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Isolation and Screening of Microorganisms for *R*-(+)-Limonene and (−)-β-Pinene Biotransformation

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Abstract This work is focused on the biotransformation of *R*-(+)-limonene and (−)-β-pinene to bioflavor production. To carry out the present study, 405 microorganisms were tested for their ability to bioconvert the substrates. From the isolated microorganisms, 193 were selected in the prescreening using mineral medium for limonene degradation. At the screening step, eight strains were able to convert *R*-(+)-limonene and 15 to transform (−)-β-pinene, both in α-terpineol. The highest concentration in α-terpineol from *R*-(+)-limonene was about 3,450 mg/L for *Penicillium* sp. isolated from eucalyptus steam. From (−)-β-pinene, the highest product concentration of 675.5 mg/L was achieved using an *Aspergillus* sp. strain isolated from orange tree stem.

Keywords Screening · Biotransformation · *R*-(+)-limonene · (−)-β-Pinene · α-Terpineol · Monoterpenes

Introduction

Flavors and fragrances are extremely important in food, cosmetic, chemical, and pharmaceutical industries. Most part of available flavor compounds is obtained by chemical synthesis or extraction, but the growing aversion of the consumer toward chemicals related mainly to the health preoccupations has induced the flavor companies to direct their attention toward flavor compounds of biological origin, so-called natural or bioflavors [1, 2]. Natural flavor compounds, obtained by biotransformation, tend to substitute the

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synthetic products. This fact can be considered true due to the advantages of the biotransformation process compared to chemical synthesis, besides the high potential offered by the microbial transformation in producing new compounds of flavor with different applications in industries [3–7].

Terpenes occur largely in nature and are obtained in large scale as industrial residues. Terpenes, and especially their oxygenated derivatives, are extensively used by the flavor and fragrance industries. Via biotransformation, monoterpene precursors can be converted into their more valuable oxygenated derivatives [8].

The major constituent of the essential oils of orange and lemon is (R)-(+)-limonene, extensively used by food and pharmaceutical industries as flavor additive and solvent for resins, pigments, inks, and rubber [9, 10]. This substrate, natural, low cost, and easily available, can also be used as starter for flavors production, including perylic alcohol, carvone, carveol, menthol, and α-terpineol [11].

(−)-β-Pinene is a bicycle monoterpene hydrocarbon of low price and commonly used as substrates for biotransformation. It is the main constituent of turpentine, a residue of paper industry, and is a component of wood and leaf oils of a wide variety of conifers and other plants [11, 12].

R-(+)-α-terpineol has a floral, typically lilac odor, while (S)-(−)-α-terpineol has a characteristic coniferous odor. α-Terpineol, one of the most commonly used fragrance compounds, is mostly produced chemically and commercially available at relatively low price. A great advantage of enzymatic processes as compared to chemical synthesis is their enantiospecificity. Terpene transformations generally suffer from the volatility of the substrate and from the toxicity of terpenes toward microorganisms [13].

The screening of microorganisms is of particular interest since there is a large diversity of metabolic process and enzymes involved and an unlimited number of microorganisms present in nature. Microorganisms can modify and degrade a variety of organic molecules and complexes, and so it can be expected that one of them may be able to catalyze a specific reaction of interest [14].

Based on the aforementioned aspects, the main objective of this work was the isolation and screening of microorganisms from citric fruits and residues of fruits juice industries with ability to biotransform monoterpenes. The potential screened microorganisms were pre-identified by micro-cultivation technique.

Material and Methods

Chemicals

(−)-β-Pinene (99%, Fluka) and *R*-(+)-limonene (97%, Aldrich) were used as substrates without any pretreatment. All other chemicals or solvents were of analytical grade.

Isolation, Selection, and Identification of Microorganisms

Samples were collected from residues of citric juice industries, soil of citric fruits, leaves and citric fruits (orange, lemon, bergamot, and lime), and stems of eucalyptus and orange tree. The collected samples were stored in aseptic packing until moment of the analysis. The samples were inoculated in Petri dishes containing potato dextrose agar (PDA) medium and incubated at 30 °C for 7 days. After growth of several isolated microorganisms, they were subcultured for plates with PDA medium and incubated at 30 °C for 7 days. This

procedure was repeated until the complete isolation of the microorganisms. After this step, cultures were codified to facilitate future identification.

The colonies were transferred to agar slants with PDA (for filamentous fungi) or yeast malt agar media (for yeasts and bacteria) and incubated at 30 °C for 48 h. Selected cultures, after growth, were stored at 4 °C. The purity of the strains was verified by microscopic examination.

The microorganisms' characterization was carried out using selective medium for bacteria and yeast and incubated in Petri dishes at 28 °C for 120 h. The selective medium for bacteria was composed of 100 mL of plate count agar (PCA; 5.0 g/L of triptone, 2.6 g/L of yeast extract, 1.0 g/L of glucose, and 15.0 g/L of agar) with 500 μL of nystatin (100 U/mL) and the selective medium for yeast of 100 mL of PCA and 50 μL of chloramphenicol (52.5 mg/mL). The result was verified as the growth of culture in specific medium.

Bacteria and filamentous fungi were identified by Gram and micro-cultivation techniques, respectively. Fungi were inoculated on a slice of agar laid on a sterile glass slide and covered by a sterile coverslip. The slide was then placed in a Petri dish, and the setup was then incubated for until 7 days at 25 °C. The coverslip with the adhered hyphae was withdrawn and stained with Cotton Blue dye. The same procedure was adopted for examining spores and hyphae bound to the slide. The identification of the fungi genus was based on the macroscopic morphology of colonies and on the study of fructification structures of the strains, following the key of investigation of genera proposed by Barnett et al. [15].

The pre-grown isolated cultures, added of a crio-protector agent (glycerol 15%) were stored under strains freezing at −80 °C (MDF-U3086S-Sanyo) following the procedure proposed by Stanbury et al. [16].

Prescreening Experiments

Aliquots of 10 μL of each isolated microorganism were inoculated in microtubes containing 1,000 μL of mineral medium (3.0 g/L of $NaNO_3$, 1.0 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, and 0.01 g/L of $FeSO_4$) added of 1% (*w*/*v*) of glucose. In parallel, aliquots of 10 μL were inoculated in the same mineral medium described above with the addition of 1.5% (*v*/*v*) of limonene. The microtubes were electromagnetically stirred at 60 Hz by 5 days at 30 °C.

The optical density (600 nm) of each sample was measured at 0 and 5 days to verify the growth of the microorganisms. All tests were carried out in triplicate, and a blank experiment (without inoculation) was also carried out.

Screening Experiments

The isolated microorganisms that presented a positive result in terms of growth on limonene as sole carbon source were submitted to biotransformation reactions in orbital shaker.

After reactivation of the microorganisms, a loopful of each strain was inoculated in centrifuge tubes containing 20 mL of the culture medium (PD for filamentous fungi and YM for bacteria and yeasts) and incubated aerobically in orbital shaker (150 rpm) at 30 °C for periods of 24 to 72 h. After the microorganisms' growth, the tubes were centrifuged at 3,500 rpm for 10 min, and the supernatant was withdrawn. Sterile distilled water was added to a final volume of 20 mL. The tubes were then stirred to resuspend the cells and centrifuged again. The supernatant was then discarded and the precipitated cells (approximately 2 g) were transferred to an Erlenmeyer containing 30 mL of mineral medium added by 1.5% (*v*/*v*) of *R*-(+)-limonene or (−)-β-pinene.

Experiments of biotransformation were started after inoculation, and the flasks were kept in orbital shaker at 30 °C and 150 rpm for 8 days. All experiments were carried out in parallel with controls, in the same conditions without the presence of microorganism. Experiments were performed in triplicate runs in closed stoppered glass flasks in order to avoid the substrate and product evaporation.

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 ethyl acetate (AcOEt). The final solution was dried over anhydrous sodium sulfate.

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 to 50 °C for 3 min, increased at 5 °C/min at 130 °C, and then increased at 15 °C/min at 210 °C by 5 min. Helium was the carrier gas, and the injection and detector temperatures were 250 °C. The dried solution (0.5 μL) 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 1:3).

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 quantitative analyses were carried out in a gas chromatograph (GC Shimadzu 2010) with automatic injector and flame ionization detector. A capillary polar column (RTx-Wax, Restec, 833551, 30 m×0.25 mm×0.25 μm) was used at the same experimental conditions described above for GC/MS analysis. The compounds were identified by injection of the external standards compared to the retention times. The quantification was carried out by the standard curve of the compound of interest, evaluating the relative area from the compound of interest and the standard curve. (−)-β-pinene (99%, Fluka), *R*-(+)-limonene (97%, Aldrich), and α-terpineol (90%, Aldrich) were used as external standards.

Production Kinetics

After growth and inoculum preparation, aliquots of 2 g of biomass were transferred aseptically to Erlenmeyer with 30 mL of mineral medium and incubated for 10 days at 30 °C and 150 rpm for product biotransformation determination. Cell induction was carried out using 0.1% of substrate and added to the inoculum after one third of the incubation time, 72 h for filamentous fungi and 30 h for yeast.The kinetics was followed at each 24 h until 10 days. The procedure of extraction and identification of substrate and products was performed as described before.

Statistical Analysis

The results were treated by analysis of variance followed by Tukey's post hoc test using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). All analyses were performed considering a confidence level of 95% ($p<0.05$).

Results and Discussion

Isolation, Screening, and Identification of Microorganisms

Figure 1 presents the number of microorganisms isolated from each source. By this figure, we can see that a total of 405 microorganisms were isolated from different sources, such as

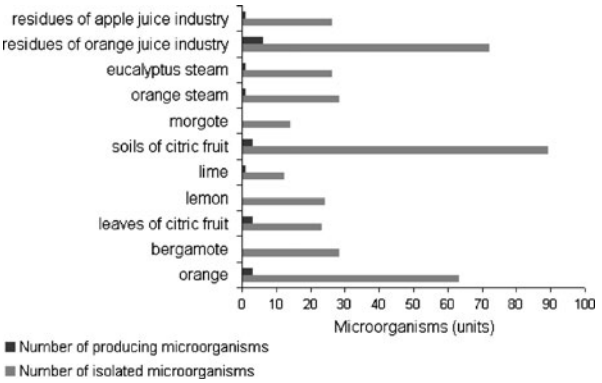


Fig. 1 Number of microorganisms isolated from each source

juice industry residues, soils of citric fruits, citric fruits and leaves, orange, and eucalyptus stem. From Fig. 1, one can also observe that most microorganisms were isolated from soils cultivated by citric fruits, residues of orange juice industry, and oranges. A previous inspection of the isolated microorganisms in optic microscope and selective medium permitted us to verify that 93 strains belong to yeast class, 45 to bacteria, and 267 to filamentous fungi. In summary, we can find that the most part of the microorganisms was isolated from residues of orange juice industry, soils cultivated by citric fruits, leaves of citric fruits, and orange.

The analysis of the results indicated that the isolated filamentous fungi presented higher potential for bioconversion than yeasts and bacteria. The best strains for bioconversion were identified by micro-cultivation technique based on the shape of fructification body. By this procedure and by comparison of macro- and microscopic aspects, three of the filamentous fungi able to bioconvert limonene and β-pinene were identified as belonging to the *Penicillium* genera (presence of paintbrush-like conidia). From all isolated α-terpineol-producing filamentous fungi, one was classified as *Aspergillus* based on the presence of hyaline and dark septated mycelium [17].

Penicillium and *Aspergillus* are common contaminants of tropical and subtropical regions, predominating among other fungi [18]. Other screened and identified filamentous fungi were classified as *Fusarium* sp. and *Paecilomyces* sp. Two bacteria were identified by Gram technique as *Bacillus* Gram-positive.

Works in the literature that reference studies on isolation, identification, and selection of microorganisms with the ability of bioconvert monoterpenes are still few. Van Rensburg et al. [8] isolated 100 of yeasts and yeast-like fungi from monoterpene-rich environments with enrichment media containing non-carbohydrate carbon sources and tested for their ability of convert (−)piperitone and (+)limonene. Demyttenaere et al. [9] found that 60 fungal strains, grown as sporulated surface cultures, were screened for their ability to bioconvert the substrate (R)-(+)-limonene. Bicas and Pastore [19] isolated a total 238 strains; 70 were able to grow well in YM medium and 50 μL (0.1%, *v*/*v*) of *d*-limonene added to preselect limonene-resistant strains.

Prescreening Experiments

From the 405 isolated and tested using mineral medium for limonene degradation microorganisms, all were able to grow in this medium added by glucose. A number of 193 strains grown in the medium added by limonene were submitted to biotransformation

of *R*-(+)-limonene and (−)-β-pinene in an orbital shaker. From these 193 microorganisms selected in the prescreening with the potential to bioconvert monoterpenes, 15 belong to the bacteria genera, 68 to yeasts, and 110 to filamentous fungi.

The next step of this work consisted in evaluating all strains in a biotransformation using limonene as sole carbon source in mineral medium and 1% (*v*/*v*) of *d*-limonene (ten *Bacillus* Gram-negatives, 55 *Bacillus* Gram-positives, one *Coccus* Gram-positive, and four yeasts).

Screening Experiments

Tables 1 and 2 present the microorganisms able to bioconvert *R*-(+)-limonene and (−)-β-pinene, respectively, the product concentration (mg/L) and process conversion (%). From these tables, one can verify that from the 110 filamentous fungi selected in the prescreening step, only two present ability of transforming the substrate limonene (04.06.01 and 01.01.17). Related to the yeasts, from the 68 preselected, six produced α-terpineol using limonene as substrate (05.01.35, 03.03.03, 01.04.03, 03.02.02, 01.04.02, and 01.08.11). Microorganisms coded as 04.06.01 and 05.01.35, isolated from eucalyptus stem and residue of orange juice industry, respectively, demonstrated a great potential for bioconversion of limonene to α-terpineol.

The bioconversion of limonene to α-terpineol as the main end product has been described using a wide range of microorganisms: *Cladosporium* strain [20], *Penicillium* sp. isolated from orange peel [21], *Penicillium digitatum* [8, 9], *Pseudomonas gladioli* [22, 23], and *Escherichia coli* expressing a thermostable limonene hydratase [24].

P. digitatum gives a significant improvement on the yield in the bioconversion of limonene to α-terpineol (3.2 mg/mL) compared to the other strains. This yield was obtained using a sequential substrate induction [11].

Tan and Day [25] investigated the bioconversion of (4R)-(+)-limonene to (4R)-(+)-α-terpineol by the immobilized fungal mycelia of *P. digitatum*. The fungi were immobilized in calcium alginate beads. α-Terpineol production was correspondent to 12.83 mg/g beads per day, leading to a 45.81% bioconversion of substrate.

Adams et al. [27] used five strains of *P. digitatum* and obtained 93% of conversion in (R)-(+)-α-terpineol with a high enantioselectivity (ee>99%) using (R)-(+)-limonene added

Table 1 Results of screening for *R*-(+)-limonene biotransformation for α-terpineol production.

Microorganism code/Source	Microorganism	α-Terpineol	
		Concentration (mg/L)	Conversion (%)
04.06.01/Eucalyptus stem	*Penicillium* sp.	3,449.6	19.16a (±1.12)
05.01.35/Orange juice industry residue	Yeast	1,690.1	9.39b (±0.89)
03.03.03/Soils of citric fruits	Yeast	1,271.9	7.06c (±0.93)
01.04.03/Leaves of citric fruits	Yeast	1,195.3	6.64c (±0.69)
03.02.02/Soils of citric fruits	Yeast	690.6	3.84d (±0.55)
01.04.02/Leaves of citric fruits	Yeast	666.7	3.70d (±0.38)
01.08.11/Soils of citric fruits	Yeast	520.2	2.89d (±0.51)
01.01.17/Orange fruit	*Paecilomyces* sp.	56.3	0.31e (±0.04)

Means followed by equal letters do not differ between them by Tukey test ($p<0.05$)

Table 2 Results of screening for (−)-β-pinene biotransformation for α-terpineol production.

Microorganism code/Source	Microorganism	α-Terpineol	
		Concentration (mg/L)	Conversion (%)
04.05.08/Orange tree stem	*Aspergillus* sp.	675.5	3.75a (±0.28)
01.04.02/Leaves of citric fruits	Yeast	281.3	1.56b (±0.14)
01.04.03/Leaves of citric fruits	Yeast	237.2	1.32b (±0.23)
01.04.07/Leaves of citric fruits	Yeast	175.1	0.97c (±0.08)
01.08.11/Soils of citric fruits	Yeast	140.2	0.78cd (±0.09)
01.01.17/Orange fruit	*Paecilomyces* sp.	97.5	0.54de (±0.05)
06.01.12/Apple juice industry residue	*Fusarium* sp.	74.1	0.41e (±0.03)
05.01.44/Orange juice industry residue	*Bacillus* Gram-positive	60.3	0.33e (±0.05)
05.01.23/Orange juice industry residue	Yeast	50.9	0.28e (±0.05)
05.01.07/Orange juice industry residue	Yeast	39.3	0.22e (±0.03)
01.07.07/Lime	*Penicillium* sp.	38.5	0.21e (±0.04)
01.01.18/Orange juice	*Bacillus* Gram-positive	35.6	0.19f (±0.03)
05.01.15/Orange juice industry residue	*Penicillium* sp.	32.6	0.18f (±0.02)
01.10.09/Orange fruit	*Fusarium* sp.	31.1	0.17f (±0.02)
05.01.02/Orange juice industry residue	Yeast	0.4	0.01g (±0.01)

Means followed by equal letters do not differ between them by Tukey test ($p<0.05$).

by 0.4% of EtOH as substrate and 8 h of reaction. Toniazzo et al. [28] tested *Aspergillus niger* and *A. niger* ATCC 9642 in the limonene biotransformation and obtained a conversion in α-terpineol of 0.68±0.19% and 0.13±0.07%, respectively. Maróstica Junior and Pastore [3] investigated the microorganisms *Penicillium* sp. 2025, *Aspergillus* sp. 2038, and *Fusarium oxysporum* 152B for the biotransformation of *R*-(+)-limonene using two agroindustrial residues (liquid cassava waste and orange essential oil). The best *R*-(+)-α-terpineol yields were achieved when the strains were grown in cassava media and the mycelia then transferred to a new flask containing mineral medium and orange essential oil as the sole carbon and energy source. One of the strains tested, *F. oxysporum* 152B, converted *R*-(+)-limonene to *R*-(+)-α-terpineol, yielding nearly 450 mg/L after 3 days of reaction.

Bicas et al. [26] optimized the process conditions for the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *F. oxysporum* 152b using response surface methodology. Best results were obtained using 0.5% (*v*/*m*) of *R*-(+)-limonene in pure distilled water as culture medium with an inoculum/culture medium ratio of 0.25 (*m*/*m*) and 72-h cultivation at 26 °C/240 rpm. Under these conditions, the concentration of *R*-(+)-α-terpineol in the culture medium reached 2.4 g/L.

From Table 2, one can observe that from the 110 prescreened filamentous fungi, six bioconverted the substrate β-pinene to α-terpineol (04.05.08, 01.01.17, 06.01.12, 01.07.07, 05.01.15, and 01.10.09). From the preselected strains of yeasts and bacteria, seven (01.04.02, 01.04.03, 01.08.11, 01.04.07, 05.01.23, 05.01.07, and 05.01.02) and two (05.01.44 and 01.01.18) were able to produce α-terpineol. A strain of *Aspergillus* (04.05.08) presented the highest conversion of β-pinene to α-terpineol (675.5 mg/L).

The analysis of Tables 1 and 2 permit us to verify that microorganisms coded as 01.04.02, 01.04.03, 01.08.11, and 01.01.17 were able to produce α-terpineol from the two

tested substrates. This fact can be explained by the great diversity of metabolic processes and enzymes involved in the cell growth, making the microorganisms able to degradate a variety of complex organic molecules [14].

The bioconversion of β-pinene to α-terpineol has been scarcely described in the literature. Van Dyk et al. [29], using *Hormonema* sp., obtained pinocamphone from (−)-β-pinene. Yoo and Day [30] and Yoo et al. [31], using *Pseudomonas* sp., obtained limonene, ρ-cymene, α-terpinolene, camphor, terpinen-4-ol, α-terpineol, endo-borneol, and ρ-cimene-8-ol. *A. niger* ATCC 9642 [27] was also used as catalysts for this biotransformation, and the compound obtained was α-terpineol with low conversion (up to 4%).

Kinetic Study

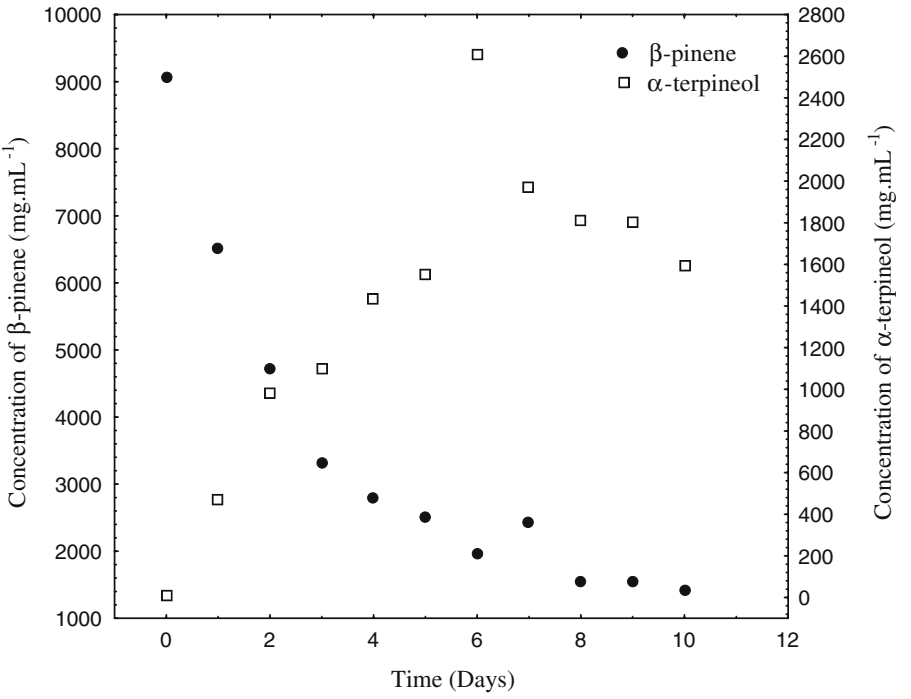
The kinetic of substrate consumption and α-terpineol production was carried out using limonene and β-pinene as substrates and the screened microorganisms coded as 05.01.35 and 04.05.08, respectively. The kinetic curves were built with induced and non-induced cells and both substrates.

Using *R*-(+)-limonene as substrate and the yeast coded as 05.01.35, the highest production of α-terpineol occured on the sixth day of reaction, with a concentration of approximately 2,000 and 900 mg/L for cells induced by limonene and non-induced, respectively. These results are presented in Fig. 2a, b. From these, we could observe that the cell induction during the inoculum growth by limonene leads to an enhancement of 1.72-fold in α-terpineol conversion. This parameter is important for optimizing the reaction production. Tan and Day [25] related the activity of the microorganism enhanced by a factor of 12 after the induction of a sequential addition of the substrate in the conversion of the racemic mixture of limonene to *R*-(+)-α-terpineol. Yoo et al. [31] studied the progress curves for the formation of major bioconversion products versus time by β-pinene-grown cells using 1% β-pinene as sole carbon source. The accumulation of the *p*-menthene derivatives, *p*-cymene, limonene, and α-terpinolene increased to a maximal concentration, 198 mg/L of *p*-cymeme, 98 mg/L of α-terpinolene, and 64 mg/L of limonene after 24 h of fermentation. Oxygenated products such as α-terpineol accumulated to 64 mg/L after 24 h of fermentation.

Figure 3a, b presents the results obtained when using (−)-β-pinene as substrate and *Aspergillus* sp. (04.05.08). The highest α-terpineol production was obtained after 7 days of reaction. At this experimental condition, a product concentration of 726 and 707 mg/L, for induced and non-induced cells, respectively, was observed. Here, the cell induction seems not to favor the product formation. Toniazzo et al. [32] obtained higher α-terpineol production for the biotransformation of (−)β-pinene from *A. niger* ATCC 9642, adding the substrate in five steps without cell induction and using ethanol as co-solvent.

The observation of Figs. 2 and 3 permit us to verify a reduction on substrate bioconversion after 7 and 8 days of reaction, respectively. Takahashi et al. [33] show that this reduction can be related to a possible product metabolization. The substrate concentration diminushed until the tenth day for both substrates for induced and non-induced cells. This fact may be a result of the lower levels of nutrients in the culture medium, making the microorganisms use the substrate as carbon source or by the enzyme inhibition by the acumulus of the product in the reaction medium.

The literature points out that a common problem in monoterpene biotransformation is the toxicity of these compounds for the microorganisms [9, 11]. To reduce this effect, the method of cell induction by the substrate has been employed, allowing the microorganism adaptation with lower amounts of substrate [11, 13].



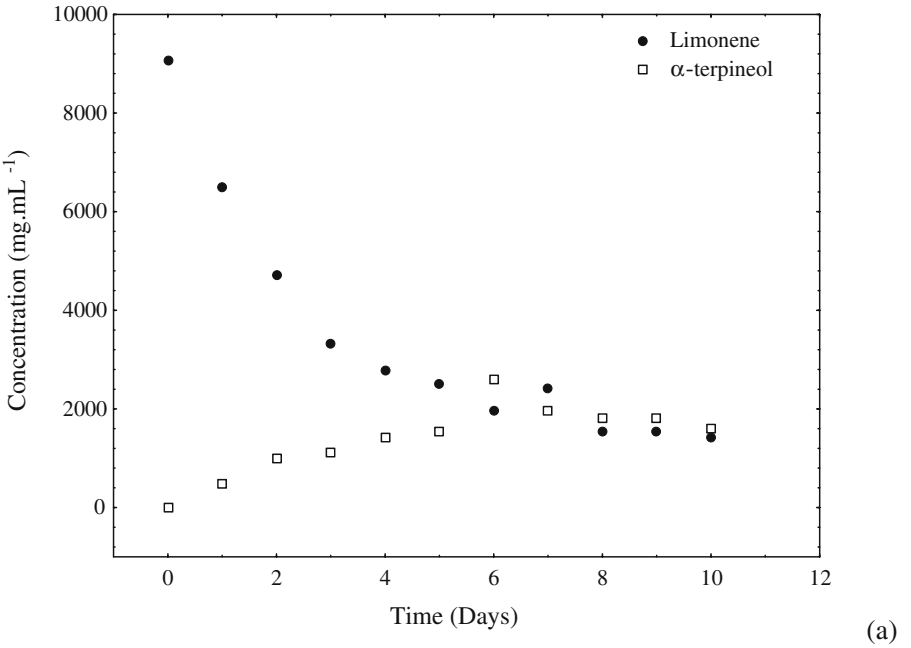
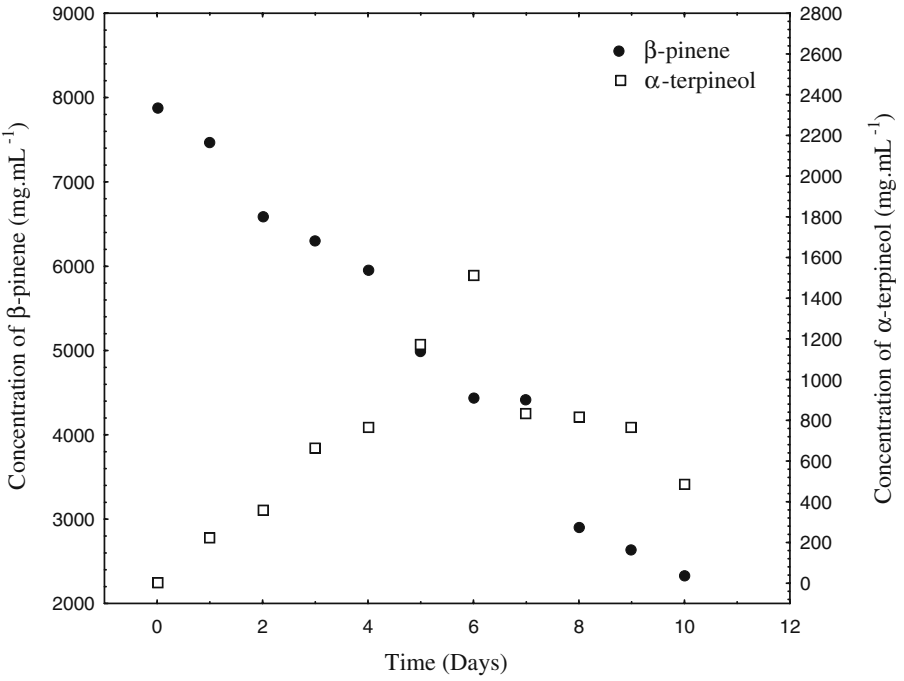
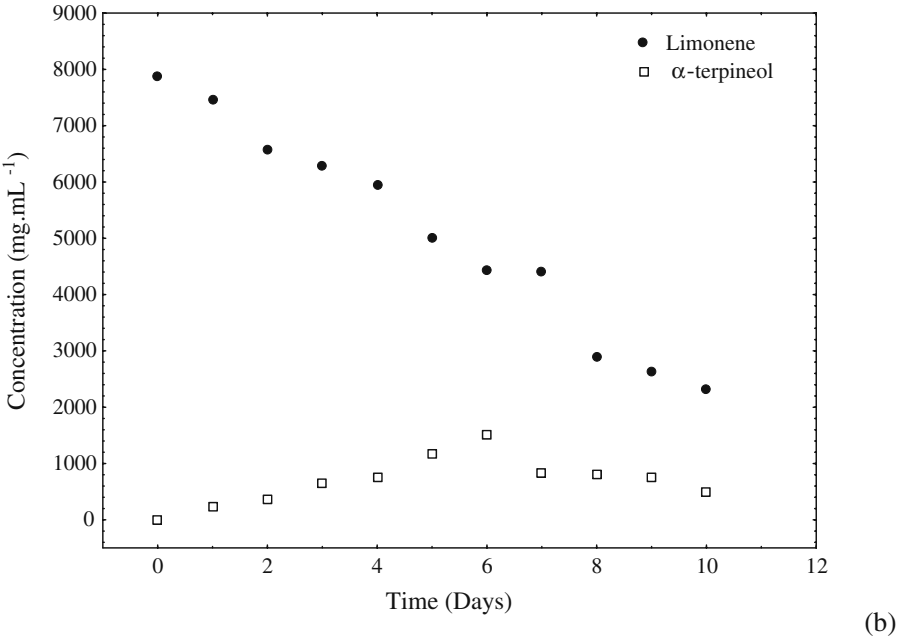


Fig. 2 Limonene and α-terpineol concentration on *R*-(+)-limonene biotransformation using the induced yeast isolated and coded as 05.01.35 (**a**) and non-induced yeast isolated and coded as 05.01.35 (**b**)





(b)

Fig. 2 (continued)

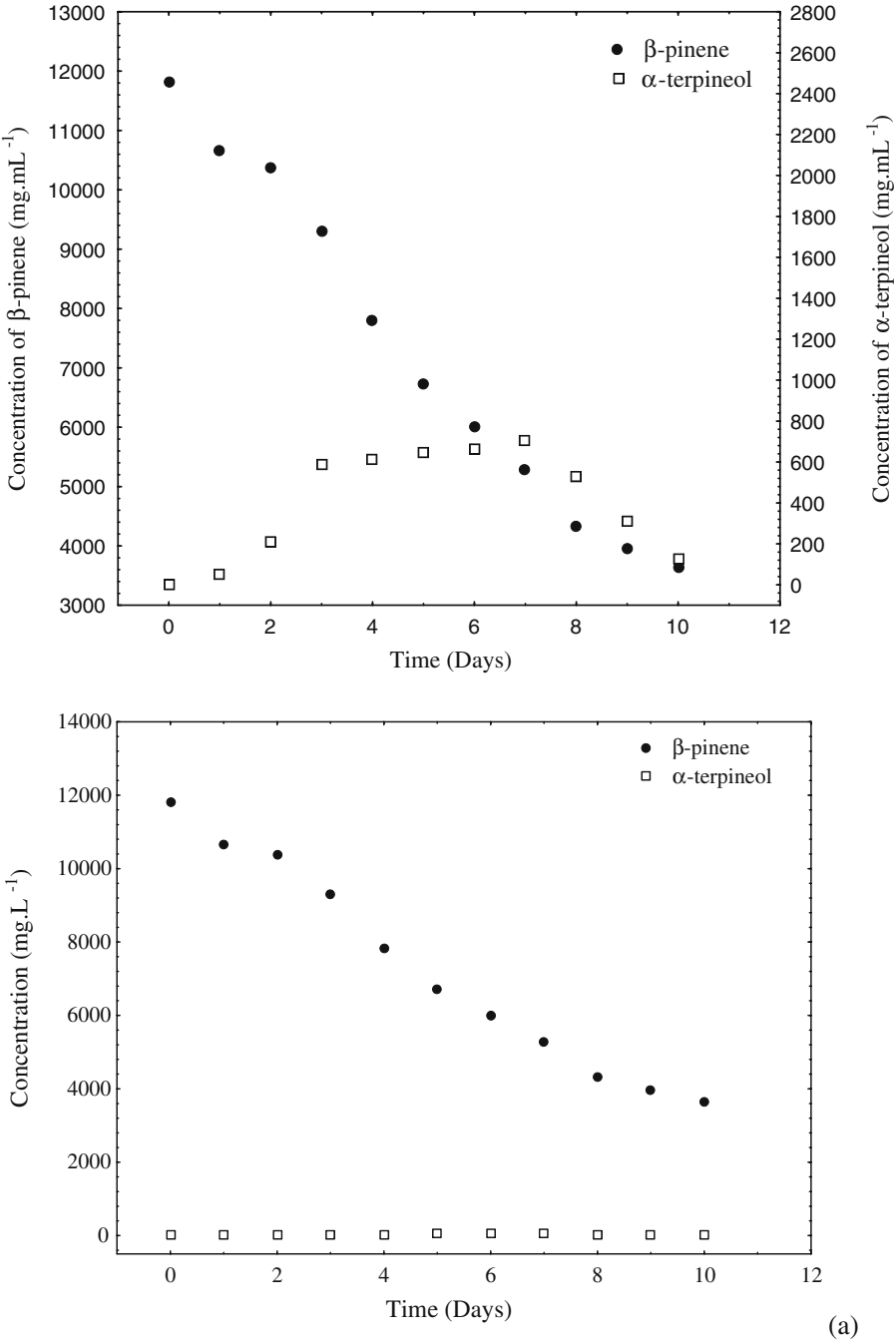
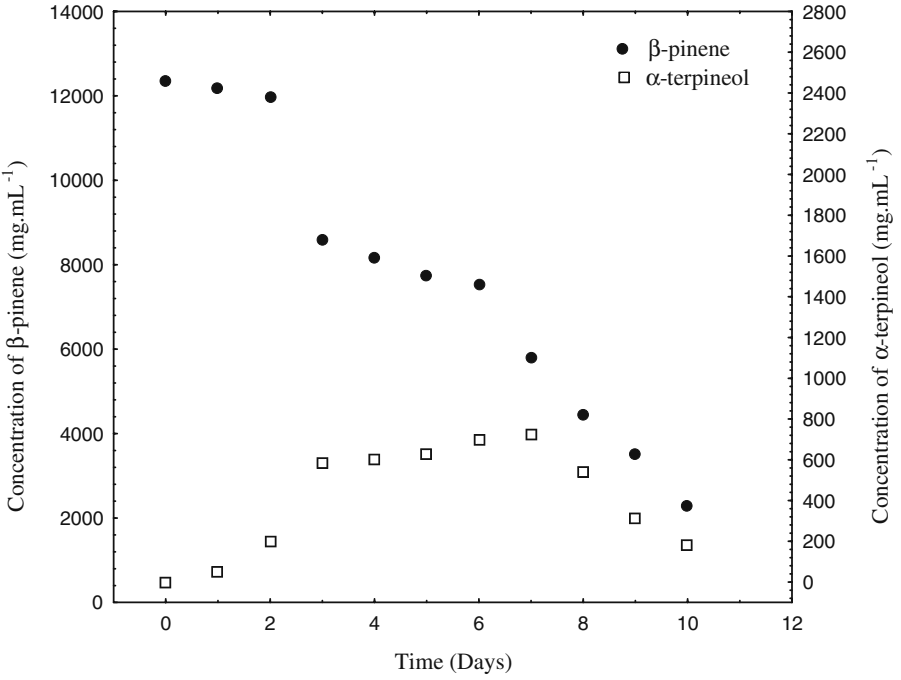
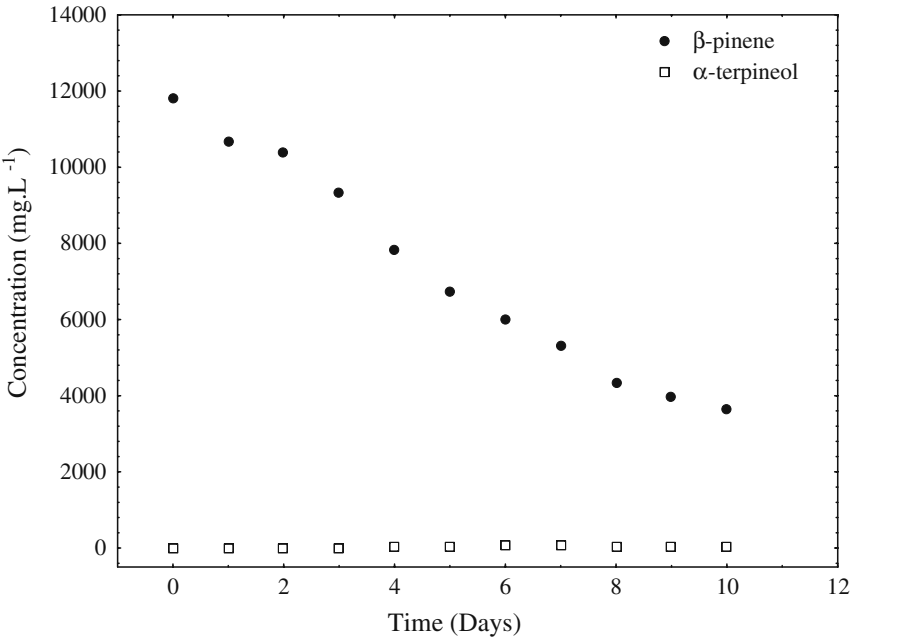


Fig. 3 β-pinene and α-terpineol concentration on (−)-β-pinene biotransformation using the induced filamentous fungi isolated and coded as 04.05.08 (**a**) and non-induced filamentous fungi isolated and coded as 04.05.08 (**b**)





(b)

Fig. 3 (continued)

Conclusions

A total of 405 microorganisms were isolated, and 193 strains were able to use the substrate limonene in the prescreening step. The results in the screening showed that eight strains were able to convert *R*-(+)-limonene and 15 the substrate (−)-β-pinene in α-terpineol. Six strains able to convert *R*-(+)-limonene belong to yeast class, and two were filamentous fungi. In the bioconversion of (−)-β-pinene to α-terpineol, seven strains were yeasts, two bacteria, and six belong to fungi class.

The high conversion in α-terpineol from *R*-(+)-limonene was found as 3,450 mg/L for *Penicillium* sp. (coded as 04.06.01 and isolated from eucalyptus stem). Using (−)-β-pinene as substrate, *Aspergillus* sp. (coded as 04.05.08 and isolated as orange tree stem) led to a conversion of 675.5 mg/L.

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