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Original

Microorganisms screening for limonene oxidation

Seleção de microrganismos para oxidação de limoneno

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Abstract

Limonene is a monoterpene obtained in large amounts from essential oils and is used as a raw material for the synthesis of flavors and fine chemicals. Several pathways or routes for the microbial degradation of limonene making use of the cytochrome P450-dependent monooxygenases have been described. In this study, we present a fermentative screening of microorganisms in order to verify their ability to perform the desirable conversion. In parallel, the PCR technique was used to select the microorganisms that contain the *lim*C gene, which is responsible for the conversion of carveol to carvone. The microorganisms selected by PCR were not able to bioconvert limonene. From this result, we can suppose that these strains do not have the gene that codifies the enzyme responsible for the transformation of limonene into carveol. The results obtained in the fermentative screening showed that 4 microorganisms were able to bioconvert limonene into carveol. In addition, the amplification results showed the presence of fragments of 800 pb, expected for the *limC* gene. Therefore, the results obtained in the bioconversion and evaluation of the *limC* gene did not allow a correlation showing that these strains do not contain all the enzymes responsible for the conversion of limonene to carvone.

Keywords: *CDH (carveol dependent dehydrogenase); carveol; carvone; PCR; fermentative screening.*

Resumo

O limoneno é um monoterpeno obtido em grandes quantidades a partir de óleos essenciais, podendo ser utilizado como matéria-prima na síntese de *flavours* e compostos para química fina. Várias rotas para a degradação microbiológica do limoneno fazendo uso da monoxigenase dependente do citocromo P450 têm sido propostas. Neste trabalho, são apresentados dados obtidos no *screening* fermentativo de microrganismos visando verificar suas habilidades em realizar a conversão desejada. Em paralelo, a técnica do PCR foi também utilizada visando selecionar microrganismos que apresentam o gene *lim*C, responsável pela conversão de carveol a carvona. Os microrganismos selecionados por PCR não foram capazes de bioconverter o limoneno. A partir dos resultados obtidos pode-se supor que estas cepas não possuem o gene que codifica a enzima responsável pela etapa de transformação do limoneno a carveol. Os resultados obtidos no *screening* fermentativo mostraram que 4 microrganismos foram capazes de bioconverter o limoneno. Além disso, o resultado da amplificação mostrou a presença de fragmentos de 800 pb, esperados para o gene *limC*. Desta forma, os resultados obtidos na bioconversão e avaliação do gene *limC* gene não permitiram obter uma correlação, mostrando que as cepas testadas não possuem todas as enzimas responsáveis pelas etapas de conversão do limoneno a carvona.

Palavras-chave: *CDH (dehidrogenase dependente do carveol); carveol; carvona; PCR; screening.*

1 Introduction

The essential oils are a source of volatile terpenes, substances widely distributed in nature. Spearmint (*Mentha spicata*) and peppermint (*Mentha piperita*) are species used for the commercial production of essential oils. Their distinct characteristics are based on the oxygenation position of the constituent monoterpenoid ρ-mentane. Peppermint produces almost exclusive monoterpenes with oxygen C3 position (such as (-)-menthol, which is responsible for the cold sensation of the peppermint). The spearmint produces a monoterpene with oxygen at the C6 allylic position (such as (-)-carvone, which is responsible for the typical note of the spearmint) (LAWRENCE, 1981). The precursor olefin of the bioconversion reaction, (-)-4S-limonene, is hydroxylated exclusively at C3 position producing (-)-trans-isopiperitenol, which is subsequently converted into a complex mixture of compounds of peppermint oil. In spearmint, the limonene is hydroxylated exclusively at C6 position producing (-)-trans-carveol and, following the oxidation, (-)-carvone. This region-specific hydroxylation is mediated by two cytochrome P450 enzymes, limonene-6-hydroxylase (CYP71D18) and limonene-3-hydroxylase (CYP71D13) (SCHALK; CROTEAU, 2000).

Kubota et al. (2005) showed that the cytochrome P450 (CYP153A), heme alkane hydroxylase, from *Alcanivorax* sp.

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**A quem a correspondência deve ser enviada*

mediates the oxidation reactions of various hydrocarbons, including not only n-alkanes but also a cycle-alkane and aromatic hydrocarbons. This P450 gene was also used as a scaffold in the study in order to build the 5'- and 3'-arm segments for cassette Polymerase Chain Reaction (PCR) for expressing functionally new P450 genes isolated from various environments. *Escherichia coli* cells expressing these self-sufficient P450 chimerical genes able to convert n-alkanes, cycle-hexane, 1-octene, n-butyl-benzene, and 4-phenyl-1-butene to 1-alkanols, cycle-hexanol, 1,2-epoxyoctane, 1-pheny-4-butanol, and 2-phenethyl-oxirane, respectively. Many studies have been carried out about the oxygenases cytochrome P450 SINCE these enzymes play an important role in the metabolism of the terpenoids mould and stabilize the oxygenation of many types of carbon skeleton, These enzymes begin the subsequent redox transformation and reactions of the conjugated that contribute significantly for the structural diversity and the great number of natural products derivative of this class (LUPIEN et al., 1999).

Cytochrome P450-dependent hydroxylases play a pivotal role in the biosynthesis of several hundred naturally occurring oxygenated monoterpenes (e.g., menthol, carvone, thujone, pinocamphone), which are largely responsible for the flavor and fragrance of plant-derived essential oils (BAUER; GARBE; SURBURG, 1990). Most oxygenated monoterpenes are formed by the initial cyclization of the precursor geranyl-diphosphate to an olefin that defines the structural family of the metabolites produced, followed by the regio- and stereoespecifc cytochrome P450–dependent allylic hydroxylation of the parent olefin, which determines the oxygenation pattern of the derived metabolites (KARP; CROTEAU, 1988).

Several pathways for the microbial degradation of limonene have been described so far, but most of them have not been substantiated by biochemical studies. The best studied microbial degradation pathway for limonene involves the hydroxylation at the C_7-methyl group resulting in the formation of perillyl alcohol. Two enzymes of this pathway, perillyl alcohol dehydrogenase and perillyl aldehyde dehydrogenase, have been partially purified (BALLAL; BHATTACHARYYA; RANGACHARI, 1967, 1968; DHAVALIKAR; BHATTACHARYYA, 1966; TRUDGILL, 1986). Another proposed pathway mentions that the conversion of carveol to carvone is performed by the carveol dehydrogenase (CDH) (van der WERF; SWARTS; de BONT, 1999).

Isolated *Rhodococcus erythropolis* DCL14 (van der WERF; SWARTS; de BONT, 1999), a strain able to grow on limonene as sole carbon and energy source that contains a novel degradation pathway for limonene. Cells grown on limonene and carveol were found to contain a DCPIP–dependent carveol dehydrogenase (CDH) that converts carveol into carvone. The *R. erythropolis* CDH *lim*C gene was identified in an operon encoding the enzymes involved in limonene degradation. The CDH nucleotide sequence revealed an open reading frame of 831 base pairs (bp) encoding a 277-amino acid protein with a deduced mass of 29,531 Da.

In this report, we describe the development of a biotechnological process of alyllic oxidation of limonene by screening microorganisms so as to verify the ability to perform the desirable conversion. In parallel, the PCR technique was also used to select the microorganisms that contain the *lim*C gene, responsible for the conversion of carveol to carvone.

2 Materials and methods

2.1 Cell production

A total of 17 microorganisms were used in this study. The strains of microorganisms belonging to different genera were obtained from different institutions, as shown in Table 1. All the strains were generously supplied by those respective institutions. Table 1 also presents the culture medium used for each microorganism. Fungi were cultivated in PD, YM, Malt, and Czapek Dox at 25 °C for 72 hour; except for *Paecilomyces variotii* that was cultivated for 120 hours. Yeasts were cultivated in YM at 25 °C for 24 hours and the bacteria were cultivated in LB at 28 °C for 24 hours. Stock cultures of organisms were maintained in medium slants and stored at 4 °C after growth.

2.2 Screening experiments

For the biotransformation experiments, the reactions were started 3 days after inoculation for the fungi and 24 hours for bacteria and yeasts by adding 300 µL of the substrate directly to the culture flasks with 30 mL of medium. The substrates were added as solutions in absolute ethanol (1:1 v/v). It was used 1% (v/v) of substrate in 1% (v/v) of EtOH, at 25 °C and 150 rpm for 6 days in all experiments. The media and cultivation time used for each microorganism are presented in Table 1. All experiments were carried out in parallel with controls and under the same experimental conditions without the presence of microorganism. The experiments (duplicate runs were conducted for each experimental condition) were performed for each microorganism in closed stoppered glass flasks in order to avoid the evaporation of the substrate and product.

2.3 Extraction and identification of biotransformation products

The cells were removed by filtration for fungi and centrifugation for bacteria and yeasts. The product recovery was performed by liquid-liquid extraction with 3 × 12 mL of ethyl acetate (Et_2O). After the extraction, the solution volume was completed to 25 mL. The final solution was dried over anhydrous sodium sulphate.

The reaction products were identified by GC/MS (Shimadzu QP5050A) using a capillary column DB-WAX (30 m, 0.25 mm, 0.25 µm). The column temperature was programmed at 50 °C for 3 minutes, increased by 5 °C/minute to 130 °C, and then increased by 15 °C/minute to 210 °C for 5 minutes. Helium was the carrier gas, and the injection and detector temperatures were both 250 °C. 0.5 µL of the dried solution was injected into the GC/MS system. The apparatus operated with a flow rate of 1 mL/minute in electronic impact mode of 70 eV and in split mode (split ratio 1:10).

The identification of the compounds was accomplished by comparing the mass spectra with those from the Wiley library

Table 1. Microorganisms tested in the screening and composition of different growth media.

Code	Microorganism	Culture medium	Time cultivation (hour)
1	*Aspergillus niger*[1]	PD[5]	72
5	*Aspergillus niger* ATCC 16404[2]	PD[5]	72
12	*Aspergillus niger* ATCC 9642[2]	YM[6]	72
19	*Aspergillus niger* ATCC 1004[2]	PD[5]	72
17	*Aspergillus oryzae* ATCC 1003[2]	PD[6]	72
3	*Penicillium citrinum* ATCC 28752[2]	Malt[7]	72
7	*Penicillium digitatum* ATCC 26821[2]	PD[5]	72
10	*Penicillium notatum* ATCC 9478[2]	PD[5]	72
15	*Penicillium brevicompactum*[3]	PD[5]	72
29	*Penicillium camembertii* (CT) ATCC 4845[2]	PD[5]	72
31	*Penicillium verrucosum*[3]	PD[5]	72
33	*Penicillium simplicissimum*[3]	PD[5]	72
35	*Penicillium duclauxii* ATCC 9121[2]	PD[5]	72
13	*Paecilomyces variotii* ATCC 22319[2]	Czapek Dox[8]	120
21	*Pseudomonas putida*[1]	LB[9]	24
25	*Pseudomonas aeruginosa* ATCC 27853[4]	LB[9]	24
27	*Candida* sp. ATCC 34147	LB[9]	24

[1]Instituto de Microbiologia of the Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil), [2]Fundação Instituto Oswaldo Cruz (Rio de Janeiro, Brazil), [3]These microorganisms were isolated from the reject of the industry of babassu for Freire, Gomes and Bom (1997) [4]Laboratório de Biotecnologia Vegetal of the Universidade Regional Integrada (Erechim, Brazil). [5]PD (300 g infusion from potates and 20 g glucose); [6]YM (3 g yeast extract, 3 g malt extract, 5 g peptone and 10 g glucose); [7]Malte (30 g malt extract and 3 g peptone); [8]Czapec Dox (0,5 g KCl, 1 g KH_2PO_4, 2 g $NaNO_3$, 30 g sucrose, 0,1 g $FeSO_4.H_2O$, 0,5 g $MgSO_4.7H_2O$) and [9]LB (10 g tryptone, 5 g yeast extract and 10 g NaCl), all the media were prepared in 1000 mL of distilled water.

and by additional comparison of the GC retention time of standard compounds.

2.4 Genomic DNA extraction

The DNA extraction from bacteria and yeast was performed according to Sambrook, Fritsch and Maniatis (1989) with some modifications. Cultures of each strain were grown according to the conditions presented previously (Table 1) 1.5 mL of each culture was centrifuged (Eppendorf Centrifuge Model 5403) for 5 minutes at 21.467 × g to collect the bacterial or yeast cells, and the supernatant was discarded. Each pellet was mixed in 600 µL of buffer containing 25 mM Tris ([Hydroxymethyl] aminomethanol–Sigma) - HCl, pH 8.0, 10 mM EDTA (Ethylenediaminetetracetic Acid, Disodium Salt, and Dihydrate–Na_2 EDTA.$2H_2O$–Gibco BRL), and 50 mM glucose were then added and mixed. Next, 50 µL of lysozyme (2 mg.mL^{-1}, Sigma) was added, and the mixture was left in repose for 10 minutes. Afterwards, 66 µL of 10% (m/v) sodium dodecyl sulfate (SDS) and 3 µL of 2-mercaptoethanol was added to each tube, which was vortexed briefly and then incubated at 65 °C for 30 minutes.

To each tube, it was added 190 µL of 3 M sodium acetate and mixed by inversion. Next, the mixture was incubated for 30 minutes at 4 °C and then centrifuged for 5 minutes at 21.467 × g. The supernatant containing DNA was precipitated with one volume of ice-cold isopropanol mixed by inversion, incubated at -20 °C for 10 minutes, and then centrifuged for 5 minutes at 21.467 × g. The supernatant was discarded and the air dried, and re-suspended in 200 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

The DNA extraction from fungi was based on the methodology developed by Roeder and Broda (1987). Cultures of each strain were grown according to the conditions presented previously (Table 1). Liquid nitrogen was added to 300 mg of mycelia in a mortar, and the cells were ground in a pestle. In sum, the powdered mycelium was transferred to an Eppendorf tube and 700 µL of buffer (3% (v/v) SDS, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 1% (v/v) 2-mercaptoethanol) were added. Each sample was incubated for 1hour at 65 °C. Then, the tubes content were removed and cooled, extracted with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged at 21.467 × g for 10 minutes. This procedure was repeated three times. After this, it was added 120 µL of 3 M pH 5.2 sodium acetate and 600 µL of ice-cold isopropanol mixed by inversion. The mixture was incubated in a freezer for 30 minutes and centrifuged for 5 minutes at 21.467 × g. The DNA precipitate was re-suspended in 500 to 700 µL of TE buffer, washed twice in 500 µL of 70% ethanol, air dried, and re-suspended in 200 µL of TE buffer.

2.5 DNA quantification

For the evaluation of DNA concentration, 1000 µL of water MilliQ sterile were added in an assay tube and 20 µL of sample. The DNA concentration was estimated by measuring the optical density at 260 nm using a spectrophotometer (Agilent 8453) and checked by electrophoresis on a 0.8% agarose gel (Gibco BRL) in TBE buffer 1X(0.89 M Tris, 0.89 M of H_3BO_3 and 0.08M of EDTA).

2.6 PCR amplification reaction

The PCR reactions were conducted in total volumes of 25 µL. The reaction mixture contained 50 mM Tris-HCl pH 9.0 buffer; 50 mM KCl (Life Technologies, São Paulo, Brazil), dNTPs mix (200 mM of each nucleotide (Life

Technologies, São Paulo, Brazil)), 10 ρmol of the primer 1 (GGGACAATGGCAAGAGTAGAA), and 10 ρmol of the primer 2 (TCCTGCATCGACGGGAAT) (these primers were selected to design the sequence obtained at GenBank for the *lim*C gene), 3 mM of MgCl2, 1,5 U of Taq DNA polymerase, Recombinant Invitrogen (Life Technologies, São Paulo, Brazil), and 40 ng of DNA.

2.7 PCR amplification

The amplification was performed in a thermal cycler, MJ Research Inc., Watertown, MA (model PTC100TM Programmable Thermal Controller) as follows: one initial cycle for three minutes at 94 °C, followed by 40 cycles for 1 minute at 94 °C, 1 minute at 50 °C and 2 minutes at 72 °C and one cycle of 5 minutes at 72 °C followed by cooling at 4 °C.

2.8 Electrophoresis of the amplifying fragments

The amplification products were separated by electrophoresis in 1.4% agarose gels in buffer TBE 1X(0.89 M Tris, 0.89 M of H_3BO_3 and 0.08 M EDTA) in a horizontal electrophoresis cube. The run was performed with constant voltage of 90 v. DNA Lambda digested with *Eco*RI and *Hind* III of the Gibco BRL was included as a molecular size marker. The gels were visualized by staining with ethidium bromide and the banding patterns were photographed under UV light. The gels were photographed using the digital system photographic GEL-PRO (Media Cybernetics, Silver Spring, MD).

2.9 DNA extraction of gel

The material amplified by PCR was extracted from the gel using the QIAquick (Gel Extraction Kit (Quiagen®)) kit and estimated on agarose gel 0.8%.

3 Results and discussion

A screening by fermentative process was performed in order to select the strains able to convert limonene into oxygenated products, especially carvone.

Limonene was one of the first terpenic compounds used in biotransformation studies. Dhavalikar and Bhattacharyya (1966) proposed three routes (Figure 1) for the biotransformation by microorganisms (a, b and c). Later, a new route was proposed by Kraidman et al. (1969) and evaluated by Kraidman, Mukherjee and Hill (1986) for the biotransformation by filamentous

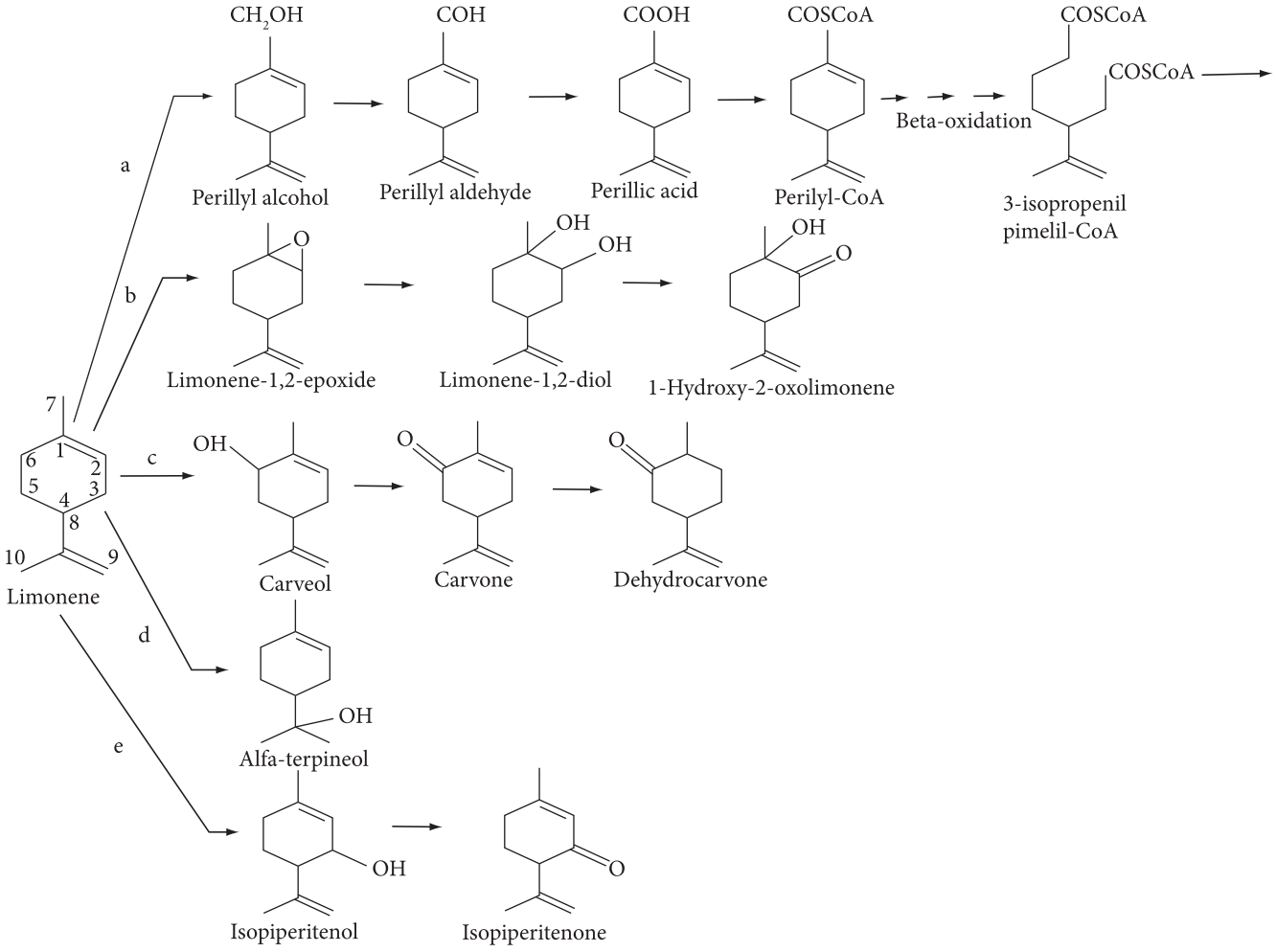

Figure 1. Proposed routes for limonene biotransformation by microorganisms.

fungus (d). van der Werf; Swarts and de Bont, (1999) studied another route (e) which shows the bioconversion of limonene to isopiperitenol and then, to isopiperitenone.

Kieslich et al. (1986) presented the results of an extensive research, in which 800 strains of many microorganism species were tested in relation to their potencial use in the biotransformation of terpenic compounds. The authors observed that 320 strains were able to convert limonene into products of known metabolic ways.

Chang and Oriel (1994) isolated a thermophilic bacterium from orange peel, *Bacillus stearothermophilus* supposing it was adapted to limonene, the main component from the orange peel oil. Perylic alcohol and α-terpineol were identified as major biotansformation products; nevertheless many other products were also obtained. The obtention of several products is not desirable in a bioconversion resulting in high separation costs and lower conversion for the product of interest.

The limonene transformation by *Pleurotus sapidus* to *cis*/trans-carveol and carvone was performed by cultivation of pre-cultures in the presence of limonene obtaining a conversion of 1.2 and 0.8%, respectively (ONKEN; BERGER, 1999).

Based on the results presented in the literature, one can observe that there are many studies related to the limonene bioconversion; nevertheless, references about a systematic study to obtain better selectivity and conversions are scarce in the literature. Due to these aspects, in the present work, the PCR technique was also used with the main objective of selecting strains that present the *lim*C gene, responsible for conversion of carveol to carvone, for a further study of over expression. The ability, versatility, and sensitivity of PCR make this technique interesting for genetic-molecular studies involving many individuals of any live organism. Many traditional methods of cloning, sequencing, and the polymorphism analysis of DNA were accelerated or substituted by using the various forms of PCR techniques.

3.1 Screening experiments

After the screening experiments, some strains were able to perform the desired biotransformation. Table 2 shows the microorganisms that presented ability for transformation, the end products, and the reaction conversion (%) obtained in relation to the normalized peak areas. The strains selected were *Aspergillus niger, Penicillium simplicissimum, Aspergillus niger* ATCC 9642, and *Pseudomonas putida*.

The metabolites recovered were α-terpineol and cycle-hexanemethanol for *A. niger,* α-terpineol for *A. niger* ATCC 9642, *cis*-carveol for *P. simplicissinum*, dehydrocarveol, and perillyl alcohol for *P. putida.*

From the results obtained in the fermentative screening, it is relevant to mention that, in spite of the low conversions obtained, *cis*-carveol and dehydrocarveol are precursors to carvone production, an important and high value compound for flavor industries (TECELÃO; van KEULEN; FONSECA, 2001).

3.2 Evaluation of the presence of limC gene

The gene of CDH enzyme (Dichlorophenolindophenol–dependent of carveol dehydrogenase), responsible for the conversion of carveol to carvone, was designed as *lim*C. This operon is related to the limonene degradation, specifically in the conversion of carveol to carvone step. The nucleotides sequence of CDH presented an ORF with 834 pairs of bases. To confirm that this gene is related to the route of limonene degradation, experiments of cloning and sequencing were performed by using the genetic material of *Rhodococcus erythropolis* DCL14 (van der WERF; SWARTS; de BONT, 1999).

In the present study, the amplification by PCR technique was performed by using PCR primers design from the sequence obtained in GenBank for the *lim*C gene. The results obtained in the amplifications made it possible to correlate the strains that present the fragment of interest and the results obtained in the fermentative screening.

The results of the amplification showed the presence of fragments between 700 and 900 bp, and the desired fragment was 834 bp (van der WERF; SWARTS; de BONT, 1999). The fragments were identified and are presented in Figure 2, numbers 1 to 7. These fragments were obtained in strains of *Aspergillus niger* ATCC 16404, *Aspergillus niger* ATCC 9642, *Aspergillus oryzae* ATCC 1003, *Penicillium notatum* ATCC 9478, *Penicillium camembertii* (CT) ATCC 4845, and *Paecilomyces variotii* ATCC 22319. These six strains were chosen through the visualization of agarose gel.

The obtained fragments close to 830 bp (numbers 1 to 7) were isolated from agarose gel and purified for a new amplification (Figure 3). In this new gel, 9 fragments were taken, which were repurified and stocked for sequencing analysis (unpublished results).

Among the evaluated strains, it was not possible to correlate the microorganisms that presented the fragment of interest with the strains able to bioconvert limonene into carveol. The metabolic route of interest can be visualized in Figure 4, which shows the respective enzymes responsible for the studied steps.

Table 2. Microorganisms selected, end products and conversion obtained from the fermentative screening.

Microorganism	Products (relative %)				
	α-terpineol	Cycle-hexanemethanol	*Cis*-carveol	Dehydrocarveol	Perillyl alcohol
A. niger	0.68 ± 0.19	0.64 ± 0.27	-	-	-
A. niger ATCC 9642	0.13 ± 0.07	-	-	-	-
P. putida	-	-	-	0.07 ± 0.01	0.31 ± 0.01
P. simplicissimum	-	-	0.18 ± 0.05	-	-

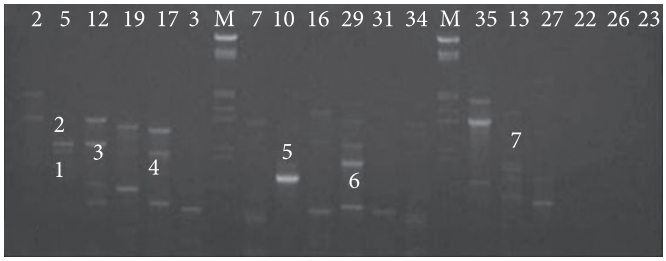

Figure 2. Agarose gel (1,4%) demonstrative obtained with primers 1 and 2. Where M is molecular size marker in base pairs (phase λ DNA digested wit EcoRI and Hind III, Gibco, BRL); and 1 represents *Aspergillus niger*, 3 *Penicillium citrinum* ATCC 28752, 5 *Aspergillus niger* ATCC 16404, 7 *Penicillium digitatum* ATCC 26821, 10 *Penicillium notatum* ATCC 9478, 12 *Aspergillus niger* ATCC 9642, 13 *Paecilomyces variotii* ATCC 22319, 15 *Penicillium brevicompactum*, 17 *Aspergillus oryzae* ATCC 1003, 19 *Aspergillus niger* ATCC 1004, 29 *Penicillium camembertii* (CT) ATCC 4845, 31 *Penicillium verrucosum*, 33 *Penicillium simplicissimum*, 35 *Penicillium duclauxi* ATCC 9121, 21 *Pseudomonas putida*, *Pseudomonas aeruginosa* ATCC 27853, and 27 *Candida* sp. ATCC 34147.

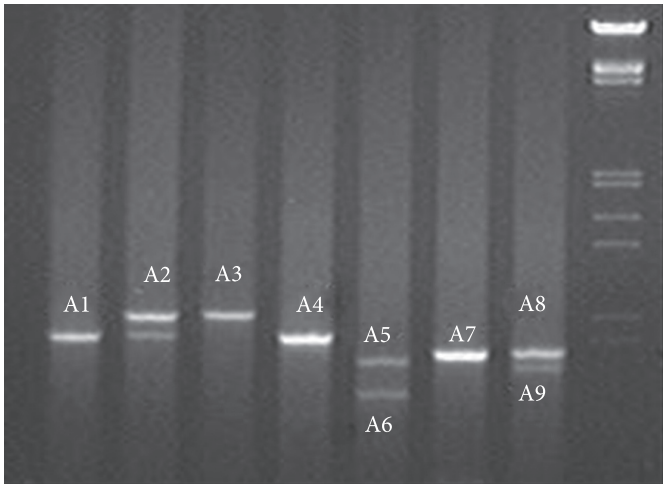

Figure 3. Agarose gel (1,4%) demonstrative obttained after one second amplification with the microorganisms selected. M is the molecular size marker in base pairs (phase λ DNA digested wit EcoRI and Hind III, Gibco, BRL); and 5 *Aspergillus niger* ATCC 16404, 10 *Penicillium notatum* ATCC 9478, 12 *Aspergillus niger* ATCC 9642, 13 *Paecilomyces variotii* ATCC 22319, 17 *Aspergillus oryzae* ATCC 1003 and 29 *Penicillium camembertii* (CT) ATCC 4845. A1, A2, A3, A4, A5, A6, A7, A8, and A9 were used to identify the bands selected.

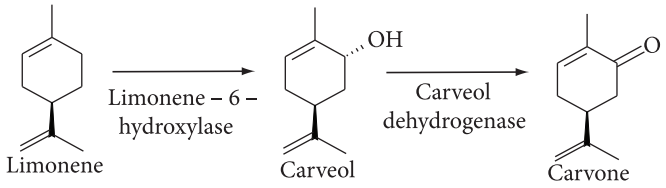

Figure 4. Biosynthetic route to carvone.

The strains of *Penicillium simplicissimum* and *Pseudomonas putida* were able to convert limonene into cis-carveol and dehydrocarveol, respectively. Nevertheless, these strains did not present the 834 bp fragment, as it can be observed in Figure 2. Thus, we can conclude that these microorganisms do not present the gene responsible for the conversion of carveol to carvone.

The selected microorganisms that presented the fragment of approximately 800 bp were not able to bioconvert limonene. From this result, we can suppose that these strains do not have the gene that codifies the enzyme responsible for the transformation of limonene to carveol. It can be said that they have only the gene able to convert carveol into carvone.

The results obtained in the bioconversion process and study of the *limC* gene presence does not allow a correlation. Among the evaluated microorganisms, none was able to produce carvone, only products of limonene degradation route, showing that these strains do not have all the enzymes responsible for the steps of conversion of limonene to carvone.

4 Conclusion

The results obtained in the fermentative screening showed that 4 microorganisms were able to bioconvert the substrate limonene. The metabolites recovered were α-terpineol and cycle-hexanemethanol (1,8–terpin) for *A. niger,* α-terpineol for *A. niger* ATCC 9642, dehydrocarveol, perillyl alcohol for *P. putida,* and cis-carveol for *P. simplicissimum.*

The amplification results obtained by using the PCR technique show the presence of fragments close to 800 pb, expected for the *limC* gene. These fragments were obtained for the strains of *Aspergillus niger* ATCC 16404, *Aspergillus niger* ATCC 9642, *Aspergillus oryzae* ATCC 1003, *Penicillium notatum* ATCC 9478, *Penicillium camembertii* (CT) ATCC 4845, and *Paecilomyces variotii* ATCC 22319. The results obtained in the bioconversion process and evaluation of the *limC* gene presence did not enable us to perform a correlation showing that these strains do not contain all enzymes responsible for the steps of conversion of limonene to carvone.

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