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Optimization of α-Terpineol Production by the Biotransformation of *R*-(+)-Limonene and (−)-β-Pinene

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Abstract The main objective of this work is to present the optimization of the biotransformation of *R*-(+)-limonene and (−)-β-pinene aiming at the production of α-terpineol by strains of fungal and yeasts previously isolated by our research group using the methodology of experimental design. New optimized experimental data on α-terpineol production by the biotransformation of *R*-(+)-limonene and (−)-β-pinene using newly isolated microorganisms are reported in this work. Conversion of about 1,700 mg/L was achieved when *R*-(+)-limonene was used as substrate and the newly isolated strain 05.01.35 as microorganism at the central point of the experimental design, corresponding to a substrate concentration of 1.75%, mass of inoculum of 2 g, and substrate to ethanol volume ratio of 1:1. The same experimental condition led to higher conversions when (−)-β-pinene was used as substrates and the strains coded as 04.05.08 and 01.04.03 as microorganism. Here, conversions of about 770 mg/L were achieved.

Keywords Experimental design · α-Terpineol · *R*-(+)-Limonene · (−)-β-Pinene

Introduction

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 largely in nature and are obtained in large scale as industrial residues [1]. They are extensively used by the flavor and fragrance industries. Via biotransformation, monoterpene precursors can be converted into their more valuable oxygenated derivatives [2].

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Terpenes are secondary metabolites of plants that are produced, in part, as a defense against microorganisms and insects, in addition to their pollinator-attractive properties [3].

The major constituent of the essential oils of orange and lemon is the (*R*)-(+)-limonene, extensively used by food and pharmaceutical industries as flavor additive, solvent for resins, pigments, inks, and rubber [4, 5]. This substrate, natural, low cost, and easily available can also be used as starter for flavor production, including perylic alcohol, carvone, carveol, menthol, and α-terpineol [6].

Pinenes are constituents of the wood and oil of an extensive variety of plants, and can also be obtained as subproduct of paper industries. (−)-β-Pinene is a bicycle monoterpene hydrocarbon of low price and commonly used as substrate 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 [6, 7].

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 compound, 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 towards microorganisms [8].

The use of biotransformation processes has significant advantages over chemical reactions and in the last 10 years has been increasing constantly. Biocatalysts have also attracted much attention from the viewpoint of green chemistry [9].

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, but biotechnological improvements to enhance the production are indispensable [10, 11].

In the scientific literature, the optimization of the production of natural aroma compounds using response surface methodology can be found only in few works [11–13]. Taking into account this information, the main objective of this work was established as presenting the optimization of the biotransformation of *R*-(+)-limonene and (−)-β-pinene aiming at the production of α-terpineol by strains of *Aspergillus* sp. and yeasts previously isolated by our research group using the methodology of experimental design. Additionally, comparing the results obtained here with that published previously [14], here, we have tested different newly isolated microorganisms whereas in the other work, only microorganisms identified and easily available (ATCC) were used. Additionally, in the present study, a systematic study was carried out by means of using of a well-recognized tool (experimental design). The use of factorial design and response surface methodology can overcome some drawbacks (high spend time, cost-ineffective, and not allowed to evaluate the interaction effects among variables) [15]. In this sense, factorial design technique has been successfully used to optimize and evaluate effect of process parameters in the biotechnological processes.

Material and Methods

Microorganism and Chemicals

The fungi strains employed in this study were previously isolated from the south of Brazil in orchards and industries of citric fruits and were identified as *Aspergillus* sp. (coded as

04.05.08 and 01.04.03). The yeasts strains tested here were also previously isolated by our research group and coded as 05.01.35, 03.03.03, 01.04.02, and 01.04.03) [16].

Other used compounds were: *R*-(+)-limonene (97%, Aldrich), (−)-β-pinene (99%, Fluka), α-terpineol (90%, Aldrich), ethyl acetate (99.5%, Vetec), anhydrous sodium sulfate (99%, Vetec), and absolute ethyl alcohol (99.5%, Nuclear). All chemicals were used without any pretreatment.

Inoculum

After reactivation of the microorganisms, a loopful of each strain was inoculated in centrifuge tubes containing 20 mL of the culture medium. PD (4 g L^{-1} potato infusion solids, 20 g L^{-1} glucose) for filamentous fungi and YM (10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract) for yeasts and incubated aerobically in orbital shaker (150 rpm) at 30 °C for periods of 30 to 72 h. The cell induction was carried out using 0.1% of the substrate. The substrates, *R*-(+)-limonene (using strains with the codes 05.01.35 and 03.03.03) and (−)-β-pinene (strains coded as 04.05.08, 01.04.02, and 01.04.03), were added to the inoculum after 1/3 of the incubation time. 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 were transferred to an Erlenmeyer flask containing 30 mL of mineral medium added by substrate.

Biotransformation Procedure

Experiments of biotransformation were started after inoculation, and the flasks were kept in orbital shaker at 30 °C and 175 rpm for 6 days with the substrate *R*-(+)-limonene and 7 days with the substrate (−)-β-pinene. Experiments were performed in duplicate runs in closed-stoppered glass flasks in order to avoid the substrate and product evaporation.

A central composite rotatable design (CCRD) 2^3 was carried out to each tested microorganism, and the studied variables were: substrate concentration (0.01 to 3.85 wt%), ratio between substrate and ethanol (0 to 1:2.7 *v*/*v*), and inoculum (0.64 to 7.36 g for filamentous fungi and 0.32 to 3.68 g for yeasts). Results were analyzed by the software Statistica® 6.0. A significance level of 95% ($p<0.05$) was used in each central composite rotatable design performed in this work.

Extraction and Quantification of the Products

At the end of each experimental run, cells were removed by filtration for fungi and centrifugation for yeasts. The product recovery was performed by liquid–liquid extraction with ethyl acetate. The final solution was dried over anhydrous sodium sulfate.

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 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 interest compound, evaluating the relative area from the interest compound and the standard curve. (−)-β-pinene (99%, Fluka), *R*-(+)-limonene (97%, Aldrich), and α-terpineol (90%, Aldrich) were used as external standards.

Results and Discussion

Table 1 presents the CCRD 2^3 matrix and respective responses in terms of α-terpineol production using *R*-(+)-limonene as substrate. One can see from this table that higher conversion using both microorganisms (03.03.03 and 05.01.35) was obtained in the central points of the experimental design. This condition corresponds to 1.75 wt% of *R*-(+)-limonene, 1:1 (*v*/*v*) of ethanol, and 2 g of inoculum and production of around 1,277 mg/L and 1,725 mg/L using the microorganisms coded as 03.03.03 and 05.01.35, respectively.

Data presented in Table 1 were statistically evaluated, and Eqs. 1 and 2 present the empirical models for each microorganism in terms of the independent variables evaluated. These models were validated using analysis of variance, where we could observe that the determination coefficient (R^2) of 0.87 and 0.82 for the microorganisms 03.03.03 and 05.01.35, respectively, and the calculated *F* higher than the listed one for both cases permitted us to validate the empirical models and verify that the processes were optimized in the variables studied range.

$$\begin{aligned}\alpha - \text{terpined} = {} & 1,254.93 - 82.82 \cdot \times 1 - 363.07 \cdot \times 1^2 + 44.98 \cdot \times 2 - 361.56 \cdot \times 2^2 \\ & + 1.01 \cdot \times 3 - 360.83 \cdot \times 3^2 - 195.78 \cdot \times 1 \cdot \times 2 + 103.84 \cdot \times 1 \cdot \times 3 \\ & - 100.26 \cdot \times 2 \cdot \times 3 \end{aligned} \quad (1)$$

$$\begin{aligned}\alpha - \text{terpined} = {} & 1,687.69 - 50.82 \cdot \times 1 - 482.86 \cdot \times 1^2 - 34.87 \cdot \times 2 - 483.14 \cdot \times 2^2 \\ & + 75.51 \cdot \times 3 - 481.10 \cdot \times 3^2 - 99.29 \cdot \times 1 \cdot \times 2 + 96.12 \cdot \times 1 \cdot \times 3 \\ & - 102.82 \cdot \times 2 \cdot \times 3 \end{aligned} \quad (2)$$

Where: ×1 is substrate concentration, ×2 is the substrate to ethanol volume ratio, and ×3 is the inoculum mass.

Results presented in Table 1 can be compared to that obtained by our research group in a previous work related to the screening of microorganisms for *R*-(+)-limonene and (−)-β-pinene biotransformation. One can notice that in the optimized experimental condition obtained here and in that from the screening step [16], similar α-terpineol production was obtained. In spite of this experimental result, it is worth to mention that from the

Table 1 CCRD 2^3 (real and coded values) for α-terpineol production using *R*-(+)-limonene as substrate

Experiment	Substrate concentration (%)	Substrate/ethanol ratio (*v*/*v*)	Inoculum mass (g)	Microorganism	
				03.03.03	05.01.35
				α-terpineol (mg/L)	
1	0.5 (−1)	0 (−1)	1 (−1)	174.78	290.49
2	3.0 (+1)	0 (−1)	1 (−1)	51.97	283.42
3	0.5 (−1)	1:2 (+1)	1 (−1)	934.79	744.36
4	3.0 (+1)	1:2 (+1)	1 (−1)	16.07	2.46
5	0.5 (−1)	0 (−1)	3 (+1)	197.62	737.09
6	3.0 (+1)	0 (−1)	3 (+1)	477.38	776.82
7	0.5 (−1)	1:2 (+1)	3 (+1)	543.81	441.99
8	3.0 (+1)	1:2 (+1)	3 (+1)	53.25	422.26
9	0.01 (−1.68)	1:1 (0)	2 (0)	0.00	0.00
10	3.85 (+1.68)	1:1 (0)	2 (0)	72.83	21.16
11	1.75 (0)	0 (−1.68)	2 (0)	50.31	9.48
12	1.75 (0)	1:2.7 (+1.68)	2 (0)	31.01	10.08
13	1.75 (0)	1:1 (0)	0.32 (−1.68)	66.71	23.60
14	1.75 (0)	1:1 (0)	3.68 (+1.68)	18.72	7.49
15	1.75 (0)	1:1 (0)	2 (0)	1277.94	1716.38
16	1.75 (0)	1:1 (0)	2 (0)	1261.37	1719.70
17	1.75 (0)	1:1 (0)	2 (0)	1294.31	1738.65

optimization experiments carried out here, we could establish a range of operational variables that leads to the highest conversions, making easy the process control. A detailed discussion about this experimental range will be presented after, according to the presentation of the results obtained in this work.

From the specialized literature, one can cite the results obtained by Tan and Day [6] who investigated the bioconversion of (4R)-(+)-limonene to (4R)-(+)-α-terpineol by immobilized fungal mycelia of *Penicillium digitatum*. The fungi cells were immobilized in calcium alginate beads. α-Terpineol production was correspondent to 12.83 mg/g beads/day, leading to a 45.81% bioconversion of substrate.

Adams et al. [17] 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 by 0.4% of EtOH as substrate and 8 h of reaction. Toniazzo et al. [18] tested *Aspergillus niger* and *A. niger* ATCC 9642 in the limonene biotransformation and achieved a conversion in α-terpineol of 0.68%±0.19 and 0.13%±0.07, respectively. Maróstica Junior and Pastore [10] investigated the microorganisms *Penicillium* sp. 2025, *Aspergillus* sp. 2038, and *Fusarium oxysporum* 152B for biotransformation of *R*-(+)-limonene using two agroindustrial residues (liquid cassava waste and orange essential oil) as substrates. The best yields in *R*-(+)-α-terpineol 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 of 450 mg/L after 3 days of reaction.

Bicas et al. [13] 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.

The microorganisms coded as 04.05.08, 01.04.02, and 01.04.03 were previously screened [16] as potential to bioconvert the substrate (−)-β-pinene into α-terpineol and used here aiming at the process optimization.

Table 2 presents the matrix of the CCRD (real and coded values) and the response in terms of α-terpineol production. One can see from this table that using *Aspergillus* sp. 04.05.08 and 01.04.03, higher production was also obtained in the central point of the experimental design, about 761 and 763 mg/L, respectively.

Using the microorganism coded as 01.04.02, higher conversion was obtained in run 8 of CCDR, corresponding to the use of 3.0% of substrate, 1:2 (*v*/*v*) of ethanol, and 3 g of inoculum, achieving α-terpineol production of 389.75 mg/L.

All results presented in Table 2 were statistically treated. Using the microorganisms 04.05.08 and 01.04.03, empirical models for each system were validated. Eqs. 3 and 4 present the behavior of the α-terpineol production using (−)-β-pinene as substrate in terms of substrate concentration, substrate to ethanol volume ratio, and inoculum mass. The analysis of variance showed R^2 values of 0.97 and 0.87 for the strains coded as 04.05.08

Table 2 CCRD 2^3 (real and coded values) for α-terpineol production using (−)-β-pinene as substrate

Experiment	Substrate concentration (%)	Substrate/ethanol ratio (*v*/*v*)	Inoculum mass (g)	Microorganism		
				04.05.08[a]	01.04.02	01.04.03
				α-terpineol (mg/L)		
1	0.5 (−1)	0 (−1)	1 (−1)	24.20	8.280	7.51
2	3.0 (+1)	0 (−1)	1 (−1)	17.81	5.656	10.73
3	0.5 (−1)	1:2 (+1)	1 (−1)	258.07	371.24	596.18
4	3.0 (+1)	1:2 (+1)	1 (−1)	37.79	257.54	225.91
5	0.5 (−1)	0 (−1)	3 (+1)	16.30	5.29	3.72
6	3.0 (+1)	0 (−1)	3 (+1)	46.39	1.47	7.25
7	0.5 (−1)	1:2 (+1)	3 (+1)	187.83	371.64	712.32
8	3.0 (+1)	1:2 (+1)	3 (+1)	35.00	389.75	444.23
9	0.01 (−1.68)	1:1 (0)	2 (0)	0.00	–	0.00
10	3.85 (+1.68)	1:1 (0)	2 (0)	83.84	–	0.53
11	1.75 (0)	0 (−1.68)	2 (0)	3.12	–	0.23
12	1.75 (0)	1:2.7 (+1.68)	2 (0)	52.57	–	0.95
13	1.75 (0)	1:1 (0)	0.32 (−1.68)	207.18	–	1.37
14	1.75 (0)	1:1 (0)	3.68 (+1.68)	10.24	–	1.08
15	1.75 (0)	1:1 (0)	2 (0)	770.97	287.56	775.17
16	1.75 (0)	1:1 (0)	2 (0)	775.78	278.93	786.63
17	1.75 (0)	1:1 (0)	2 (0)	735.43	277.85	727.96

[a] For this strain of *Aspergillus*, a double of inoculum mass was used

and 01.04.03, respectively, and in both cases, the calculated F was higher than the listed ones.

$$\alpha - \text{terpined} = 756.55 - 15.28 \cdot \times 1 - 240.72 \cdot \times 1^2 + 36.43 \cdot \times 2 - 245.71 \cdot \times 2^2 - 28.09 \cdot \times 3 - 217.06 \cdot \times 3^2 - 49.60 \cdot \times 1 \cdot \times 2 + 12.99 \cdot \times 1 \cdot \times 3 - 11.71 \cdot \times 2 \cdot \times 3 \quad (3)$$

$$\alpha - \text{terpined} = 763.25 - 46.22 \cdot \times 1 - 270.33 \cdot \times 1^2 + 142.96 \cdot \times 2 - 270.22 \cdot \times 2^2 - 40.89 \cdot \times 3 + 28.28 \cdot \times 3^2 - 80.64 \cdot \times 1 \cdot \times 2 + 12.81 \cdot \times 1 \cdot \times 3 + 42.72 \cdot \times 2 \cdot \times 3 \quad (4)$$

where ×1 is substrate concentration, ×2 is the substrate to ethanol volume ratio, and ×3 is the inoculum mass.

Related to the microorganism coded as 01.04.02, experiments related to the axial points (−1.68 and 1.68) were not carried out, since a first order model was validated. Eq. 5 presents the empirical model that describes the α-terpineol production in terms of the independent studied variables. The analysis of variance for this model presented a calculated F higher than the listed one and a determination coefficient of 0.90.

$$\alpha - \text{terpined} = 205.03 - 12.75 \cdot \times 1 + 171.19 \cdot \times 2 + 15.68 \cdot \times 3 - 11.14 \cdot \times 1 \cdot \times 2 + 16.33 \cdot \times 1 \cdot \times 3 + 17.47 \cdot \times 2 \cdot \times 3 \quad (5)$$

where ×1 is substrate concentration, ×2 is the substrate to ethanol ratio, and ×3 is the inoculum mass.

The statistical analysis permitted us to build Figs 1a and b, where one can verify that the ethanol concentration affected positively ($p<0.05$) the α-terpineol production. In this sense, a study using different concentrations of ethanol was carried out. The concentration of the substrate and the inoculum mass was kept in the inferior level of the CCDR presented in Table 2, since the effects of both variables were not statistically significant. Table 3 presents the results obtained in terms of α-terpineol production for all ethanol concentrations tested in this step. The analysis of variance followed by the Tukey's test showed that the ethanol concentrations of 1:1 and 1:2 are equal at 95% of confidence level, and higher concentrations probably caused an inhibition on cell growth and consequently a reduction in product conversion.

The bioconversion of β-pinene to α-terpineol has been scarcely described in the literature. van Dyk et al. [19], using *Hormonema* sp., obtained pino-camphone from (−)-β-pinene. Yoo and Day [20] and Yoo et al. [21], 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 [21] was also used as catalysts for this biotransformation, and the compound obtained was α-terpineol with low conversion (up to 4%).

Conclusions

New optimized experimental data on α-terpineol production by the biotransformation of *R*-(+)-limonene and (−)-β-pinene using newly isolated microorganisms are reported in this

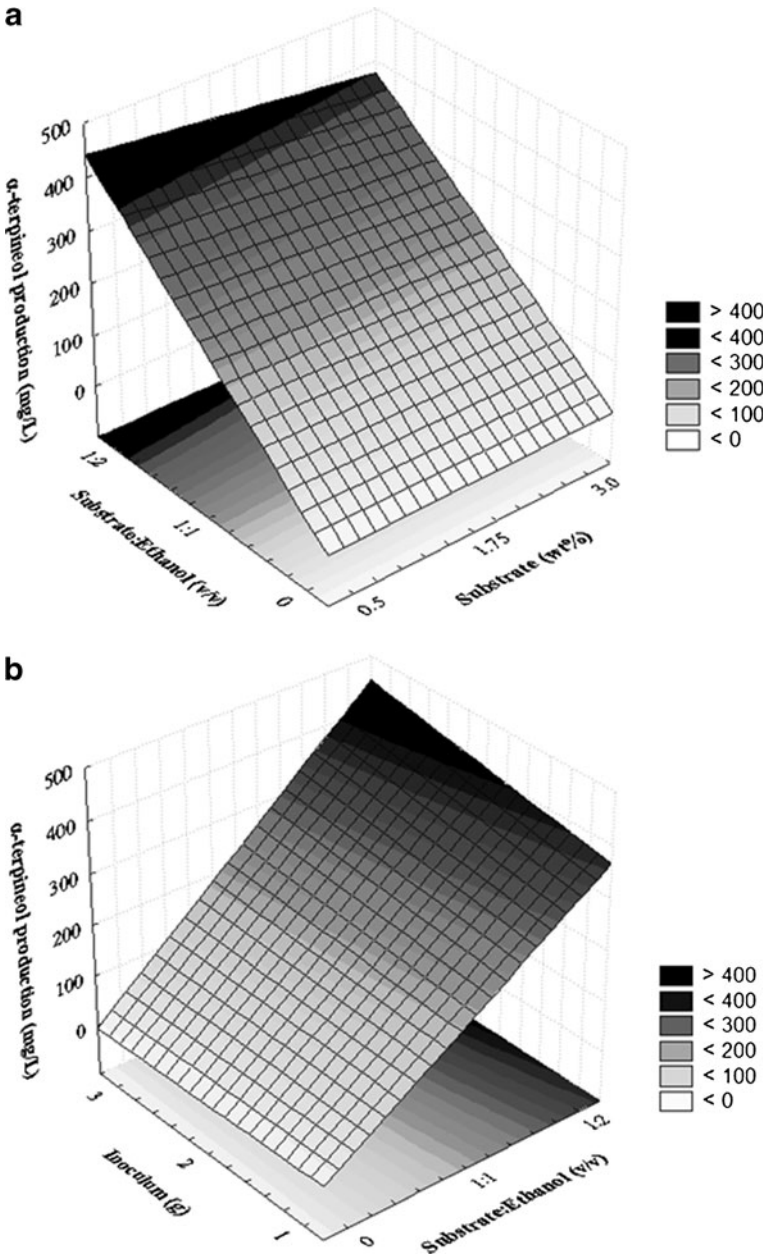

Fig. 1 Response surface for α-terpineol production using (−)-β-pinene as substrate and the microorganism coded as 01.04.02 as a function of **a** substrate/ethanol volume ratio and substrate concentration and **b** inoculum mass and substrate/ethanol volume ratio

Table 3 Evaluation of ethanol concentration for α-terpineol production using (−)-β-pinene as substrate and the microorganism coded as 01.04.02

Experiment	Substrate concentration (%)	Substrate/ethanol ratio (*v*/*v*)	Inoculum mass (g)	α-terpineol (mg/L)[a]
1	0.5	1:1	1	469.67±7.07a
2	0.5	1:2	1	450.97±8.79a
3	0.5	1:3	1	307.55±11.17b
4	0.5	1:4	1	287.89±2.18b
5	0.5	1:5	1	305.60±8.71b

[a] Mean and standard deviation of triplicates. Means with different lowercase letters in the same column differ significantly ($p<0.05$) in Tukey's test

work, showing a promising perspective of rationally bioproducing this important biotechnological compound. Results pointed out here showed that the strategy adopted for the experimental design proved to be useful in evaluating the effects of the studied variables on the product conversion. Conversion of about 1,700 mg/L was achieved when *R*-(+)-limonene was used as substrate and the newly isolated strain 05.01.35 as microorganism at the central point of the experimental design, corresponding to a substrate concentration of 1.75%, mass of inoculum of 2 g, and substrate to ethanol volume ratio of 1:1. The same experimental condition led to higher conversions when (−)-β-pinene was used as substrates and the strains coded as 04.05.08 and 01.04.03 as microorganism. Here, conversions of about 770 mg/L were achieved.

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