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Microorganism Screening for Limonene Bioconversion and Correlation With RAPD Markers

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Abstract

The use of microorganisms for biotransformations of monoterpenes has stimulated the biotechnological market. Aiming at the highest efficiency in the process of strains screening, the application of molecular biology techniques have been proposed. Based on these aspects, the objective of this work was to select different strains able to convert limonene using fermentative process and random amplified polymorphic DNA (RAPD) markers. The results obtained in the fermentative screening, from 17 strains tested, pointed out that four microorganisms were able to convert limonene into oxygenated derivatives. The RAPD study showed a polymorphism of 96.02% and a similarity from 16.02 to 51.51%. Based on this it was possible to observe a high genetic diversity, even among strains of same species, concluding that the RAPD was not able to correlate the genetic characteristics of the microorganism with the results obtained from the biotransformation process.

Index Entries: Bioconversion; limonene; RAPD; screening.

Introduction

Terpenes and especially their oxygenated derivatives are extensively used in the flavor industry. Limonene (4-isopropenyl-1-methylcyclohexene), a monocyclic monoterpene, is a low-priced monoterpene and in most cases the major compound in essential oils of citrus fruits *(1–3)*. The production of flavors through a biotechnological route offers a number of advantages. One important attribute of microbial biocatalysis is the ability

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to synthesize products that can be labeled as natural, if derived from natural substrates, and added to foods without being considered as additives. Another important attribute is to convert monoterpene precursors or intermediates into higher value products for flavors and fragrance industries *(1,2)*. Screening of microorganisms, plants, or animal cells represent a natural way to obtain new enzymes. The microorganisms, in this case, are of particular interest owing to the large diversity of metabolic processes and enzymes involved, and the unlimited number of microorganisms in nature that can be tested. They modify and degrade a variety of organic molecules and complexes, and so, it can be expected that some of them may be capable of catalyzing specific reactions *(4)*, such as biotransformation of limonene.

In this sense, the fermentative screen can be performed in order to confirm the strains that are able to convert limonene into oxygenated monoterpenes. As examples, Dhavalikar and Bhattacharyya *(5)* and Dhavalikar et al. *(6)* isolated strains of *Pseudomonas* able to grow on limonene as the sole carbon and energy source. Dehydrocarvone, carvone, carveol, 1,2-*cis*-diol-limonene among other compounds were accumulated during growth. Bowen *(7)* used the *Penicillium digitatum* strain, isolated from orange, and the strain was able to convert limonene into several compounds. Noma et al. *(8)* used the fungus *Aspergillus niger* to biotransform limonene, obtaining *trans*-1,2-diol-(+)-limonene as a major reaction product and, in smaller quantities, (+)-*cis*-carveol, (–)-carvone, and α-terpineol, among others. From these results, it can be observed that at least a few microorganisms are able to transform limonene into cis/trans-carveol and carvone *(5,7)*. In order to obtain a better efficiency in the screening process, techniques of molecular biology have been used *(9–11)*. In this sense, the random amplified polymorphic DNA (RAPD) is technically simple and automated. It requires small amounts of DNA, no previous information on the target genome sequence and the level of polymorphism obtained is usually high *(12)*. RAPD markers, which are based on the amplification of discrete DNA fragments in the genome by the use of oligonucleotide primers with random sequences, have been widely used to identify physiological strains of fungi *(9)*. The adoption of RAPD markers in detection, diagnostic, and determination of genetic diversity is related to the simplicity of the method, which has high sensitivity, low cost and is safe and fast. The RAPD technique has been proven to be useful for measuring and characterizing the genetic variability of microorganisms, plants, and animal cells *(13,14)*. The objective of this work was to use RAPD markers to analyze different strains reported in literature as able to biotransform limonene. Simultaneously, fermentations have been performed to bioconvert limonene, with the purpose of comparing the results with those obtained from the molecular biology study. Based on these aspects, this work screened 17 strains of microorganisms for their ability to bioconvert limonene.

Table 1
Microorganisms Tested in the Screening and Composition of Different Growth Media

Code	Microorganism	Culture medium	Time cultivation (h)
1	*A. niger*[a]	PD[e]	72
5	*A. niger* ATCC 16404[b]	PD[e]	72
12	*A. niger* ATCC 9642[b]	YM[f]	72
19	*A. niger* ATCC 1004[b]	PD[e]	72
17	*A. oryzae* ATCC 1003[b]	PD[f]	72
3	*P. citrinum* ATCC 28752[b]	Malt[g]	72
7	*P. digitatum* ATCC 26821[b]	PD[e]	72
10	*P. notatum* ATCC 9478[b]	PD[e]	72
15	*P. brevicompactum*[c]	PD[e]	72
29	*P. camembertii* (CT) ATCC 4845[b]	PD[e]	72
31	*P. verrucosum*[c]	PD[e]	72
33	*P. simplicissimum*[c]	PD[e]	72
35	*P. duclauxii* ATCC 9121[b]	PD[e]	72
13	*Paecilomyces variotii* ATCC 22319[b]	Czapek Dox[h]	120
21	*P. putida*[a]	LB[i]	24
25	*P. aeruginosa* ATCC 27853[d]	LB[i]	24
27	*Candida* spp. ATCC 34147	LB[i]	24

[a]Instituto de Microbiologia of the Universidade Federal do Rio de Janeiro (Rio de Janeiro Brazil).
[b]Fundação Instituto Oswaldo Cruz (Rio de Janeiro Brazil).
[c]These microorganisms were isolated from the reject of the industry of babassu for Freire et al. *(22)*.
[d]Laboratório de Biotecnologia Vegetal of the Universidade Regional Integrada (Erechim, Brazil).
[e]PD (300 g infusion from potates and 20 g glucose).
[f]YM (3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g glucose).
[g]Malte (30 g malt extract, and 3 g peptone).
[h]Czapec Dox (0.5 g KCl, 1 g KH_2PO_4, 2 g $NaNO_3$, 30g sucrose, 0.1 g $FeSO_4 \cdot H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$).
[i]LB (10 g tryptone, 5 g yeast extract, and 10 g NaCl), all the media were prepared in 1000 mL of distilled water.

Materials and Methods

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 the respective institutions. Table 1 also presents the cultive medium and cultivation times used for each microorganism. Fungi were cultivated in potato dextrose (PD), yeast malt (YM), Malt, and Czapek Dox at 25°C for 72 h; except for *Paecilomyces variotii* that was cultivated for 120 h. Yeasts were cultivated in YM at 25°C for 24 h and bacteria were cultivated in Luria

Bertani (LB) at 28°C for 24 h. Stock cultures of organisms were maintained in medium slants and stored at 4°C after growth.

Screening Experiments

For biotransformation experiments the reactions were started 3 d after inoculation for fungi and 24 h after inoculation for bacteria and yeasts by adding 300 µL of substrate directly into culture flasks containing 30 mL of medium. These were then maintained for 6 d in an orbital shaker operating at 25°C and 150 rpm. The substrate was added as solutions in absolute ethanol (1:1 v/v); all experiments used 1% (v/v) of substrate in a background of 1% (v/v) of EtOH. All experiments were carried out in duplicate in parallel, blanks were run using control media, under the same conditions, without the microorganism present. The experiments were performed for each microorganism in closed stopped glass flasks in order to avoid substrate and product evaporation.

Extraction and Identification of Biotransformation Products

At the end of the experiments, 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 extraction, the solution volume was brought up to 25 mL with the additional Et_2O. The final solution was then dried using anhydrous sodium sulphate. The reaction products were identified by gas chromatography (GC)/mass spectrometer (MS) (Shimadzu QP5050A), using a capillary DB-WAX column (30 m, 0.25 mm, 0.25 µm). The column temperature was programmed at 50°C for 3 min, then increased at 5°C/min up to 130°C and then further increased at 15°C/ min up to 210°C and then held at this temperature for 5 min. Helium was the carrier gas, and the injection and detector temperatures were set to 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/min 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 and by additional comparison of the GC retention time of standard compounds. The products were semi-quantified by the percent of area of each peak in relation to total peak area.

Genomic DNA Extraction

DNA extraction of bacteria and yeast was performed according to Sambrook et al. *(15)* with some modifications. Cultures of each strain were grown as previously presented (Table 1). Almost 1.5 mL of each culture was centrifuged (Eppendorf Centrifuge Model 5403) for 5 min 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 m*M* Tris ([Hydroxymethyl] aminomethanol – Sigma)-HCl, pH 8.0, 10 m*M* ethylenediaminetetracetic

acid (EDTA), (Disodium Salt, Dihydrate – Na_2 EDTA·$2H_2O$ – Gibco BRL) and then 50 m*M* glucose was added and the solution further mixed. Then, 50 µL of lysozyme (2 mg/mL, Sigma) was added, and the mixture was maintained in repose for 10 min. Afterwards, 66 µL of 10% (w/v) sodium dodecyl sulfate (SDS) and 3 µL of 2-mercaptoethanol was added to each tube, which was then briefly vortexed before being incubated at 65°C for 30 min. Thereafter, 190 µL of 3 *M* sodium acetate was added to each tube and mixed by inversion. This mixture was then incubated at 40°C for 30 min (in a refrigerator) and then centrifuged for 5 min at 21.467*g*. The supernatant was collected for use. The DNA was precipitated using one volume of ice-cold isopropanol mixed in by inversion, and then incubating at –20°C (in a freezer) for 10 min before centrifuging for 5 min at 21.467*g*. The supernatant was discarded. The recovered precipitate was air dried and then resuspended in 200 µL of TE buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA).

The DNA extraction of fungi was based on the method reported by Roeder and Broda *(16)*. Cultures of each strain were grown as previously presented (Table 1). Liquid nitrogen was added to 300 mg of mycelia in a mortar, and the cells were ground with a pestle. In brief, the powdered mycelium was transferred to an Eppendorf tube and 700 µL of buffer (3% [v/v] SDS, 50 m*M* EDTA, 50 m*M* Tris-HCl, pH 8.0, 1% [v/v] 2,-mercaptoethanol) were added. Each sample was incubated for 1 h at 65°C. Then, the tubes'contents were removed and cooled, extracted with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged at 21.467*g* for 10 min. This procedure was repeated three times. Afterwards, 120 µL of 3 *M* sodium acetate pH 5.2 and 600 µL of ice-cold isopropanol were added and the solution was mixed by inversion, incubated at –20°C for 30 min and then centrifuged for 5 min at 21.467*g*. The suspension was resuspended at 500–700 µL of TE buffer, washed twice in 500 µL of 70% ethanol, air dried, and resuspended in 200 µL of TE buffer.

DNA Quantification

For DNA concentration 980 µL of Milli-Q sterile water and 20 µL of sample were added to an assay tube. DNA concentration was estimated by measuring the optical density at 260 nm, using a spectrophotometer (Agilent 8453), and this estimate was checked by performing electrophoresis on a 0.8% agarose gel (Gibco BRL) in TBE 1 × (0.89 *M* Tris, 0.89 *M* of H_3BO_3 and 0.08 *M* EDTA).

Amplification Reaction

For amplification procedure, the reaction reported by Williams et al. *(10)*, was used with some modifications. RAPD reactions were conducted in total volumes of 25 µL. The reaction mixture contained buffer (50 m*M* Tris-HCl pH 9.0, 50 m*M* KCl) (Life Technologies, São Paulo, Brazil), dNTPs mix (200 m*M* of each nucleotide), 0.2 m*M* of primer, 3 m*M* $MgCl_2$, 0.25 m*M*

Table 2
Total and Polymorphic Numbers of Fragments Obtained for Each Primer Used for the 17 Microorganisms

Primer	Sequence (5′ to 3′)	Total fragments	Polymorphic fragments
OPA-03	AGTCAGCCAC	17	17
OPA-04	AATCCGGCTG	30	30
OPA-13	CCACACCAGT	31	31
OPA-20	GTTGCGATCC	22	21
OPF-09	CCAAGCTTCC	12	6
OPH-18	GAATCGGCCA	20	20
OPH-19	CTGACCAGCC	27	27
OPY-03	ACAGCCTGGT	18	18
OPW-19	CAAAGCGCTC	24	23
TOTAL		201	193
Polymorphism (%)			96.02

Triton-X-100, 1.5 U of recombinant Taq DNA polymerase (Invitrogen), and 40 ng of DNA.

Random RAPD Primers

The kits OPA, OPF, OPH, OPW, and OPY from the Operon Technologies (Alameda, CA), were used with 20 primers of each one, in order to identify the primers that showed the better results, estimate the numbers, intensities, sizes of each band, reproducibility as well as to generate polymorphism, selecting nine primers (Table 2).

Amplification Procedure RAPD

The amplification was performed in a thermo cycles MJ Research Inc., Watertown, MA (model PTC100TM Programmable Thermal Controller) as follows: one initial cycle for 3 min at 92°C, followed by 40 cycles comprising denaturation (one min at 92°C); annealing (1 min at 35°C); and extension (two min at 72°C) and then a final extension for 3 min at 72°C.

Electrophoresis of the Amplifying Fragments

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) on an electrophoresis horizontal cube using a constant voltage of 90 Volts. DNA Lambda was digested with *Eco*RI and *Hind* III (Gibco BRL- Life Technologies, São Paulo, Brazil) was included as a molecular size marker. Gels were visualized by staining with ethidium bromide (SIGMA Chemical CO, St Louis, MO) and banding patters were photographed over UV light. The gels were photographed using a GEL-PRO digital photographic system (Media Cybernetics, Silver Spring, MD).

Analysis of RAPD Products

To determine the genetic variability, the matrix formed by the presence or absence of bands was analyzed using NTSYS version 1.7 software (Numerical Taxonomy System for Multivariate Analysis). The dendrogram were constructed by the algoritm UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) using the Jaccard coefficient of similarity *(17)*.

Results and Discussion

The strains used in this work, as presented in Table 1, were selected based on results reported in the literature related to bioconversion of monoterpenes.

Screening Experiments

The screening experiments confirmed that some strains were able to perform the desired biotransformation of limonene. Table 3 shows the subset of microorganisms that exhibited the ability for transformation and the end products they produced. Because the proposed reactions are slow, we believe the oxygen in the headspace of the closed flasks was sufficient to enable biotransformation. However, this was a screening effort and the evaluation of oxygen limitations was outside the scope of this work. Further investigations are being carried out to verify oxygen requirements and limitations in 2-L bioreactors equipped with oxygen monitoring and control.

The strains selected were *Aspergillus niger, Penicillium simplicissimum*, *A. niger* ATCC 9642 and *Pseudomonas putida*. The metabolites recovered after biotransformation of limonene 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*. Figure 1 presents the structures of these different metabolites. It is relevant to note that in spite of the low conversions obtained, *cis*-carveol, and dehydrocarveol are precursors to carvone production, an important and high value compound in the flavor industries *(18)*. Experiments are underway in our group to optimize the process conversion process, to improve carveol production.

RAPD Analysis

Based on the results obtained in the fermentative screening, the RAPD technique was used in order to test if genetic characteristics could be correlated with the microbial capacity of limonene bioconversion. These experiments used the nine primers from Operon Technologies listed in Table 2. The amplification reactions with the nine primers generated a total of 201 fragments, 193 (96.02%) of them being polymorphic (Table 2) with sizes ranging from 50 to 2200 bp (Fig. 2). The average number of fragments per primer was 22.33. Figure 2 shows the variability observed for different species analyzed using the primer OPA-04.

Table 3
Microorganisms Selected, End Products and Concentration of the Fermentative Screening

	Products (% ratio of product in relation to all products and substrate present in the sample)				
Microorganism	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 ± 0.00	0.31 ± 0.01
P. simplicissimum	–	–	0.18 ± 0.05	–	–

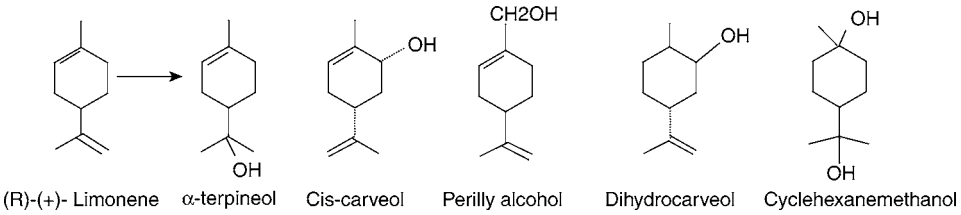

Fig. 1. Structures of metabolites recovered after biotransformation of limonene.

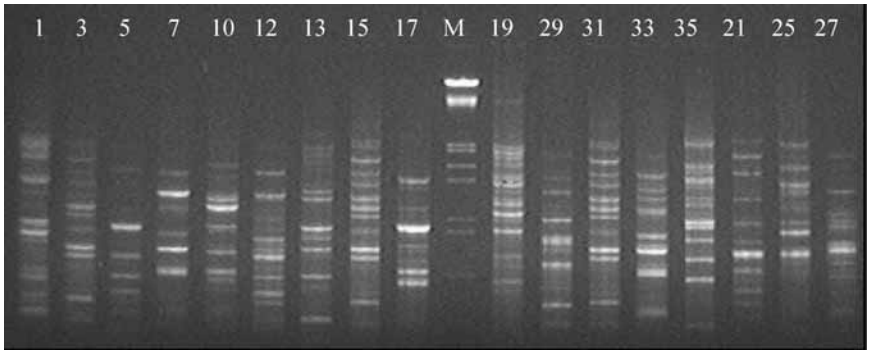

Fig. 2. RAPD patterns obtained with the OPA-04 primer in agarose gel 1.4%, where M is molecular size marker in base pairs (phase λ DNA digested wit *Eco*RI and *Hind*III, Gibco, BRL); and 1 represents *A. niger*, 3 *P. citrinum* ATCC 28752, 5 *A. niger* ATCC 16404, 7 *P. digitatum* ATCC 26821, 10 *P. notatum* ATCC 9478, 12 *A. niger* ATCC 9642, 13 *Paecilomyces variotii* ATCC 22319, 15 *P. brevicompactum*, 17 *A. oryzae* ATCC 1003, 19 *A. niger* ATCC 1004, 29 *P. camembertii* (CT) ATCC 4845, 31 *P. verrucosum*, 33 *P. simplicissimum*, 35 *P. duclauxi* ATCC 9121, 21 *P. putida*, *P. aeruginosa* ATCC 27853, and 27 *Candida* spp. ATCC 34147.

Similarity indices (Jaccard coefficient) among the studied microorganisms varied from 0.1527 to 0.6578 with a mean value of 0.2445 (Table 4). The similarity from 15.27% to 65.78% among the studied microorganisms can be considered low. These results indicate a high variability among the 17 strains of microorganisms tested. Low similarity between fungi and bacteria was expected, but it was not anticipated that fungi from the same species would have genotypic profiles exhibiting so much variability (Fig. 3).

The high variability among the strains prevented correlating RAPD analysis results with the capacity of limonene bioconversion. It was not possible to select the microorganisms tested by application of the RAPD technique. Different microorganisms, independent of genetic differences among them, are able to convert limonene, making it possible to suggest that several genes might be involved and probably no amplified fragment corresponds to the genes involved in desired bioconversion *(19)*. The high genetic difference among the studied microorganisms, even within single species, suggests that it is not possible to discard any microorganism. The biocatalytic a hydroxylation converts these molecules into different metabolites,

Table 4
Similarity of the Studied Microorganisms

Strain number	Mean	Minimum value	Maximum value	Standard deviation	Variation coefficient (%)
17	0.2445	0.1527	0.6578	0.0632	0.2584

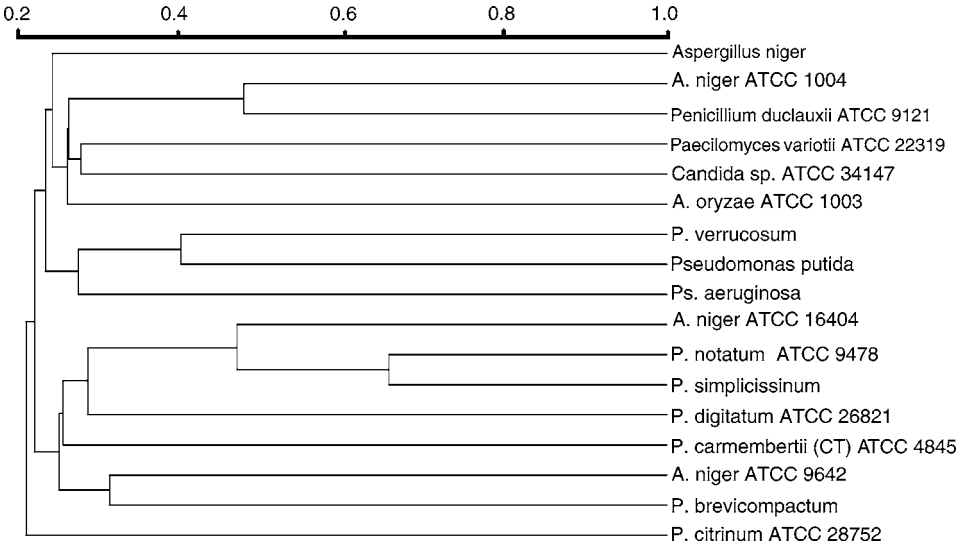

Fig. 3. Dendrogram generated by the UPGMA method from the RAPD data showing the relationship among the clusters of the strains *Aspergillus*, *Penicillium*, *Paecilomyces*, *Candida*, and *Pseudomonas*. Similarities were calculated using the Jaccard coefficient.

easier to be excreted by the cell. Thus, different microorganisms may present such metabolic routes as a way to survive *(20)*. Another interesting aspect of the enzymatic complex responsible for biotransformation is that their substrates induce the enzyme synthesis. This auto-induction can supply a mechanism of detoxification *(21)*.

Conclusions

The results obtained in the fermentative screening showed that, from 17 strains of microorganisms, four were able to bioconvert limonene into oxygenated monoterpenes. The metabolites recovered (and their relative abundance) were α-terpineol (0.68% ± 0.19) and cycle-hexanemethanol (0.64% ± 0.27) for *A. niger*, α-terpineol (0.13% ± 0.07) for *A. niger* ATCC 9642, dehydrocarveol (0.07% ± 0.00) and perillyl alcohol (0.31% ± 0.01) for *P. putida* and *cis*-carveol (0.18% ± 0.18) for *P. simplicissimum*.

The molecular biology study showed that it was not possible to establish a correlation between the genetic characteristics and the microbial

capacity for limonene bioconversion. From a mean similarity of genomic profile of 24.45% it can be concluded that a high genetic variability exists among the strains. It was expected that there would be a low similarity between fungi and bacteria, but it was not expected that fungi within the same species would exhibit such dissimilar genotypic profiles.

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