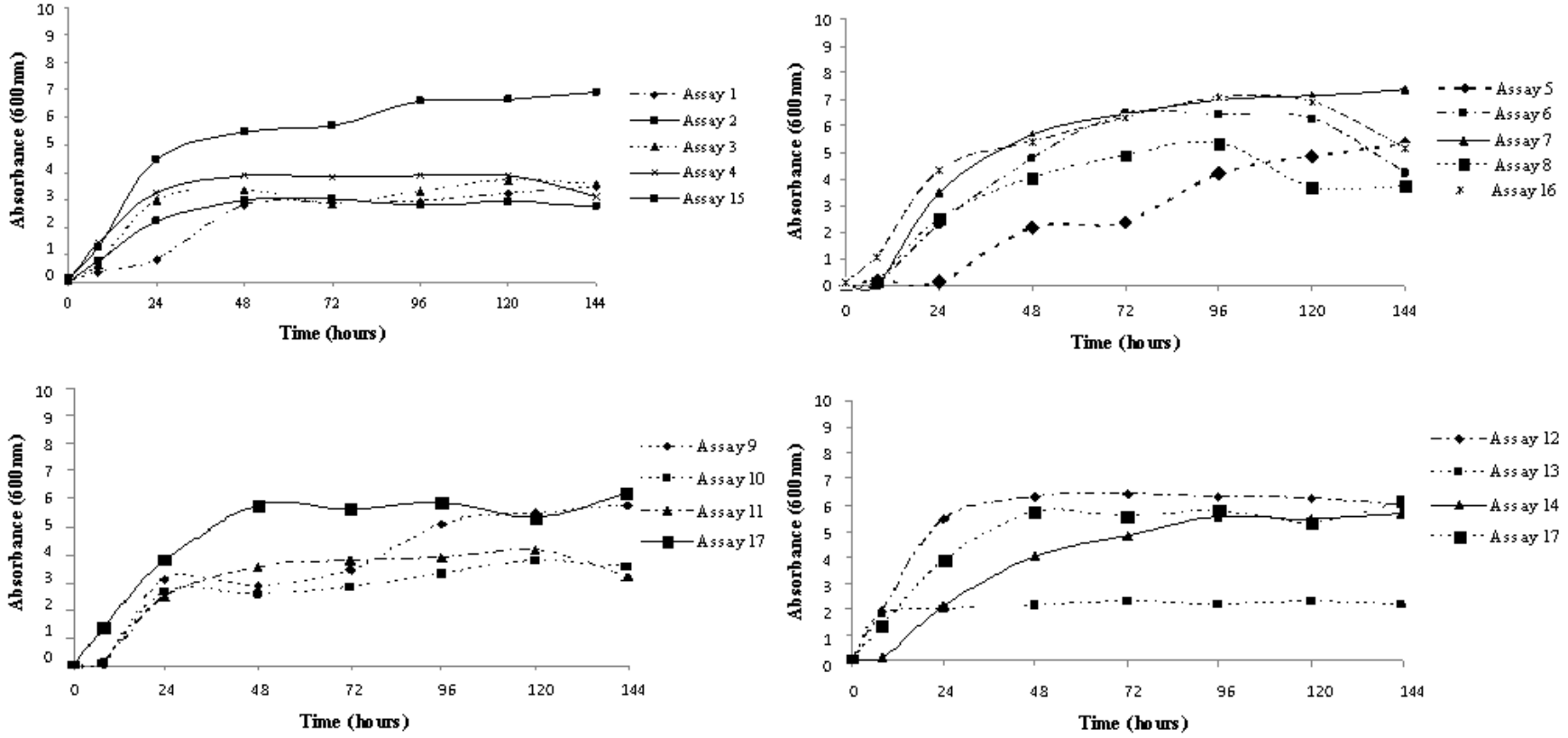

Figure 2 - Absorbance results.

Considering the intervals tested (Table 1) a model for these three variables based on the following formula was proposed:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_{12} + b_{22}x_{22} + b_{33}x_{32}$$

Where: Y = dependent variable (absorbance), b_0 is the average, b_1, b_2 and b_3 are the linear regression coefficients for the variables x_1, x_2 and x_3, respectively, b_{11}, b_{22} and b_{33} are the regression coefficients for the quadratic variables x_1, x_2 and x_3, respectively, and b_{12}, b_{13} and B_{23} are the regression coefficients of the interactions of the variables x_1 and x_2, and x_3 and x_1, x_2 and x_3, respectively. The variables x_1, x_2 and x_3 are, respectively, temperature (°C), agitation (rpm) and cassava wastewater (%) in coded values (Table 1).

Based on the responses obtained in the DCCR, the regression coefficients statistically significant for the response ($p < 0.05$) are shown in Table 7. In order to verify the validity of the model, analysis of variance (ANOVA) were performed.

Table 7 - Regression coefficients for the response.

Parameters	Regression Coefficients	Standard Error	t(7)	*p*-value
Average	**4.391954**	**0.362468**	**12.11680**	**0.000006**
x_1	0.156368	0.170218	0.91863	0.388845
x_1^2	**-0.654649**	**0.187350**	**-3.49426**	**0.010071**
x_2	**0.866837**	**0.170218**	**5.09251**	**0.001411**
x_2^2	-0.283453	0.187350	-1.51296	0.174053
x_3	-0.042656	0.170218	-0.25059	0.809325
x_3^2	**-0.967968**	**0.187350**	**-5.16663**	**0.001300**
$x_1 \cdot x_2$	**-0.526838**	**0.222401**	**-2.36887**	**0.049689**
$x_1 \cdot x_3$	-0.063138	0.222401	-0.28389	0.784708
$x_2 \cdot x_3$	0.045663	0.222401	0.20532	0.843168

[a] The parameters are described in Table 1. Parameters in bold are statistically significant for the model ($p < 0.05$).

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

$$\hat{Y} = 4.39 + 0.86\, x_2 - 0.52\, x_1 x_2 - 0.65\, x_1^2 - 0.96\, b_{33} x_3^2$$

The ANOVA table (Table 8) indicate that a quadratic model adjusted for the responses was satisfactory. The F-value (12.88) was about four times higher than the listed

value (considering p <0.05), while the *p*-value of the model was less than 0.0001. Although not ideal, the value of R^2 = 0.85 is perfectly acceptable for biological systems (Rodrigues; Iemma, 2005) and reflects a reasonable adjustance of the model results with the results predicted.

Table 8 - Analysis of variance (ANOVA) for the quadratic model proposed in the equation.

Variation Source	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	***F*-value**	***p*-value**
Regressão	23.907	5	4.7814	12.88	>0.0001
Resíduo	4.083	11	0.3712		
Total	27.99	16	1.7493		
$R^2 = 0.8541$		$F_{0.05(5;\ 11)} = 3.20$		$F_{0.1(5;\ 11)} = 2.45$	

The model obtained (Equation) can be used and the results are shown in Figures 3, 4 and 5.

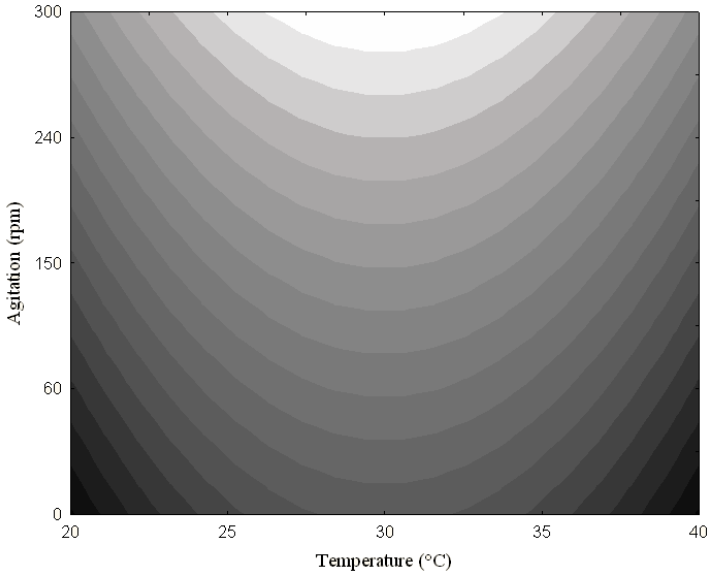

Figure 3 - Contour plot for biomass production in 24 hours of fermentation, depending on the temperature and agitation.

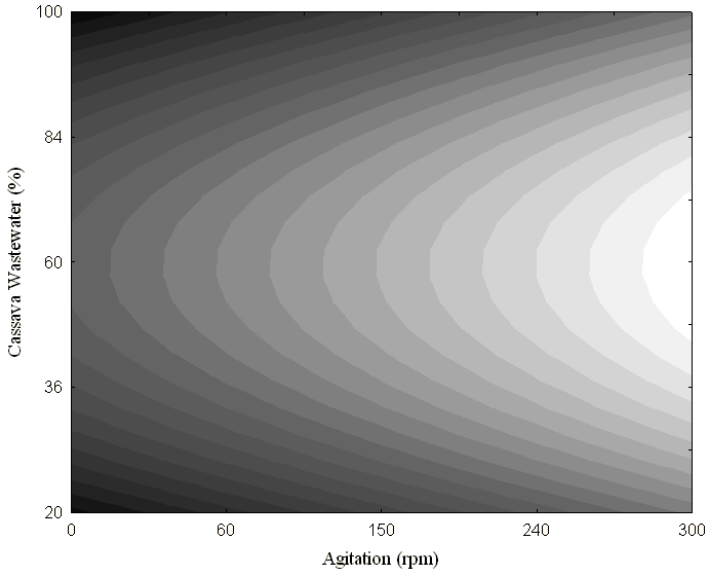

Figure 4 - Contour plot for biomass production in 24 hours of fermentation, depending on the agitation and cassava wastewater concentration.

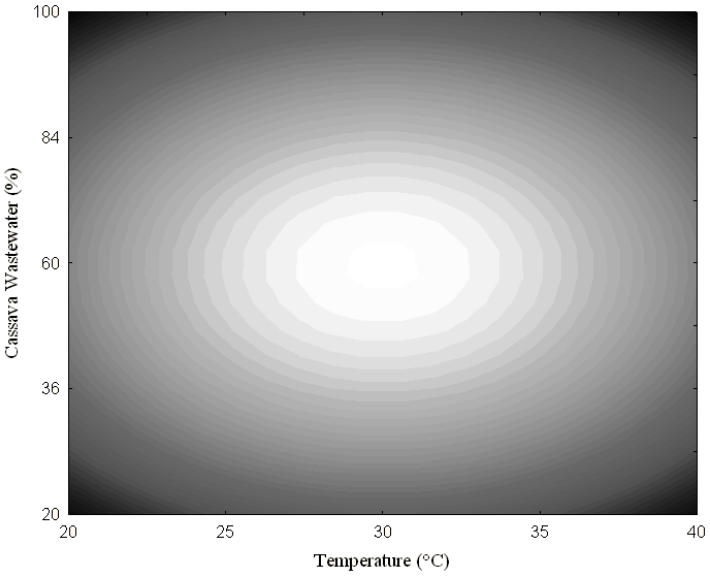

Figure 5 - Contour curve for biomass production in 24 hours fermentation, depending on the temperature and cassava wastewater concentration.

The optimum conditions to improve the biomass production of the strain LB398APD are: 300 rpm, 30 °C and 60 % of cassava wastewater, obtaining approximately 4.6 $g.L^{-1}$ after

24 hours of fermentation. Six assays were performed at the same conditions to validate the model, showing compatible results for the absorbance (Mean=5.18, Standard Error=0.16) and dry mass (Mean= 4.2g.L^{-1}, Standard Error=0.08).

The strategy adopted in the experiment clearly demonstrated the potential use of the cassava wasterwater in the biotechnological production of biomass, to use in biotransformation procedure. However, to verify the real possibility of the use in the biotransformation of β-carotene, an experiment envolving this by-product was conduced. The results are similar to those obtained with yeast-malt medium, with a production ~10 mg.L^{-1} of β-cyclocitral (110 hour), a volatile terpene derived from β-carotene.

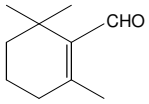

Figure 6 - Structure of β-cyclocitral.

3.2. Glycerol

In the glycerol assays, it was chosen a sequential planning strategy using a 2^{4-1} design and three replicates at the central point. In this case, only the absorbance results were considered mainly because the growth of the strain in this substrate was low and was not possible to analyze its dry mass.

The results of biomass production using glycerol are markedly lower when compared to cassava wastewater. The assay 3 (conditions: 5 g.L^{-1} glycerol, 10 g.L^{-1} peptone, 20 °C and 300 rpm) showed, in general, the best results in this experiment (absorbance in 48 h ~ 1.25). However, when comparing the results obtained with cassava wastewater, the growth of biomass is about seven times lower for the same time.

Thus, the biomass growth was evaluated at 24 and 48 hours to verify the possibility of using the biomass in a longer period of fermentation. The results with 24 hours of fermentation showed that biomass production was affected only by peptone and agitation. The results with 48 hours of fermentation showed that only the peptone was significant for the system.

Tabela 9 – 2^{4-1} experimentos for the biomass production by the LB398APD strain using glycerol (absorbance values).

	G	P	T	A	0h	8h	24h	48h	72h	96h	120h	144h
1	-1	-1	-1	-1	0.00	0.01	0.09	0.07	0.09	0.09	0.10	0.10
2	1	-1	-1	1	0.00	0.08	0.08	0.11	0.12	0.12	0.12	0.14
3	-1	1	-1	1	0.00	0.54	1.25	1.32	1.16	1.28	1.21	1.05
4	1	1	-1	-1	0.00	0.24	0.50	0.77	0.85	0,99	0.68	0.65
5	-1	-1	1	1	0.00	0.05	0.06	0.06	0.04	0.04	0.03	0.04
6	1	-1	1	-1	0.00	0.01	0.02	0.03	0.04	0.02	0.02	0.00
7	-1	1	1	-1	0.00	0.07	0.14	0.47	0.85	0.87	0.84	0.81
8	1	1	1	1	0.00	0.72	1.3	1.01	0.79	0.78	0.70	0.66
9	0	0	0	0	0.00	0.56	0.70	0.76	0.63	0.49	0.34	0.35
10	0	0	0	0	0.00	0.57	0.71	0.78	0.64	0.50	0.34	0.35
11	0	0	0	0	0.00	0.58	0.71	0.78	0.66	0.52	0.35	0.37

Table 10 - Estimates of the effects on the biomass growth after 24 hours of fermentation.

Factors	Effect	Standard Error	t(6)	*p*-valor
Mean	**0.505**	**0.099**	**5.114**	**0.002**
Glycerol (%)	0.089	0.231	0.386	0.712
Peptone (%)	**0.734**	**0.231**	**3.172**	**0.019**
Temperature (°C)	-0.099	0.231	-0.428	0.683
Agitation (rpm)	**0.489**	**0.231**	**2.110**	**0.079**

[a] Parameters in bold are statistically significant for the model (p <0.10).

Table 11 - Estimates of the effects on the biomass growth after 48 hours of fermentation.

Factors	Effect	Standard Error	t(6)	*p*-valor
Mean	**0.5606**	**0.0722**	**7.7651**	**0.0002**
Glycerol (%)	-0.0029	0.1693	-0.0173	0.9868
Peptone (%)	**0.8258**	**0.1693**	**4.8776**	**0.0028**
Temperature (°C)	-0.1748	0.1693	-1.0323	0.3417
Agitation (rpm)	0.2922	0.1693	1.7257	0.1352

[a] Parameters in bold are statistically significant for the model (p <0.10).

With the high *p*-value for the glycerol, in both fermentation times, the experiment was not completed with the design, because the initial intention of using glycerol as carbon source had not been achieved in the proposed experiment. However, some studies have shown interesting results when glycerol was used in the system. Glycerol was reported in different bioconversion processes, with applications in production of 1,3-propanediol, dihydroxyacetone, succinic acid, propionic acid, ethanol, citric acid, pigments, polyhydroxyalcanoate and biosurfactants (Da Silva; Mack; Contiero, 2009). About the last

compound, diverses papers describe the potential of the by-product into convert biosurfactants, *e.g*. the basidiomycete yeast *Pseudozyma Antarctica* JCM 10317, applied to produced mannosylerythritol lipids (MELs) (Morita *et al.*, 2007) and *Y. lipolytica* to produce biosurfactants and citric acid (Amaral *et al.*, 2009).

Finally, studies envolving optimization of the culture medium were conduced in some researchs groups. Imandi and co-workers (2007) optimized the production of citric acid by the *Yarrowia lipolytica* NCIM 3589 strain using glycerol as carbon source. Through the response surface methodology they estimated that the concentration of yeast extract, glycerol and saline solution are important factors to increase the production of citric acid (optimum concentration: 0.27 $g.L^{-1}$ of yeast extract, 54.41 $g.L^{-1}$ of glycerol and saline at 13.7 %). Further studies, using the same srain, evaluated a process optimization for citric acid production from raw glycerol using response surface methodology envolving central composite design (CCD). The optimal condition were found to be carbon concentration 38.77 $g.L^{-1}$, nitrogen concentration 0.401 $g.L^{-1}$, and salt solution concentration 12.3 % ($v.v^{-1}$) (Kumari, Babu; Rao; 2008). Unfortunately, in the presente paper, was not possible the use of glycerol by strain LB398APD in the conditions tested.

4. CONCLUSION

The optimum conditions for development of biomass by the LB398APD strain were successfully obtained using cassava wastewater as culture media, considering that this residue represents an environment risk and its uses in biotechnological process are an alternative to provide a conscientious use of this by-product. When the optimal conditions are used (60 % cassava wastewater, 30 °C and 300 rpm), the production of biomass obtained was approximately $4.6g.L^{-1}$. On the other hand, biomass production using glycerol as carbon-

source was not satisfactory. However, others conditions should be considered to improve its production, as substrate concentration and alternative nitrogen sources.

5. ACKNOWLEDGEMENTS

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CAPÍTULO 6

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EVALUATION OF FUNCTIONAL PROPERTIES OF CAROTENOID-DERIVED AROMA COMPOUNDS

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ABSTRACT

A considerable number of apparently carotenoid-derived compounds has been identified in nature, many of them playing an important role as flavor and fragrance substances. Studies *in vitro* and *in vivo* with carotenoids are abundant in the literature. However, few papers report the potencial of the carotenoid-derived compounds in anti-proliferative and anti-microbial studies. In this paper, anti-proliferative effect of six aroma compounds (β-ionone, β-damascenone, β-damascone, α-ionone, pseudoionone and β-ciclocytral) against eleven cancerous cell lines were performed and compared to the positive control doxorubicin. On the other hand, disk diffusion method was used for the evaluation of the anti-microbial activity using five pathogenics micro-organisms. The results obtained for the anti-proliferative and anti-microbial tests will encourage future *in vivo* studies using these carotenoid-derived compounds.

Key-words: carotenoid-derived aroma compounds, antimicrobial and antitumoral properties.

1. INTRODUCTION

Terpene is any of a large and varied class of hydrocarbons, with the molecular formula $(C_5H_8)n$, that are produced by a wide variety of plants. Carotenoids are terpenoids, and be considered an important precursor of a variety of compounds: the C_{20}-retinoids, the C_{15}-phytohormones, and the C_9 to C_{13}-aromas. Among the last type, carotenoid-derived aroma compounds are of great interest to the food and perfume industry (Winterhalter; Rouseff, 2002) and its biotechnological synthesis were stimulated recently due a extraction from natural products is often difficult and expensive (Waché; Ratuld; Belin, 2002).

Cancer is a major public health problem in developed and developing countries (Jemal *et al.*, 2008). Therefore, new compounds with antiproliferative activity are sought for the prevention and treatment of cancers. Carotenoids include substances able to be converted into vitamin A, such as α- and β-carotene, as well other compounds, which cannot be converted into vitamin A but have more potent antioxidant properties, such as lycopene. Together with vitamins A, C and E, the carotenoids are believed to be important in cancer prevention because of their properties as antioxidants or their ability to affect cell differentiation or proliferation (Cramer *et al.*, 2001).

Experimental research performed since 1960s demonstrated that high dose of retinoids could inhibit carcinogenesis in numerous animal models (Moon *et al.*, 1994). Therefore, epidemiologic studies were carried out in an effort to evaluate the effects of dietary vitamin A on cancer occurrence, at nutritional intake levels and sice 1984, human intervention trials have been undertaken with β –carotene, and the results of several of them have recently been obtained (Astorg, 1997).

Studies demonstrating the potential of carotenoids to prevent cancer diseases were done in many researches groups, however few studies involving derived aroma compounds can be found in the literature. Some works suggest that the β-ionone could be further

developed for prevention and treatment of colon cancer (Janakiram *et al.*, 2008), human breast cancer (Duncan *et al.*, 2004), mammary tumor (Liu *et al.*, 2008; Liu *et al.*, 2004; Liu *et al.*, 2005, Yu; Anderson; Elson, 1995), melanoma and leukemia (Tatman *et al.*, 2002), and present a potential as alternative to antimicrobial growth enhancers (Lee *et al.*, 2003).

Considering that of approximately 25,000 terpene structures reported very few have been investigated from a functional perspective, the purpose of this paper are elucidate six carotenoid-derived aroma compounds to use as functional ingredient in addition its importance in fragrances and aromas industry.

2. MATERIALS AND METHODS

2.1. Chemicals

The chemicals used were: β-cyclocitral (~90 %, Sigma-Aldrich), damascenone (~95 %, Sigma-Aldrich), α-ionone (~90 %, Sigma-Aldrich), β-damascone (~90 %, Sigma-Aldrich), β-ionone (~95 %, Sigma-Aldrich) and pseudoionone (~90 %, Sigma-Aldrich). All of the solvents were analytical grade.

2.2. Microorganisms

Escherichia coli ATCC 1065, *Candida albicans* CCT 0776, *Staphylococus aureus* ATCC 6538, *Salmonela choleraesuis* ATCC 10708 and *Pseudomonas aureginosa* ATCC 10145 were used in this investigation. The cultures were routinely maintained in nutrient agar at 4 °C.

2.3. Determination of the anti-microbial activity

The bacteria cultures were grown in Brain Heart Infusion (BHI) liquid medium at 37 °C. After 24 h of growth, each microorganism, at a concentration approximated of 106 cells/mL, was inoculated on the surface of plates containing the same medium (BHI), with agar. Subsequently, filter paper discs (6 mm in diameter) were added with different concentrations of the carotenoid derived aroma compounds (1:1, 1:5 and 1:10). The plates were incubated at 30 °C overnight for 24 h. After this period, the inhibition zone was measured in millimeter.

2.4. Anti-proliferative activity *in vitro*

2.4.1. Cell lines and cultivation

The human cell lines from different histological and embryonic origins were employed, all assigned by the National Cancer Institute (NCI-USA). Human tumoral cells : U = UACC-62 (melanoma); M = MCF-7 (breast adenocarcinoma); A = NCI-ADR/RES (breast adenocarcinoma, multidrug resistant phenotype); 7 = 786-O (renal adenocarcinoma); P = PC-3 (prostate adenocarcinoma); O = NIH:OVCAR-3 (ovarian adenocarcinoma); K = K-562 (chronic myeloid leukemia); 4 = NCI-H460 (lung adenocarcinoma); H = HT-29 (cólon adenocarcinoma); V = VERO (green monkey kidney), 2 = U251 (glioma).

The stock cultures were conserved in RPMI-1640 culture medium (Gibco) with 5 % of fetal bovine serum (FBS) (Gibco) and no antibiotics; the latter was added (gentamicin, 50 $\mu g.mL^{-1}$) only in the cells submitted to the anti-proliferative assay. The cells were kept in 25 cm^2 flasks, with 5 mL of RPMI/FBS medium and replicated weekly. Each flask was incubated at 37 °C in a humid atmosphere enriched with 5 % of CO_2.

For the adhered cells, which form a cell monolayer in the flask, the cell suspension was prepared by exhausting the culture medium followed by 10 consecutive washings with

Hank's buffer (Sigma), the addition of 500 μL of trypsin and finally the RPMI/FBS medium. For non-adhered cells (K-562), the cell suspension was prepared by transferring to a novel 25 cm^2 flask a previously determined volume, being the final volume adjusted to 5 mL.

2.4.2. Determination of the anti-proliferative activity

For the evaluation of the anti-cancer activity, 100 μL of the cell suspension in RPMI/FBS/getamicin were transferred to each of the 96 wells from a microplate and than incubated at 37 °C for 24 h in a humid atmosphere enriched with 5 % of CO_2.

A stock solution of each sample of the terpenes (diluted at 0.1 $g.mL^{-1}$ in DMSO) was diluted at different concentrations (0.25, 2.5, 25 and 250 $mg.L^{-1}$ in RPMI) and transferred (1,000 μL) to the respective well from the plate (T). A control group (C) presented no sample addition and another group (T_0), also with no sample addition, was read at time zero. The plate was than incubated at 37°C for 48 h in a humid atmosphere enriched with 5 % CO_2. Subsequently, 50 μL of a trichloroacetic acid solution 50 % was added and the plate was kept at 4 °C for 30 min to fix the living cells. After washing and drying, the number of cells was determined by the spectrophotometric quantification (540 nm) of the total cell proteic conctent, using for each well 50 μL of sulphorodamin B (0.4 $g.mL^{-1}$, in acetic acid 1%, for 30 min at 4 °C) as indicator of the amount of viable cells (Skehan; Scudeiro, 1990). Doxorubicin was employed as positive control.

Considering the curves% Inhibition (or cell death) *versus* Sample concentration, the values of TGI (Total Growth Inhibition) were calculated, as indicated by Equation 1:

$$100\ x\ (T - T_0) / (C - T_0) = 0 \qquad \textbf{(Equation 1)}$$

Where T_0 and T correspond to, respectively, the values of absorbance in the moment just before sample addition (time zero) and after the treatment; C indicates the values of absorbance of the cells in the absence of sample (control) after the same period of treatment.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity

Terpenes are secondary metabolites of plant cells and are widely used in flavour and perfumery industries as well as in food preservation (Derfer; Derfer, 1983). In some species they possess effective *in vivo* antimicrobial properties against pathogenic bacteria (Scortichini; Rossi, 1991; Ahamed *et al.*, 1993; Habtemaria; Gray; Waterman, 1993), fungi (Harrigan *et al.*, 1993; Rana *et al.*, 1997) and viruses (Fujioka; Kashiwada, 1994). The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Cowan, 1999).

Considering the potential antimicrobial uses of the terpenes, this paper describe the microbial inhibition caused for some carotenoid-derived aroma compounds against five pathogenics microorganisms (yeast, gram positive and gram negative bacteria). The growth inhibition zones measured by disc diffusion method are presented in Table 1. 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).

Table 1 – Antimicrobial properties of carotenoid-derived aroma compounds using the disc diffusion method. The diameter of the zone of inhibition includes the paper disc (5 mm).

Compound		**Diameter of inhibition zone (mm)**				
		E. coli ATCC 1065	*C. albicans* CCT 0776	*S. aureus* ATCC 6538	*S. choleraesuis* ATCC 10708	*P. aureginosa* ATCC 10145
β-Ionone	1:1	-	10.0	15.0	10.0	12.0
	1:5	-	7.0	12.0	8.0	8.0
	1:10	-	-	10.0	-	-
α-Ionone	1:1	8.0	12.0	11.0	9.0	9.0
	1:5	7.0	11.0	7.0	8.0	8.0
	1:10	-	-	-	-	-
β-Damascone	1:1	-	-	20.0	15.0	14.0
	1:5	-	-	10.0	9.0	10.0
	1:10	-	-	-	6.0	8.0
β-Damascenone	1:1	12.0	2.0	15.0	14.0	8.0
	1:5	7.0	8.0	-	9.0	-
	1:10	-	-	-	-	-
β-Cyclocitral	1:1	16.0	22.0	16.0	22.0	25.0
	1:5	14.0	17.0	14.0	13.0	22.0
	1:10	-	9.0	8.0	11.0	16.0
Pseudoionone	1:1	9.0	9.0	10.0	10.0	8.0
	1:5	8.0	8.0	9.0	8.0	8.0
	1:10	7.0	7.0	7.0	6.0	8.0
DMSO	-	0.0	0.0	0.0	0.0	0.0
Chlorine	-	20.0	20.0	20.0	20.0	20.0

Negative Control = DMSO (dimethyl sulfoxide); Positive Control: Chlorine.

All the assayed samples proved to be sensible to the activity of the compounds, with inhibition diameter of 6 to 22 mm, displayning consequently great effectiveness of all aroma compounds. Considering the upper concentration tested, the results obtained with terpenes clearly showed that β-cyclocitral presented the strongest antibacterial and antifungical effect for all strains, excepted for *Staphylococus aureus*, which obtained a best result with β-damascone.

Another works demonstrate the potential use of the terpenes against the same pathogenic microorganisms. Inouea and co-workers (2004) demonstrated the antibacterial activities of terpene alcohols with long aliphatic chains against *Staphylococus aureus* on the basis of damage to cell membranes. The activity appeared to depend not only on the length of the aliphatic chain but also the configurations of functional groups and double bonds. In your turn, carvone was shown to inhibit the transformation of *Candida albicans* to a filamentous form at concentrations far lower and more biologically relevant than the concentrations necessary to inhibit growth. This morphological transformation is associated with *C. albicans* pathogenicity; hence these naturally occurring monoterpenes are potential lead compounds in the development of therapeutic agents against *C. albicans* infection (McGeady *et al.*, 2002). Tests of *C. albicans* strains showed that thymol interferes with the starting phases of biofilm production as well as with mature *C. albicans* biofilms (Braga *et al.*, 2008). Finally, seven citrus essential oils were screened by disc diffusion assay for their antibacterial activity against strains of *Salmonella*. Analysis of GC-MS revealed that the orange essence oil was composed principally of *d*-limonene, 94 %, and myrcene at about 3 %. The authors concluded that all the essential oils from citrus offer a potential as antimicrobial natural (O'Brian *et al.*, 2008).

3.2. Anti-proliferative activity *in vitro*

Cancer is a group of diseases characterized by unregulated growth and spread of abnormal cells, which can result in death if not controlled (American Cancer Society, 2010). It has been considered one of the major causes of death worldwide, accounting for 7.4 million deaths (about 13 % of all deaths) in 2004 (WHO, 2009).

Terpenes are natural products that have medicinal properties and biological activity. In general, some volatile compounds derived from terpenes have been shown significant effects in some cancers, involving *in vitro* and *in vivo* studies. Limonene is one of the most abundant naturally occurring monocyclic monoterpenes and has chemoprotective activity against spontaneous and chemically induced rodent tumors (Crowell; Gould, 1994; Elson *et al.*, 1988). Investigations of structureactivity relationships among monoterpenes have revealed that several limonene metabolites, perillic acid and hydroxylated compounds such as perillic alcohol, and methylated derivates, have greater antiproliferative activity than limonene *in vitro* (Crowell *et al.*, 1994). A brazilian patent involving the use of perillyl alcohol as a chemopreventive agent for regression of mammalian nervous system cell tumors and its method for administration are elucidate recently (Da Fonseca *et al.*, 2004a). However, although the elucidation of the potential of some monoterpenes have been reported, nowadays, there are few reports documenting the antiproliferative effect of carotenoid-derived aroma compounds in some tumor cells.

Focusing in the volatile terpenes derived from carotenoids, the norisoprenoid β-ionone represent the most studied compound, with envolviment in prevention and treatment of diverses types of cancer. Considering that, it was expected that the molecule produce a greater anti-tumoral action when compared to the other compounds in this study. However, the *in vitro* cytotoxicity evaluation demonstrated a high potency specially for the β-damascone and β-damascenone, showing a good concentration and effect relationship for

these aroma compounds. The β-damascone presented a special selectivity for kidney adenocarcinoma (7 = 786-O) and glioma (2= U251), with a citocida effect using very low concentrations of the terpene. The tables 2, 3 and 4 represents, repectivelly, the GI_{50} (cytostatic effect), TGI (total cytostatic effect) and LC_{50} (cytocida effect) of the compounds evaluated in this paper.

The GI_{50} values (growth inhibitory activity) refer to the compound concentration that produce a 50 % reduction of cellular growth when compared to untreated control cells. The TGI (cytostatic activity) and LC_{50} (cytotoxic activity) values parameters refer to the compound concentration for total growth inhibition and for killing 50 % of the cells, respectively.

Table 2 - Values of GI_{50} ($\mu g.mL^{-1}$) for β-Ionone, α-ionone, β-damascone, β-damascenone, β-cyclocitral and pseudoionone, in each of the cell lines tested.

	Cells										
	2	**U**	**M**	**A**	**7**	**4**	**P**	**O**	**H**	**K**	**V**
Doxorrubicin	0.031	0.060	0.056	1.49	0.025	<0.025	<0.025	0.27	0.25	0.052	0.19
β **-Ionone**	0.35	0.34	<0.25	0.34	0.35	0.30	1.86	2.55	0.64	0.36	2.13
α**-Ionone**	<0.25	<0.25	<0.25	<0.25	<0.25	26.42	1.88	2.15	<0.25	1.83	<0.25
β-**Damascone**	<0.25	<0.25	0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	0.25	<0.25
β-**Damascenone**	<0.25	0.42	0.25	<0.25	0.25	<0.25	5.70	0.25	<0.25	<0.25	<0.25
Pseudoionone	0.68	2.48	0.36	0.76	0.53	13.47	0.40	0.37	0.35	0.39	<0.25
β-**Ciclocytral**	61.98	>250	66.45	36.21	>250	29.42	163.99	80.64	>250	38.61	38.65

Human tumoral cells : U = UACC-62 (melanoma); M = MCF-7 (breast adenocarcinoma); A = NCI-ADR/RES (breast adenocarcinoma, multidrug resistant phenotype); 7 = 786-O (renal adenocarcinoma); P = PC-3 (prostate adenocarcinoma); O = NIH:OVCAR-3 (ovarian adenocarcinoma); K = K-562 (chronic myeloid leukemia); 4 = NCI-H460 (lung adenocarcinoma); H = HT-29 (cólon adenocarcinoma); V = VERO (green monkey kidney), 2= U251 (glioma). GI_{50} = cytostatic effect

Table 3 - Values of TGI ($\mu g.mL^{-1}$) for β-Ionone, α-ionone, β-damascone, β-damascenone, β-cyclocitral and pseudoionone, in each of the cell lines tested.

	Cells										
	2	**U**	**M**	**A**	**7**	**4**	**P**	**O**	**H**	**K**	**V**
Doxorrubicin	1.75	0.32	10.04	>25	0.16	1.59	0.56	4.18	13.21	19.01	2.06
β-**Ionone**	35.66	>250	27.30	>250	7.22	>250	34.36	140.47	>250	>250	>250
α-**Ionone**	2.10	6.24	18.99	15.17	6.25	81.10	31.16	82.22	53.60	>250	3.06
β-**Damascone**	<0.25	0.45	13.72	>250	<0.25	15.74	>250	13.39	16.78	>250	0.39
β-**Damascenone**	1.73	17.68	>250	>250	1.59	>250	>250	14.79	>250	>250	14.74
Pseudoionone	10.93	4.83	25.53	>250	5.82	194.23	9.61	14.71	31.72	>250	7.08
β-**Ciclocytral**	>250	>250	>250	>250	>250	197.52	>250	>250	>250	>250	193.41

Human tumoral cells : U = UACC-62 (melanoma); M = MCF-7 (breast adenocarcinoma); A = NCI-ADR/RES (breast adenocarcinoma, multidrug resistant phenotype); 7 = 786-O (renal adenocarcinoma); P = PC-3 (prostate adenocarcinoma); O = NIH:OVCAR-3 (ovarian adenocarcinoma); K = K-562 (chronic myeloid leukemia); 4 = NCI-H460 (lung adenocarcinoma); H = HT-29 (cólon adenocarcinoma); V = VERO (green monkey kidney), 2= U251 (glioma). TGI = total cytostatic effect.

Table 4 - Values of LC_{50} ($\mu g.mL^{-1}$) for β-Ionone, α-ionone, β-damascone, β-damascenone, β-cyclocitral and pseudoionone, in each of the cell lines tested.

	Cells										
	2	**U**	**M**	**A**	**7**	**4**	**P**	**O**	**H**	**K**	**V**
Doxorrubicin	>25	1.62	>25	>25	2.04	>25	9.94	>25	>25	>25	23.70
β-Ionone	>250	>250	244.78	>250	>250	>250	225.58	>250	>250	>250	>250
α-Ionone	55.93	134.42	243.88	>250	128.00	243.27	207.27	>250	>250	>250	136.83
β-Damascone	0.83	6.76	>250	>250	0.94	239.57	>250	>250	>250	>250	>250
β-Damascenone	30.80	250	>250	>250	142.16	>250	>250	127.96	>250	>250	236.28
Pseudoionone	211.75	21.43	>250	>250	32.33	>250	96.42	>250	>250	>250	203.76
β-Ciclocytral	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250

Human tumoral cells : U = UACC-62 (melanoma); M = MCF-7 (breast adenocarcinoma); A = NCI-ADR/RES (breast adenocarcinoma, multidrug resistant phenotype); 7 = 786-O (renal adenocarcinoma); P = PC-3 (prostate adenocarcinoma); O = NIH:OVCAR-3 (ovarian adenocarcinoma); K = K-562 (chronic myeloid leukemia); 4 = NCI-H460 (lung adenocarcinoma); H = HT-29 (cólon adenocarcinoma); V = VERO (green monkey kidney), 2= U251 (glioma). LC_{50} = cytocida effect.

Comparing all the terpenes in this study, β-ciclocytral requires higher concentrations to produced the same effects as the other compounds tested. On the other hand, when the results were compared in the literature against other terpenes, the results show a good protective effect of this compound. Bicas *et al.* (2009) tested *R*(+)-limonene and α-terpineol against seven tumoral cell lines, revealing concentration in order to 591 and 916 μM to produce total citostatic effetc (TGI) in the cell line responsible for lung cancer (NCI-H460). In our study, 193.41 $\mu g.mL^{-1}$ of β-ciclocytral was requered to produced the same effect.

The most studied terpene in cancer, the perillyl alcohol (POH) is a hydroxylated product of *d*-limonene, which is formed by the condensation of two isoprene molecules. POH is a naturally occurring anti-cancer compound which is effective against a variety of rodent organ-specific tumor models. This monocyclic monoterpene has shown chemopreventive and therapeutic activity in rodent mammary, skin, lung, liver, pancreatic, and colon tumor models (Bardon *et al.*, 2002). The effect of POH on cell growth, against human breast cancer cell lines was examined, showing that the terpene inhibited cell proliferation in a dose-dependent

manner in all cell lines tested. POH at a dose of 500 μM had a cytostatic effect (Yuri *et al.*, 2004). Recently, the perillyl alcohol (POH) was used as a potential adjuvant therapeutic strategy for patients with relapsing malignant gliomas. For the first time, that intranasal administration of the signal transduction inhibitor, perillyl alcohol, is a safe, non invasive, low cost and regression of tumor size in some patients is suggestive of antitumor activity (Da Fonseca, 2007; Da Fonseca *et al.*, 2006b; Da Fonseca *et al.* 2006c; Da Fonseca *et al.* 2008).

Carotenoids have a broad range of functions, especially to human health and their role as biological antioxidants. Fraser and Bramley (2004) reviewer more than 200 articles about the biossynthesis and nutritional uses of these terpenes, including cardiovascular disease and cancer. So, the β-carotene was performed using the same antitumoral assay to compare the results with the volatiles derived from carotenoids (Table 5).

Table 5 - Values of TGI ($\mu g.mL^{-1}$) for β-carotene, in each of the cell lines tested.

	Cells									
	2	**U**	**M**	**7**	**4**	**P**	**O**	**H**	**K**	**V**
Doxorrubicin	1.75	0.32	10.04	0.16	1.59	0.56	4.18	13.21	19.01	2.06
β –carotene	77,75	125,93	>250	134,13	>250	>250	71,00	182,78	>250	>250

Human tumoral cells : U = UACC-62 (melanoma); M = MCF-7 (breast adenocarcinoma); A = NCI-ADR/RES (breast adenocarcinoma, multidrug resistant phenotype); 7 = 786-O (renal adenocarcinoma); P = PC-3 (prostate adenocarcinoma); O = NIH:OVCAR-3 (ovarian adenocarcinoma); K = K-562 (chronic myeloid leukemia); 4 = NCI-H460 (lung adenocarcinoma); H = HT-29 (cólon adenocarcinoma); V = VERO (green monkey kidney), 2= U251 (glioma). TGI = total citostatic effect.

The results are extremely interesting, since they show a greater capacity *in vitro* of volatile compounds when was compared with the β–carotene. Against the lung adenocarcinoma (NCI-H460) and green monkey kidney (VERO), the TGI obtained to β-ciclocytral (197.52 and 193.41 $\mu g.mL^{-1}$, respectivelly) was significatively lower.

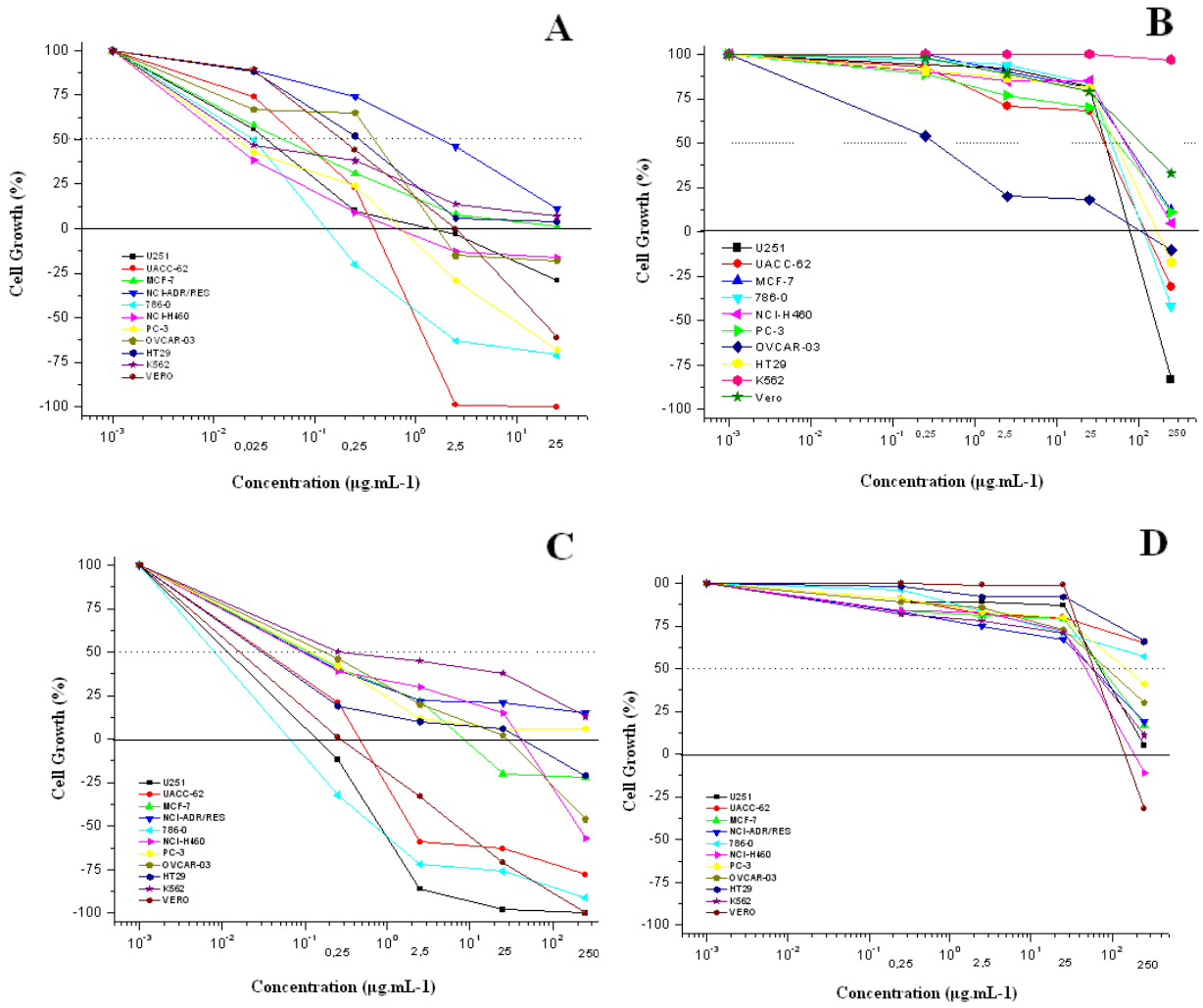

Figure 1 - Antiproliferative activity of (A) doxorrubicin; (B) β-carotene; (C) β-damascone and (D) β-ciclocytral. GI_{50} values (growth inhibitory activity): compound concentration that produce a 50 % reduction of cellular growth when compared to untreated control cells; TGI (cytostatic activity) values parameters refer to the compound concentration for total growth inhibition and LC_{50} (cytotoxic activity) values parameters refer to the compound concentration for total growth inhibition and for killing 50 % of the cells.

Overall, the data show that the carotenoid-derived aroma compounds are promising anticancer agents with multiple proliferation inhibitory effects *in vitro*. Currently the potential anticancer properties of these compounds are being studied *in vivo*.

4. CONCLUSION

Preliminary *in vitro* screening showed significant anti-cancer and anti-microbial activity of some carotenoid-derived aroma compounds The results obtained in the experiment suggested that some compounds may be useful as “functional aroma compounds”. However, citotoxic and genotoxic experiences *in vivo* are needed in order to use these aroma compounds for human and animal health.

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CONCLUSÃO GERAL

Inicialmente, o isolamento e seleção de micro-organismos potencialmente biotransformadores de carotenóides foram realizados. Dos mais de quatrocentos micro-organismos testados, aproxidamente sessenta apresentaram capacidade de gerar um halo de degradação do β-caroteno. Desta forma, as linhagens seguiram para avaliação dos compostos voláteis formados a partir da degradação deste tetraterpeno, onde os compostos foram extraídos e concentrados por SPME e analisados por CG-DIC. A linhagem LB398APD se destacou para a formação de compostos voláteis e a técnica de extração representou uma excelente ferramenta para análises dos compostos voláteis formados a partir da biotransformação do β-caroteno. Seguindo a mesma linha, outros terpenos foram avaliados como substratos, usando algumas linhagens fúngicas isoladas no início da Tese de Doutorado. Destaca-se dois importantes compostos voláteis formados a partir da biotransformação do limoneno: carveol e carvona. O trabalho descreve, até o momento, a primeira vez onde linhagens de *Paecylomyces* sp. são usadas em ensaios de biotransformação de limoneno.

A partir dos resultados obtidos na biotransformação do β-caroteno pela linhagem LB398APD, um estudo do efeito das principais variáveis envolvidas nesse processo para posterior otimização das condições de produção foi realizado. Foi demonstrado que o Planejamento Experimental, empregando matriz Plackett-Burman e um Delineamento Composto Central Rotacional foi eficiente para avaliação de 10 parâmetros principais. O uso de planejamento experimental e análise de superfície de resposta foi usada pela primeira vez para otimizar as condições de biotransformação deste tetraterpeno. Por sua vez, levando-se em consideração os custos do processo, dois resíduos (manipueira e glicerol) foram testados para o desenvolvimento de biomassa da mesma linhagem. Um planejamento completo (2^3)

para a manipueira, e um fracionado (2^{4-1}) para o glicerol foram avaliados. A manipueira apresentou excelentes resultados para o desenvolvimento da biomassa.

Por fim, uma avaliação da capacidade anti-proliferativa e anti-microbiana dos compostos de aroma derivados de carotenóides também foram realizadas. Os resultados sugerem novas possibilidades para utilização destes compostos na indústria de alimentos, uma vez que há uma demanda do mercado consumidor por produtos bioativos "naturais" que possuam papel na prevenção de doenças.

SUGESTÕES PARA TRABALHOS FUTUROS

Considerando-se os resultados aqui apresentados sugere-se:

- Avaliar linhagens de micro-organismos endofíticos isolados de frutas exóticas brasileiras quanto a capacidade de biotransformação de β-caroteno;
- Realizar a biotransformação usando outros carotenóides como substratos;
- Aprofundar metodologia analítica para experimentos de biotransformação (validação, recuperação dos compostos voláteis, etc);
- Acompanhar, através de cromatografia líquida de alta eficiência, o processo de biotransformação para verificar a taxa de conversão do terpeno em compostos de aromas;
- Usar substratos extraídos de fontes naturais (buriti, pupunha, etc), para redução dos custos do bioprocesso;
- Aprofundar os estudos na atividade antimicrobiana e antitumoral dos compostos de aromas derivados de carotenóides, assim como avaliar sua capacidade antioxidante;
- Realizar ensaios *in vivo* com os compostos de aromas que apresentaram melhores resultados.