Appl Biochem Biotechnol (2010) 162:2221–2231
DOI 10.1007/s12010-010-8996-y

Microbial Oxidation of (-)-α-pinene to Verbenol Production by Newly Isolated Strains

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Received: 1 January 2010 / Accepted: 19 May 2010 /
Published online: 6 June 2010

Abstract Verbenol is a bicyclicbicycle secondary allylic alcohol, with pronounced camphor and mint flavor notes, mainly used as food flavoring. This compound is also used to control harmful insects, and hence has potential for using in agriculture, and is an intermediate in the synthesis of valuable perfume and medicinal substances. This work is focused on the microbial oxidation of (-)-α-pinene to verbenol production. To carry out the present study, 405 microorganisms were tested for their ability to bioconvert the substrate. From the isolated microorganisms, 193 were selected in the pre-screening using mineral medium for limonene degradation. At the screening step, 31 strains were able to convert (-)-α-pinene in verbenol. The highest concentration in verbenol from (-)-α-pinene was about 125.6 mg/L for yeast isolated from orange juice industrial residue.

Keywords Screening · Biotransformation · (-)-α-Pinene · Verbenol · Monoterpenes

Introduction

The use of biotransformation has significant advantages over chemical reactions and in the last 10 years has been increasing constantly since biocatalysts have attracted much attention from the viewpoint of green chemistry [1].

Natural flavor compounds, obtained by biotransformation, tend to substitute the 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 [2, 3].

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Terpenes are unsaturated hydrocarbons derived from isoprene units. They are widely distributed in nature and their oxygenated derivatives, usually named terpenoids, are important flavor compounds. Terpenes occur widely in nature and are obtained in large scale as industrial residues [4]. 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 [5].

Pinenes are constituents of the wood and oil of an extensive variety of plants and can also be obtained as sub-product of paper industries. The α-pinene is a bicycle bicyclicalmonoterpene hydrocarbon of low price and is commonly used as substrates for biotransformation, largely employed in fragrance and flavor industries as raw material for the synthesis of high-value products [6, 7].

α-Pinene is the principal constituent of turpentine from most conifers and is a component of the wood and leaf oils of a wide variety of other plants [8].

Verbenol 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol, $C_{10}H_{16}O$, is a bicyclice secondary allylic alcohol, with pronounced camphor and mint flavor notes, used as a food flavoring [9]. Verbenol is also used to control harmful insects, and hence has potential for use in agriculture, and is an intermediate in the synthesis of valuable perfume and medicinal substances [10, 11]. In spite of all these applications, just a few works were found in the literature concerning the microbial oxidation of terpenes to verbenol production.

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 [12].

Based on the above-mentioned aspects, the main objective of this work was the isolation and screening of microorganisms from citric fruits and residues of fruit juice industries with the ability to biotransform the substrate (-)-α-pinene.

Material and Methods

Chemicals

R-(+)-limonene (97%, -Aldrich), (-)-α-pinene (>98%, Fluka) were used as substrates and (*S*)-*cis*-verbenol (95%, Aldrich) as internal standard. The reagents were used without any pre-treatment. All other chemicals or solvents were of analytical grade.

Isolation, Selection, and Identification of Microorganisms

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

Colonies were transferred to agar slants with PDA medium (for filamentous fungi) or yeast malt agar (YMA) 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 characterization of the microorganisms was carried out using selective medium for bacteria and yeast, incubated in Petri dishes at 28 °C for 120 h. The selective medium for bacteria was composed by 100 mL of plate count agar (PCA)CA 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 by 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. [13].

The pre-growth isolated cultures added of a cryo-protector agent (glycerol 15%) were stored under freezing at -80 °C (MDF-U3086S-Sanyo), following the procedure proposed by Stanbury et al. [14].

Pre-screening Experiments

Aliquots of 10 μL of each isolated microorganism were inoculated in microtubes containing 1000 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 24–72 h. After the growth of the microorganisms, the tubes were centrifuged at 3,500 rpm for 10 min, and the supernadant was withdrawn. Sterile distilled water was added to a final volume of 20 mL. 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 (-)-α-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.

The product recovery was performed by liquid – liquid extraction with ethyl acetate (AcOEt). The final solution was dried over anhydrous sodium sulphate.

The reaction products were identified by gas chromatography/mass spectrometry (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 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 interest compound, evaluating the relative area from the interest compound and the standard curve. (-)-α-Pinene (purity >98%, Fluka) and (*S*)-*cis*-Verbenol (95%, Aldrich) were used as external standards.

Kinetic Study

The kinetic was driven with induced and non-induced cells with the substrate. Cell induction was carried out using 0.1% of substrate, added to the inoculum after 1/3 of the incubation time, 30 h for yeast.

After growth and inoculum preparation, aliquots of 2 g of biomass were transferred aseptically to Erlenmeyer with 30 mL of mineral medium added by 1.5% (v/v) of (-)-α-pinene and incubated for 10 days at 30 °C and 150 rpm for product biotransformation determination. The kinetic was followed every 24 h for 10 days. The procedure of extraction and identification of substrate and products were performed as described before.

Statistical Analysis

Results obtained along the work 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. A total of 405 microorganisms were isolated from different sources such as juice industrial residues, soils of citric fruits, citric fruits and leaves, orange, and eucalyptus stem. Most microorganisms were isolated from soils cultivated by citric fruits, residues of orange juice industry, and

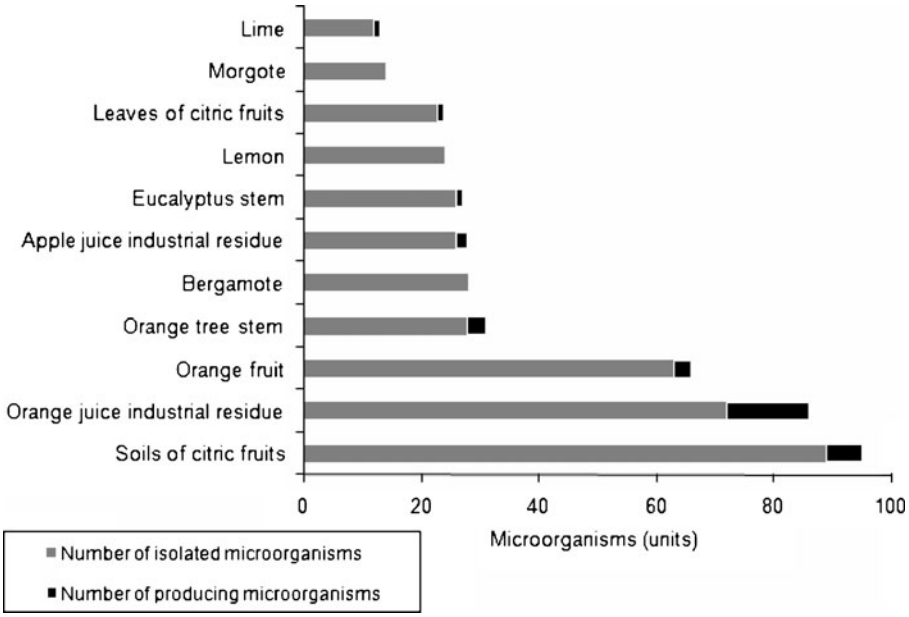

Fig. 1 Number of microorganisms isolated from each source

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.

We can verify, by analysis of the results, that filamentous fungi presented higher potential to bioconvert monoterpenes. However, for conversion of α-pinene to verbenol, yeasts presented a higher number of positive results, 14 strains, followed by 11 strains selected from the filamentous fungi. These potential microorganisms were mainly isolated from residues of orange juice industry, soils of citric fruits, orange fruit, and orange tree stem.

The selected strains of filamentous fungi were identified by micro-cultivation technique, based on the shape of fructification body and by comparison of macro- and microscopic aspects, the coloration of upper and lower parts, formation of agglomerates, and mycelium density.

From the isolated filamentous fungi, four were identified as belonging to the *Fusarium* genera, based on specific characteristics [15], three were identified as *Penicillium* genera (presence of paintbrush like conidia) and two as *Aspergillus*, based on the presence of hyaline and dark septated mycelium [16]. *Penicillium* and *Aspergillus* are common contaminants of tropical and subtropical regions, predominating among other fungi [17]. Other screened and identified filamentous fungi were classified as *Rhizopus* sp. (one) and *Paecilomyces* sp. (one). Seven bacteria were identified by general characteristics of the colonies and Gram technique as Bacillus Gram positive and negative and Coccus Gram positive and negative.

Some works that reference studies on isolation of microorganisms presented in the literature carried out the identification and selection of microorganisms with ability to

bioconvert monoterpenes. van Rensburg et al. isolated 100 yeasts and yeast-like fungi from monoterpene-rich environments with enrichment media containing non-carbohydrate carbon sources and tested their ability to convert piperitone and (+)- - limonene [5]. Demyttenaere et al. [18] 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 of 238 strains, 70 were able to grow well in medium YM and 50 μL (0.1%, v/v) of *d*-limonene, added to pre-select limonene-resistant strains.

Pre-screening 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 were grown in the medium added by limonene and were submitted to biotransformation of (-)-α-pinene in orbital shaker. From these 193 microorganisms selected in the pre-screening with potential to bioconvert monoterpenes, 15 belong to the bacteria genera, 68 to yeasts, and 110 to filamentous fungi.

Some authors related that a simple strategy for screening of strains with potential for biotransformation is the use of enrichment techniques using the substrate as the sole carbon source [20–22].

Screening Experiments

Table 1 presents the microorganisms that are able to bioconvert (-)-α-pinene and the product concentration (mg/L). The table shows that from the 110 filamentous fungi selected in the pre-screening step, 11 presented the ability of transforming the substrate (-)-α-pinene (04.05.10, 03.01.05, 04.05.06, 03.01.04, 01.07.07, 05.01.15, 03.01.06, 01.10.09, 06.01.21, 01.10.17, and 06.01.12). Related to the yeasts, from the 68 pre-selected, 13 produced verbenol (05.01.05, 05.01.11, 05.01.40, 05.01.39, 05.01.07, 05.01.18, 05.01.02, 03.01.12, 03.01.01, 05.01.08, 01.04.07, 05.01.23, and 05.01.33). From the pre-selected strains of bacteria, seven were able to produce verbenol (05.01.19, 04.05.05, 04.06.03, 03.01.13, 01.10.18, 05.01.44, and 05.01.06).

The yeast coded as 05.01.05, isolated from residue of orange juice industry, demonstrated the best potential for bioconversion of (-)-α-pinene to verbenol, 125.6 mg/L, among the producing microorganisms. This result is statistically ($p<0.05$) equal (Tukey's test) to the bacterium 04.05.05, which obtain a product concentration of 119.4 mg/L.

Here, it is worth to mention the work by Toniazzo et al. [23] who was not able to biotransform (-)-α-pinene in the experimental conditions investigated, with the *Aspergillus niger* fungus. On the other side, Yoo and Day, using *Pseudomonas* sp., metabolized either α- or β-pinene via *p*-menthene derivatives such as limonene and *p*-cymene. The accumulation of *p*-cymene reached 750 mg/L of the maximum concentration during the period of fermentation after 48 h, but afterwards its concentration rapidly decreased to 144 mg/L after 72 h. The other major products, limonene, α-terpinolene, α-terpineol, and *endo*-borneol also showed patterns similar to *p*-cymeme [24]. Yoo et al. [25], using *Pseudomonas* sp., obtained camphor, terpinen-4-ol, α-terpineol, *endo*-borneol, and ρ-cimene-8-ol as fermentation products.

van Dyk et al., using *Hormonema* sp., freshly isolated from pine forest litter, hydroxylated mono- and bicyclic monoterpenes on the cyclohexane ring, and converted

Table 1 Results of screening for (-)-α-pinene biotransformation for verbenol production.

Codes/source	Microorganism	Verbenol concentration (mg/L)
05.01.05/Orange juice industrial residue	Yeast	125.56[a] (± 2.09)
04.05.05/Orange tree stem	Coccus Gram Positive	119.42[a] (± 3.86)
05.01.11/Orange juice industrial residue	Yeast	106.02[b] (± 2.35)
05.01.19/ Orange juice industrial residue	Coccus Gram negative	100.96[c] (± 0.77)
05.01.40/ Orange juice industrial residue	Yeast	96.82[c] (± 1.74)
05.01.39/ Orange juice industrial residue	Yeast	96.76[c] (± 1.90)
04.06.03/Eucalyptus stem	Coccus Gram positive	96.45[c] (± 2.09)
05.01.07/ Orange juice industrial residue	Yeast	78.13[d] (± 2.01)
05.01.08/ Orange juice industrial residue	Yeast	77.08[d] (± 2.62)
04.05.10/Orange tree stem	*Fusarium* sp.	72.89[de] (± 1.13)
05.01.18/Orange juice industrial residue	Yeast	70.26[e] (± 1.40)
05.01.02/Orange juice industrial residue	Yeast	68.95[ef] (± 1.01)
03.01.12/Soils of citric fruits	Yeast	68.56[ef] (± 0.61)
03.01.01/Soils of citric fruits	Yeast	66.10[f] (± 0.32)
03.01.13/Soils of citric fruits	Bacillus Gram negative	65.30[f] (± 0.72)
04.05.06/Orange tree stem	*Fusarium* sp.	63.98[fg] (± 1.76)
03.01.05/Soils of citric fruits	*Aspergillus* sp.	61.36[g] (± 0.68)
01.04.07/Leaves of citric fruits	Yeast	58.57[g] (± 2.02)
05.01.23/Orange juice industrial residue	Yeast	57.93[gh] (± 0.24)
05.01.33/Orange juice industrial residue	Yeast	55.10[h] (± 0.20)
03.01.04/Soils of citric fruits	*Aspergillus* sp.	49.56[i] (± 1.86)
01.01.18/Orange fruit	Bacillus Gram positive	45.44[i] (± 1.01)
05.01.44/Orange juice industrial residue	Bacillus Gram positive	43.56[ij] (± 0.19)
05.01.06/ Orange juice industrial residue	Bacillus Gram positive	40.85[j] (± 1.05)
01.07.07/Lime	*Penicillium* sp.	35.21[k] (± 1.01)
05.01.15/Orange juice industrial residue	*Penicillium* sp.	29.16[l] (± 0.14)
03.01.06/Soils of citric fruits	*Rhizopus* sp.	24.47[m] (± 0.11)
01.10.09/Orange fruit	*Fusarium* sp.	18.79[n] (± 0.06)
06.01.21/Apple juice industrial residue	*Penicillium* sp.	2.29[o] (± 0.02)
01.01.17/Orange fruit	*Paecilomyces* sp.	1.20[o] (± 0.01)
06.01.12/Apple juice industrial residue	*Fusarium* sp.	0.98[o] (± 0.01)

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

(-)-α-pinene. The two products formed from (-)-α-pinene wereas identified as verbenone (0.3 g/L) and *trans*-verbenol (0.4 g/L) after 96 h [26].

Agrawal et al. tested variants of *Aspergillus* sp. and *Penicillium* sp. obtained after treatment with colchicine, ethyl methanesulphonate (EMS), or ultraviolet (UV) irradiation. Results indicated several levels of significant increases in their efficiency to transform α-pinene to verbenol. In case of *Aspergillus* sp., the UV-induced variant was the best performer with a 15-fold increase in biotransformation efficiency compared to the wild type. The UV-induced variant of *Penicillium* sp. was capable of eightfold increase in efficiency. Yields from 0.30 for 4.5 mg/100 mL in *Aspergillus* sp. and 0.44 for 3.6 mg/100 L in *Penicillium* sp were achieved [27].

Maróstica Jr. et al. studied the biotransformation of monoterpenic agro-industrial wastes, turpentine oil, being the largest representative α-pinene. More than 40 fungal strains were isolated from Brazilian tropical fruits, soil samples, and eucalyptus trees, and they were screened for biotransformation of the waste substrates. The selected strains were submitted to submerged liquid culture. The concentration of verbenol 72 h after the first addition of turpentine oil was of 51.3, 17, and 6.1 mg/L produced by *Mucor* sp. 2276, *Penicillium* sp., and *Mucor* sp. 2288, respectively [28].

The α-pinene bioproduction can generate a wide variety of compounds. For example, Bhattacharyya et al. [29] reported that *Aspergillus niger* formed verbenone, *cis*-verbenol, and *trans*-sobrerol from α-pinene. Busmann and Berger [30] described verbenone and *trans*-verbenol, together with myrtenol and *trans*pinocarveol, as major biotransformation products of α-pinene formed by basidiomycetes fungi. Wright et al. [31] used a strain of the bacterium *Serratia marcescens,* isolated from sewage sludge, which can oxidize the terpene hydrocarbon α-pinene to produce *trans*-verbenol as the major product, with verbenone and *trans*-sobrerol as minor products.

Kinetic Study

The kinetic of substrate consumption and verbenol production was carried out using α-pinene as substrate and the screened microorganism coded as 05.01.05. The kinetic curves were built with induced and non-induced cells with the substrate.

Highest verbenol production was achieved after 8 days of reaction. At this experimental condition, a product concentration of 120.1 and 109.5 mg/L for induced and non-induced cells, respectively, was achieved. Here, the cell induction seems to less favor the product formation.

Figure 2 (a and b) presents these results. The cell induction during the inoculum growth by (-)-α-pinene led to an enhancement of 1.1-fold in verbenol conversion. This parameter is important for optimizing the reaction production.

Yoo et al. studied the progress curves for the formation of major bioconversion products versus time by α-pinene grown cells using 1% α-pinene as a sole carbon source. The pattern of accumulation of the *p*-menthene derivatives was different. They increased in parallel with a decrease in the amount of α-pinene into the control and reached a maximum concentration, 130 mg/L of *p*-cymeme and 22 mg/L of limonene, after 24 h. Their concentrations rapidly decreased, especially *p*-cymeme, to 9 mg/L after 60 h. α-Terpinolene reached a maximum concentration (33 mg/L) at the initial stage of cell growth (after 16 h), but its concentration remained stable during the period of fermentation. Oxygenated products, such as α-terpineol and *endo*-borneol, accumulated to 39 mg/L (36 h) and 22 mg/L (3 h) throughout the period of fermentation with little fluctuation [25].

The observation of Fig. 2 permits us to verify a reduction on substrate bioconversion after 9 days of reaction. Takahashi et al. [32] show that this reduction can be related to a possible product metabolization. The substrate concentration reduced until the tenth day for induced and non-induced cells. This may be a result of the lower levels of nutrients in the culture medium, perhaps because the microorganisms use the substrate as carbon source or because of enzymes inhibition due to the acumulus of the product in the reaction medium.

The literature points out that a common problem in the monoterpenes biotransformation is the toxicity of these compounds for the microorganisms [18–33]. To reduce this effect, the method of cell induction by the substrate has been employed, allowing the microorganism adaptation with lower amounts of substrate [33, 34].

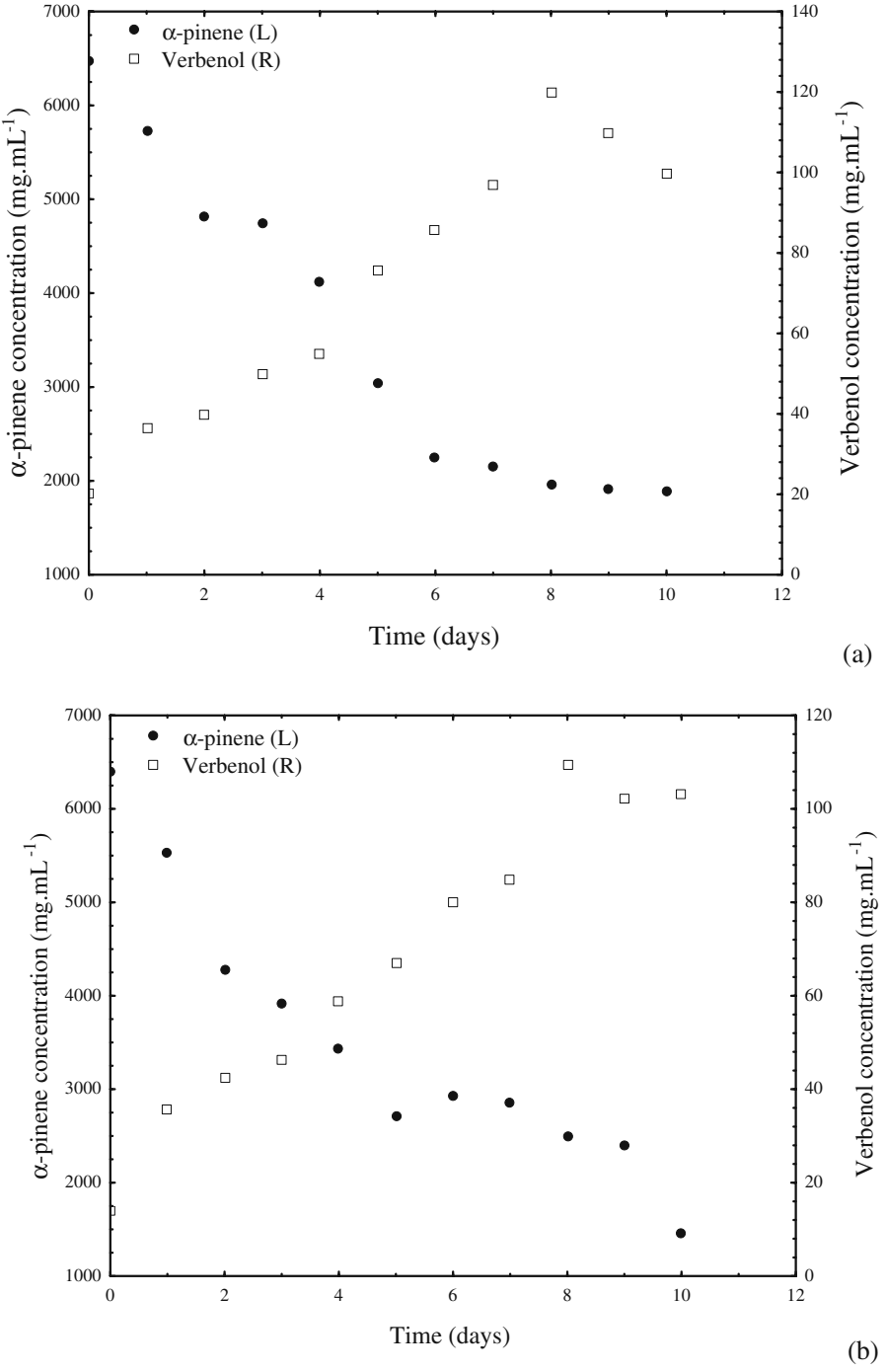

Fig. 2 (-)-α-Pinene and verbenol concentration on (-)-α-pinene biotransformation using the induced yeast isolated and coded as 05.01.05 (**a**) and the non-induced yeast isolated and coded as 05.01.05 (**b**)

Conclusion

A total of 405 microorganisms were isolated, and 193 strains were able to use the substrate limonene in the pre-screening step. The results in the screening showed that 31 strains were able to convert the substrate (-)-α-pinene in verbenol. Eleven strains that were able to convert (-)-α-pinene belong to filamentous fungi class, 13 were yeast, and seven were bacteria.

The high conversion in verbenol from (-)-α-pinene was found as 125.6 mg/L for yeast coded as 05.01.05 and isolated from residues of orange juice industry.

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