J Ind Microbiol Biotechnol (2008) 35:1061–1070
DOI 10.1007/s10295-008-0383-0

ORIGINAL PAPER

# Optimization of *R*-(+)-α-terpineol production by the biotransformation of *R*-(+)-limonene

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Received: 10 April 2008 / Accepted: 29 May 2008 / Published online: 17 June 2008


**Abstract** *R*-(+)-limonene is an abundant and non-expensive by-product of the citrus industry and is, therefore, a suitable starting material for the production of natural flavor and fragrance compounds. The biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *Fusarium oxysporum* 152b has already been reported, although the influence of the main process parameters on the production has not yet been evaluated. In this paper, a Plackett-Burman screening design was used to define the effects of the medium composition (glucose, peptone, yeast extract, malt extract and pH), the presence of a co-substrate (biosurfactant), the cultivation conditions (temperature, agitation), the substrate concentration and the inoculum/culture medium ratio on the absolute amount of *R*-(+)-α-terpineol resulting from this biotransformation. The process conditions were further optimized applying response surface methodology (RSM). The volatiles were extracted using a SPME device and were subsequently quantified by GC-FID and identified by GC-MS. The best results were obtained using 0.5% (v/m) *R*-(+)-limonene in pure distilled water as the 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 $L^{-1}$, a production almost six times greater than in earlier trials. The presence of a biosurfactant (0–500 mg $L^{-1}$) did not significantly increase the yield.

**Keywords** *Fusarium oxysporum* · Experimental design · Response surface methodology · Bioflavor · Biosurfactant

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## Introduction

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 [16]. Terpenoids, particularly mono and sesquiterpenoids, are the main flavor and fragrance impact molecules in the essential oils of higher plants [31]. *R*-(+)-limonene, for example, is one of the most abundant monocyclic monoterpenes in nature and represents more than 90% of orange peel oil, thus being an inexpensive citrus by-product available in bulk amounts [3]. As its chemical structure is similar to that of many oxygenated monoterpenoids presenting a pleasant fragrance, e.g. perillyl alcohol, carveol, carvone, menthol and α-terpineol, it may be used as a precursor in the synthesis of these flavor compounds [25]. Besides their desirable aromas, some of these oxygenated limonene counterparts have shown biological activity in vivo against certain types of tumor, not only preventing the formation or progression of the cancer, but also regressing existing malignant tumors [12, 20]. These characteristics greatly enhance the industrial interest in such compounds and, for this reason, the biotransformation process has emerged as an attractive alternative for the conversion of limonene since, when compared to the traditional chemical methods, they proceed under mild conditions, have an

elevated regio- and enantioselectivity, do not generate toxic wastes and the products obtained can be labeled as "natural" [34].

α-Terpineol is a stable alcohol commonly produced by acid-catalyzed chemical synthesis from α-pinene or turpentine oil. It is an important commercial product that is typically applied in soaps, cosmetics and flavor preparations [3]. *R*-(+)-α-terpineol has a floral, typically lilac odor, while *S*-(−)-α-terpineol occurs in conifer and lavandin oils and has a pine odor.

In the scientific literature, the biotransformation of limonene by microorganisms has been well documented [14]. Biotransformations of limonene to α-terpineol as the main product employing *Cladosporium* sp. [21], *Pseudomonas gladioli* [7] and an α-terpineol dehydratase isolated from the same strain [8], have been reported. Other publications have described the use of *P. digitatum* [39] and its immobilized cells [37], and have studied the effects of co-solvents on this transformation [1, 38]. Recently, a fungal strain identified as *Fusarium oxysporum* 152b was selected based on its high extracellular alkaline lipase production [29]. Subsequently, testing the same strain for the bioconversion of *R*-(+)-limonene, Maróstica Jr. and Pastore [24] noticed that *R*-(+)-α-terpineol accumulated in the medium. In order to reduce production costs, the authors explored agroindustrial residues as substitutes for the fungal cultivation medium (cassava wastewater) and substrate (orange peel oil).

The use of bioprocessing is a promising alternative in the recovery of terpene-derivate natural flavor compounds, but a commercial process with high productivity and low manufacturing costs must be developed [4]. In this context, biotechnological improvements to enhance the production rate, yields and recovery efficiency are indispensable [41].

The classical optimization method consists of varying the parameters one-at-a-time, maintaining the other variables constant. This strategy is usually time-consuming, requires a large number of experiments and does not consider the effects of interaction between the factors. It is thus an inadequate method for a full understanding of the process [32]. The Plackett–Burman design is a screening approach used to statistically select the significant variables of numerous factor-experiments, focusing on a reduction in the number of trials in the final design. The central composite design is a statistical methodology used to analyze the effects and interactions of the variables studied. This technique allows for the proposal of a mathematical model that describes the behavior of the factors analyzed and establishes their optimal values [30].

Optimization of the production of natural aroma compounds using response surface methodology has already been published [10, 26]. However, the authors of the present paper found no references in the literature describing the use of experimental designs and response surface methodology for the optimization of a bioprocess for the production of aromatic monoterpenoids, especially the formation of *R*-(+)-α-terpineol from *R*-(+)-limonene, although classical methods were reported for the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *Penicillium digitatum* NRRL 1202 [39], as well as the biotransformation of limonene to carvone by means of glucose oxidase and peroxidase [40] and the production of verbenone from α-pinene by *Penicillium* sp. [2]. This paper describes the optimization of the ten main process variables involved in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *Fusarium oxysporum* 152b using a Plackett–Burman matrix with 16 assays (PB-16) for the variables screened, followed by central composite design methodology.

## Material and methods

### Microorganism and chemicals

The microbial strain employed in this study was isolated from the northeast Brazilian fruits, and was identified as *Fusarium oxysporum* 152b [29]. *R*-(+)-limonene (Merck, Darmstadt, HE, Germany, >94% purity), (+)-α-terpineol (Aldrich, St. Louis, MO, USA, 90% purity) and *R*-(+)-α-terpineol (Fluka, Buchs, SG, Switzerland, ~99% purity) were kept under refrigeration (4 °C). The *Bacillus subtilis* biosurfactant was produced in the Laboratory of Bioflavors following a standard technique [27].

### Inoculum

A 48 h culture grown on agar in a Petri dish was divided amongst three 500 mL conical flasks each containing 200 mL of YM medium (10 g $L^{-1}$ glucose, 5 g $L^{-1}$ peptone, 3 g $L^{-1}$ yeast extract, 3 g $L^{-1}$ malt extract, pH 6.7) and homogenized under sterile conditions using an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After 72 h incubation at 30 °C/150 rpm, the cell mass was concentrated by vacuum filtration using a Buchner funnel with Whatman n° 1 filter paper. The separation was interrupted when the retentate reached 45% of the initial mass, which resulted in a final count of $1.6 \pm 0.2 \times 10^7$ CFU $mL^{-1}$.

### Optimization experiments

As no information was available on the influence of the main parameters involved in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol using *Fusarium oxysporum* 152b, an extensive study was carried out in order to

define the effects of the medium composition, the cultivation conditions, the substrate concentration and the inoculum/culture medium ratio, on the response (area of *R*-(+)-α-terpineol peak in GC). Thus, the strategy used was to run a Plackett–Burman screening design [18, 30] with 16 experiments (PB-16) and five center points to estimate the experimental error and select the main parameters (Tables 1, 2). A central composite design using the parameters selected was then carried out to define the optimal process conditions (Tables 4, 5). The center points of the screening design were YM broth as the culture medium with 1.0% (v/m) substrate and an inoculum/medium ratio of 0.5 (m/m), incubated at 30 °C/150 rpm (adapted from [24]), plus a concentration of the *Bacillus subtilis* LB5a biosurfactant equivalent to one CMC (critical micelle concentration; in this case, 11 mg $L^{-1}$). The central points and amplitudes of the central composite design parameters (Table 4) were chosen based on previous results.

## Biotransformation procedure

The concentrated cell mass (item 2.2) was distributed amongst 100 mL sterile screw-top flasks. Subsequently, the cultivation medium (pH adjusted prior to sterilization) and the substrate were aseptically added to totalize a final weight of 15 g, the proportion of each component varying according to the experimental design as shown in Tables 1 and 4. Each flask was incubated in a rotary shaker under their respective conditions (see Tables 1, 4). In order to define the transformation kinetics, four screening designs were performed, one for each biotransformation time: 24, 48, 72 and 96 h. As the transformation rate generally reached its maximum after 72–96 h, it was decided to carry out the central composite design twice: one for 72 h and the other for 96 h.

**Table 1** Variables and levels evaluated in the screening design

| Variables | Levels | | |
|---|---|---|---|
| | −1 | 0 | +1 |
| Medium composition | | | |
| Glucose (g $L^{-1}$) | 0 | 10 | 20 |
| Peptone (g $L^{-1}$) | 0 | 5 | 10 |
| Yeast extract (g $L^{-1}$) | 0 | 3 | 6 |
| Malt extract (g $L^{-1}$) | 0 | 3 | 6 |
| pH | 5.2 | 6.7 | 8.2 |
| Biosurfactant (mg $L^{-1}$) | 0 | 10 | 20 |
| Substrate | | | |
| Limonene (%, v/m) | 0.5 | 1.0 | 1.5 |
| Cultivation conditions | | | |
| Temperature (°C) | 20 | 30 | 40 |
| Agitation (rpm) | 0 | 150 | 300 |
| Inoculum | | | |
| Inoculum/medium proportion (m/m) | 0.25 | 0.50 | 0.75 |

## Quantification and identification of the volatile compounds

A SPME device (Supelco, Bellefonte, PA, USA) containing a fused-silica fiber (10 mm in length) coated with a 75 μm layer of CAR-PDMS was used. Before analysis the fiber was preconditioned in the injection port of the chromatograph according to the manufacturer's instructions.

The aroma compounds were extracted from the 100 mL flasks using a PTFE septum containing 50 μL of fermented medium diluted in 50 mL of a 360 g $L^{-1}$ sodium chloride solution. The system was left for 10 min at 40 °C with agitation to allow for equilibrium of the volatiles in the headspace. The fiber was exposed to the headspace for 30 min at 40 °C.

Analyses were performed using a Varian 3800 gas chromatograph (GC) equipped with a flame ionization detector (FID) (Palo Alto, CA, USA). Desorption proceeded in the injection port of the gas chromatograph for 1 min at 280 °C with the purge valve off (splitless mode). The volatile compounds were separated in a 30 m × 0.25 mm i.d. DB-Wax fused silica capillary column with a 0.5 μm film thickness (J&W Scientific, Folsom, CA, USA). The temperature program was isothermal for 2 min at 50 °C, and then rose to 200 at 5 °C $min^{-1}$ where it was held for 5 min. Helium was the carrier gas at a flow rate of 1.2 mL $min^{-1}$ and the detector temperature was 250 °C. After desorption, the fiber remained a further 10 min in the injector port to eliminate the possibility of any carry-over of analyte between samples. Quantification was performed using the external calibration curve of α-terpineol, obtained by distributing opposing concentrations of standard samples of *R*-(+)-limonene (4.23, 3.17, 2.11, 1.06 and 0.10 g $L^{-1}$ of medium) and α-terpineol (0.10, 1.12, 2.24, 3.36 and 4.20 g $L^{-1}$ of medium), in a 100 mL screw-top flask containing 3.75 g of the inoculum and 11.25 g of distilled water (mean composition at the central point of the central composite design), all carried out in duplicate. The enantiomeric identification was carried out by comparison between standard *R*-(+)-α-terpineol and the sample regarding to the retention time and spiking. It was used a Beta Dex™ 120 fused silica capillary column (Supelco; 60 m, 0.25 mm i.d., 0.25 μm film thickness) with a temperature program isothermal for 20 min at 140 °C, and then rose to 210 at 20 °C $min^{-1}$ where it was held for 5 min. The other conditions were the same as described above.

**Table 2** Plackett–Burman screening design matrix (PB-16) and the *R*-(+)-α-terpineol GC area after 24, 48, 72 and 96 h of biotransformation

| | *Glu* | *Pep* | *MEx* | *YEx* | *L* | pH | *S* | *T* | *A* | *I/M* | α-T area ($\times 10^6$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | 24 h | 48 h | 72 h | 96 h |
| 1 | +1 | −1 | −1 | −1 | +1 | −1 | −1 | +1 | +1 | −1 | 0.16 | 0.15 | 0.16 | 0.51 |
| 2 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | −1 | +1 | +1 | 1.09 | 3.58 | 12.25 | 15.82 |
| 3 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | −1 | +1 | 0.76 | 1.66 | 2.50 | 2.62 |
| 4 | +1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | −1 | 0.12 | 0.17 | 0.18 | 0.14 |
| 5 | −1 | +1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | 0.13 | 0.33 | 2.77 | 5.22 |
| 6 | +1 | −1 | +1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | 0.26 | 0.37 | 0.47 | 0.51 |
| 7 | −1 | +1 | −1 | +1 | +1 | +1 | +1 | −1 | −1 | −1 | 0.15 | 0.15 | 0.20 | 0.28 |
| 8 | +1 | −1 | +1 | −1 | +1 | +1 | +1 | +1 | −1 | −1 | 0.09 | 0.14 | 0.14 | 0.13 |
| 9 | +1 | +1 | −1 | +1 | −1 | +1 | +1 | +1 | +1 | −1 | 0.13 | 0.45 | 0.18 | 0.25 |
| 10 | −1 | +1 | +1 | −1 | +1 | −1 | +1 | +1 | +1 | +1 | 0.18 | 0.21 | 0.20 | 0.31 |
| 11 | −1 | −1 | +1 | +1 | −1 | +1 | −1 | +1 | +1 | +1 | 0.16 | 0.22 | 0.22 | 0.24 |
| 12 | +1 | −1 | −1 | +1 | +1 | −1 | +1 | −1 | +1 | +1 | 0.27 | 0.89 | 3.05 | 5.81 |
| 13 | −1 | +1 | −1 | −1 | +1 | +1 | −1 | +1 | −1 | +1 | 0.13 | 0.16 | 0.13 | 0.16 |
| 14 | −1 | −1 | +1 | −1 | −1 | +1 | +1 | −1 | +1 | −1 | 0.40 | 6.31 | 26.94 | 21.93 |
| 15 | −1 | −1 | −1 | +1 | −1 | −1 | +1 | +1 | −1 | +1 | 0.11 | 0.11 | 0.12 | 0.13 |
| 16 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 0.78 | 1.63 | 3.19 | 4.45 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.41 | 1.38 | 6.20 | 10.78 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.35 | 1.85 | 5.81 | 6.39 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.48 | 1.40 | 5.61 | 8.91 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.58 | 1.16 | 5.70 | 8.17 |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.35 | 1.64 | 6.61 | 8.30 |

*Glu* glucose, *Pep* peptone, *MEx* malt extract, *YEx* yeast extract, *L* limonene, *S* biosurfactant, *T* temperature, *A* agitation, *I/M* inoculum/medium ratio (m/m), *α-T area* *R*-(+)-α-terpineol peak area. The levels of each variable are described in Table 1

For the identification of the volatile products, the compounds adsorbed by the fiber were desorbed in the injection port of the GC under the same conditions as above. The compounds were separated using a DB-5 capillary column (J&W Scientific; 30 m, 0.25 mm i.d., film thickness 0.25 μm) installed in a Shimadzu 17A gas chromatograph coupled with a Shimadzu QP-5000 mass spectrometer (MS) (Kyoto, Kansai, Japan). The temperature program used was 60 °C, rising to 210 at 3 °C $min^{-1}$ and held for 5 min. Helium was the carrier gas with a flow rate of 1.0 mL $min^{-1}$. The GC/MS interface was maintained at 240 °C. The mass spectrometer was operated in the electron impact mode with electron energy of 70 eV and a multiplier voltage of 1.4 kV at 0.5 scan $s^{-1}$ over a range of *m/z* 35 − 350.

### Data analysis

The results were analyzed by the software STATISTICA® 5.5 A. A significance level of 10% ($P < 0.1$) was considered for the variables screened and 5% ($P < 0.05$) for the central composite design.

## Results and discussion

### Screening of the variables

The center points for the screening design were chosen based on the conditions usually applied for this biotransformation. The triplicate of the center points showed that before the biotransformation took place (0 h), there was a little *R*-(+)-α-terpineol in the medium (areas from 0.7 to 0.9 × $10^6$), probably present as a contaminant of the substrate. A blank experiment without the inoculum, carried out under the same conditions as the center points, demonstrated that the amount of *R*-(+)-α-terpineol in the medium remained practically stable in the absence of the catalyst, presenting only a slight increase between 0 and 48 h (from 0.8 to 1.1 × $10^6$), with no significant rise until 96 h, indicating that the *R*-(+)-α-terpineol produced in the process did not originate from the autooxidation of *R*-(+)-limonene (data not shown).

Table 2 illustrates that, with few exceptions, the *R*-(+)-α-terpineol area increased during the course of the reaction, reaching a maximum after 72–96 h, the same profile obtained before [24]. However, the increase observed

between 72 and 96 h might not be big enough to justify an extra 24 h of process. For this reason only these two periods were considered in the statistical analysis and for the central composite design. The statistical evaluation of the results is shown in Table 3.

The use of biosurfactants as the co-substrate was tested for the first time in this bioprocess and, due to the lack of references, it was decided to use a concentration of one CMC: the minimal concentration for the formation of micelles. The choice of CMC as the concentration parameter was made in function of the relation between this value and the efficiency of a surfactant [6, 35]. Hydrophobic compounds can be dissolved in the micelle. Typically, the solubility of surfactants remains very low until the CMC value is reached, whereas at concentrations above the CMC, it increases rapidly and almost linearly with the surfactant content [19]. Thus, the main characteristic of biosurfactants is to reduce the interfacial tension at the surface of the insoluble substrate and to increase the availability of the hydrophobic substrate to the microbe. For liquid hydrocarbons, reduced interfacial tension facilitates emulsification, increasing the surface area available for dissolution, microbial attachment, and substrate absorption [17]. Additionally, surfactants increase the membrane permeability with the formation of pores [9].

**Table 3** Estimates of the effects of the parameters analyzed after 72 and 96 h of biotransformation

| Factor | Time (h) | Effect ($\times 10^6$) | SE ($\times 10^6$) | $t(10)$ | $P$ value |
|---|---|---|---|---|---|
| **Mean** | **72** | **3.93** | **1.00** | **3.92** | **0.0029** |
| | **96** | **4.81** | **1.06** | **4.55** | **0.0011** |
| Glu | 72 | −1.85 | 2.30 | −0.81 | 0.4386 |
| | 96 | −0.87 | 2.42 | −0.36 | 0.7277 |
| Pep | 72 | −1.98 | 2.30 | −0.86 | 0.4088 |
| | 96 | −1.11 | 2.42 | −0.46 | 0.6556 |
| MEx | 72 | 1.77 | 2.30 | 0.77 | 0.4599 |
| | 96 | 0.46 | 2.42 | 0.19 | 0.8521 |
| **YEx** | **72** | **−4.79** | **2.30** | **−2.08** | **0.0637** |
| | 96 | −4.17 | 2.42 | −1.72 | 0.1161 |
| **L** | **72** | **−4.81** | **2.30** | **−2.09** | **0.0630** |
| | 96 | −4.08 | 2.42 | −1.68 | 0.1230 |
| pH | 72 | 3.54 | 2.30 | 1.54 | 0.1542 |
| | 96 | 2.52 | 2.42 | 1.04 | 0.3232 |
| *S* | 72 | 1.74 | 2.30 | 0.76 | 0.4656 |
| | 96 | 0.55 | 2.42 | 0.23 | 0.8242 |
| **T** | **72** | **−6.25** | **2.30** | **−2.72** | **0.0215** |
| | **96** | **−6.85** | **2.42** | **−2.83** | **0.0179** |
| **A** | **72** | **4.86** | **2.30** | **2.11** | **0.0608** |
| | **96** | **5.21** | **2.42** | **2.15** | **0.0569** |
| *I/M* | 72 | −1.85 | 2.30 | 0.81 | 0.4390 |
| | 96 | −0.91 | 2.42 | −0.38 | 0.7142 |

*SE* Standard error, *Glu* glucose, *Pep* peptone, *MEx* malt extract, *YEx* yeast extract, *L* limonene, *S* biosurfactant, *T* temperature, *A* agitation, *I/M* inoculum/medium ratio (m/m)

Parameters in bold are statistically significant for the response ($P < 0.1$), considering the residual SS

The levels of the variables tested in the screening design are described in Table 1. These codified values were applied in the PB-16 Plackett–Burman matrix (Table 2). In this case, a $P$ value of 0.1 is currently recommended, since it is more conservative and lowers the risk of false-excluding statistically significant parameters [30].

## Effect of the medium composition

The medium composition usually displays an important role in biotransformation processes, being one of the main factors responsible for alterations in yield [1]. The presence of co-substrates might also enhance the bioconversion performance [36, 38]. It was demonstrated that, considering the ranges tested, neither glucose, nor peptone, malt extract or the pH value presented any statistical effects on the responses after 72 or 96 h, and thus these variables were of no significant interest (at $P < 0.1$) to this process. Yeast extract showed a negative effect at 72 h ($P < 0.1$) and at 96 h ($P < 0.12$), which means that this compound hinders the biotransformation of limonene by *F. oxysporum*, lowering its activity. Therefore, according to the results (Table 3), the best medium for this process was pure distilled water with a pH between 5.2 and 8.2. The following procedures used the normal pH of the water as the standard. This result is completely comprehensible since in terpene biotransformation processes, the substrate is usually the sole carbon source, and mineral mediums (saline solutions) or buffers are typically applied as the culture medium [24, 36, 39], even though some authors have proposed there is a correlation between best fungal growth and best bioconversion yield, suggesting mediums with other carbon sources [1].

Unfortunately, the biosurfactant showed no statistical effect (at $P < 0.1$) on the response, considering the levels tested (Table 3), for either 72 or 96 h of biotransformation. Nevertheless, univariate experiments with a broad range of biosurfactant concentration levels (50, 250, 1,250 and 6,250 mg $L^{-1}$, equivalent to approximately 5, 25, 125 and 625 CMC) were carried out in duplicate to observe the behavior of the biotransformation. Apparently, the yield increased up to 25 CMC and higher concentrations only resulted in a slight increase in biotransformation (results not shown).

### *Effect of the substrate concentration*

One of the greatest challenges in biotransformation processes consists of the high cytotoxicity and high volatility

of both substrate and product [22]. When compared to bacteria, fungi seem to be more sensitive to limonene [5], possibly because this monoterpene increases the fluidity of fungal membranes, leading to a high unspecific membrane permeability and loss of membrane integrity [28]. In this study, the limonene concentration showed a negative effect on the levels tested for 72 h ($P < 0.1$) and 96 h ($P < 0.13$) of biotransformation (Table 3), which might be explained by the toxic activity of *R*-(+)-limonene on the *F. oxysporum*. In this case, smaller amounts of substrate (0.5% or less) are indicated, still within the limonene concentration usual applied in biotransformation processes (0.2–1.0%) [5]. Some authors have suggested that substrate induction might enhance the yield [1, 15, 39], however, this was not the case in the present process [24].

The system employed in this experiment, a 100 mL screw-top flask with a plastic cover, apparently reduced limonene volatilization. In earlier work using normal conical flasks, the limonene evaporated to very small concentrations after a short period of time, even when co-substrates and the subsequent addition of substrate were applied. Discounting the transformed limonene (yield ~50%), substrate recoveries of <10% after 48 h [13], and <20% after 72 h, have been reported [24]. However, in the blank experiments of the present study, the reduction in the limonene area (GC-FID) after 24, 48, 72 and 96 h was, respectively, 14, 36, 57 and 63% (data not shown).

*Effect of the cultivation conditions*

It is known that the medium temperature directly influences biological reactions. Moreover, medium agitation promotes development of the microorganism and cell-substrate interaction. However, the use of high temperatures and agitation speeds, in addition to increasing the process energy costs, might enhance the loss of substrate and product and the occurrence of side reactions. Thus, an ideal balance must be searched for in order to achieve the best results.

The temperature showed a negative effect ($P < 0.1$) on the biotransformation process (Table 3), possibly explained by inhibition of microbial growth and enzyme denaturation at temperatures close to 40 °C. Table 2 clearly shows that temperatures of 40 °C (+1 level) presented virtually no product formation. Thus in the present case, mild temperatures (lower than 30 °C) should be considered, in order to obtain the best performance. Similar results were obtained for *Penicillium digitatum* NRRL 1202 [39] and for *Pseudomonas putida* [11, 36], which showed dramatic decreases in bioconversion at temperatures above 32 and 30 °C, respectively.

Agitation, on the other hand, showed a positive effect ($P < 0.1$) in the α-terpineol area (Table 3), probably related to the increase in cell-substrate contact. Hence, the optimal agitation may be situated at values above the maximum value tested in the screening design (300 rpm) and an ampler range is suggested. To relate the need for agitation with the process economics, a repetition with no agitation should be run in the optimization design.

*Effect of the inoculum size*

The size of the inoculum is an important factor in a fermentation process, as it has a considerable effect on the subsequent stages. In industrial fermentation processes, it is a well-known fact that the age and density of the inoculum used directly influences the duration of the lag phase, specific growth rate, biomass yield, sporulation and quality of the final product, and hence the production costs [33]. A positive influence of the biocatalyst content on the maximum product recovery was already reported for the biotransformation of α-pinene oxide to isonovalal [15]. It was also suggested that the use of mycelium concentrates, as applied in this study, might significantly enhance the yield [23]. Curiously, the inoculum size had no statistically significant ($P < 0.1$) influence on the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by *F. oxysporum,* considering the levels tested. Therefore, the minimal inoculum/culture medium ratio tested (0.25, m/m) was chosen as the standard for the following trials.

Optimization using a central composite design

According to the variables screened, the significant factors ($P < 0.1$) for the process under study, considering the levels tested, which were worth considering in the further optimization design, were only temperature, agitation speed and substrate (limonene) concentration. The biosurfactant only appeared to present an effect at higher concentrations (around 25 CMC). For this reason, these four variables, which were analyzed at the levels described in Table 4, were optimized using a $2^4$ central composite design with six center points, as shown in Table 5.

**Table 4** Variables and levels evaluated in the central composite design

| Variables | Levels | | | | |
|---|---|---|---|---|---|
| | −2 | −1 | 0 | +1 | +2 |
| Cultivation conditions | | | | | |
| Temperature (°C) | 10 | 15 | 20 | 25 | 30 |
| Agitation (rpm) | 0 | 90 | 180 | 270 | 360 |
| Substrate | | | | | |
| Limonene (%) | 0.1 | 0.3 | 0.5 | 0.7 | 0.9 |
| Co-substrate | | | | | |
| Biosurfactant (mg $L^{-1}$) | 0 | 125 | 250 | 375 | 500 |

**Table 5** $2^4$ Central composite design matrix and the *R*-(+)-α-terpineol GC-FID area after 72 and 96 h incubation

| | *T* | *A* | *L* | *S* | α-T area ($\times 10^6$) | |
|---|---|---|---|---|---|---|
| | | | | | 72 h | 96 h |
| 1 | −1 | −1 | −1 | −1 | 0.81 | 2.23 |
| 2 | +1 | −1 | −1 | −1 | 19.14 | 18.36 |
| 3 | −1 | +1 | −1 | −1 | 1.21 | 16.27 |
| 4 | +1 | +1 | −1 | −1 | 21.17 | 22.37 |
| 5 | −1 | −1 | +1 | −1 | 0.68 | 1.95 |
| 6 | +1 | −1 | +1 | −1 | 7.63 | 13.30 |
| 7 | −1 | +1 | +1 | −1 | 0.19 | 0.47 |
| 8 | +1 | +1 | +1 | −1 | 18.17 | 16.66 |
| 9 | −1 | −1 | −1 | +1 | 0.88 | 9.51 |
| 10 | +1 | −1 | −1 | +1 | 16.60 | 18.75 |
| 11 | −1 | +1 | −1 | +1 | 10.52 | 20.37 |
| 12 | +1 | +1 | −1 | +1 | 20.38 | 19.65 |
| 13 | −1 | −1 | +1 | +1 | 0.37 | 2.58 |
| 14 | +1 | −1 | +1 | +1 | 9.92 | 11.76 |
| 15 | −1 | +1 | +1 | +1 | 0.20 | 0.19 |
| 16 | +1 | +1 | +1 | +1 | 15.27 | 18.53 |
| 17 | −2 | 0 | 0 | 0 | 0.18 | 0.19 |
| 18 | +2 | 0 | 0 | 0 | 17.68 | 18.55 |
| 19 | 0 | −2 | 0 | 0 | 2.35 | 4.31 |
| 20 | 0 | +2 | 0 | 0 | 19.61 | 16.58 |
| 21 | 0 | 0 | −2 | 0 | 0.20 | 7.89 |
| 22 | 0 | 0 | +2 | 0 | 9.93 | 13.00 |
| 23 | 0 | 0 | 0 | −2 | 15.33 | 18.76 |
| 24 | 0 | 0 | 0 | +2 | 19.98 | 19.86 |
| 25 | 0 | 0 | 0 | 0 | 16.63 | 21.75 |
| 26 | 0 | 0 | 0 | 0 | 16.17 | 18.77 |
| 27 | 0 | 0 | 0 | 0 | 18.76 | 19.53 |
| 28 | 0 | 0 | 0 | 0 | 18.70 | 22.54 |
| 29 | 0 | 0 | 0 | 0 | 17.78 | 19.03 |
| 30 | 0 | 0 | 0 | 0 | 18.25 | 18.11 |

The levels of each variable are described in Table 4. *T* temperature, *A* agitation, *L* limonene, *S* biosurfactant, *α-T area* *R*-(+)-α-terpineol peak area

It can be seen that the greatest increases in the *R*-(+)-α-terpineol area occurred for those points which, after 72 h, showed a response no higher than $11 \times 10^6$. All the experiments carried out at temperatures equal or inferior to 15 °C are comprised in this group, demonstrating that lower temperatures clearly reduced the biotransformation activity and retarded the process. In the region of interest (areas above $15 \times 10^6$), only a small, or even negative increase in the response (Table 5), was found after 96 h. As a result, in agreement with previous work [24], a 96 h-reaction period presented no practical advantage over 72 h, which was considered to be the optimum biotransformation time for the statistical evaluation. These data (72 h-biotransformation), were treated by the software STATISTICA® v. 5.5A, which generated the regression coefficients and respective statistical analysis of the parameters considered (Table 6).

**Table 6** The least-squares and significances of the regression coefficients of the model parameters (72 h-biotransformation)

| Parameter | RC ($\times 10^6$) | SE ($\times 10^6$) | *t*(15) | *P* value |
|---|---|---|---|---|
| **Mean** | **17.7** | **1.77** | **10.01** | **<0.001** |
| **T** | **6.18** | **0.89** | **6.986** | **<0.001** |
| **$T^2$** | **−2.48** | **0.83** | **−2.997** | **0.009** |
| **A** | **2.73** | **0.89** | **3.088** | **0.008** |
| **$A^2$** | **−1.97** | **0.83** | **−2.377** | **0.031** |
| *L* | −0.78 | 0.89 | −0.886 | 0.390 |
| **$L^2$** | **−3.45** | **0.83** | **−4.164** | **<0.001** |
| *S* | 0.60 | 0.89 | 0.680 | 0.507 |
| $S^2$ | −0.30 | 0.83 | −0.363 | 0.722 |
| $T \times A$ | 0.77 | 1.08 | 0.710 | 0.488 |
| $T \times L$ | −0.89 | 1.08 | −0.824 | 0.423 |
| $T \times S$ | −0.81 | 1.08 | −0.751 | 0.464 |
| $A \times L$ | −0.04 | 1.08 | −0.035 | 0.972 |
| $A \times S$ | 0.38 | 1.08 | 0.353 | 0.729 |
| $L \times S$ | −0.43 | 1.08 | −0.400 | 0.695 |

*RC* Regression coefficient, *SE* standard error, *T* temperature, *A* agitation, *L* limonene, *S* biosurfactant

Parameters in bold are statistically significant for the model ($P < 0.05$)

**Table 7** ANOVA of the quadratic model

| Variation source | SS ($\times 10^{14}$) | *df* | SM ($\times 10^{13}$) | *F* value | *P* value |
|---|---|---|---|---|---|
| Regression | 16.9 | 5 | 33.4 | 23.5 | <0.0001 |
| Residues | 3.46 | 24 | 1.44 | | |
| Total | 20.4 | 29 | | | |
| $R^2 = 0.83$ | | | | $F_{0.95(5,24)} = 2.62$ | |

*SS* Sum of squares, *df* degrees of freedom, *SM* mean square

In order to verify the validity of the model, an analysis of variance (ANOVA) was performed considering only the statistically significant ($P < 0.05$) variables (parameters in bold in Table 6) for the analysis. The ANOVA table (Table 7) demonstrated that the quadratic model adjusted for the process responses was satisfactory. The calculated *F* value was nine times higher than the respective listed value, while the *P* value of the model was lower than 0.0001. Although it is not ideal, a value for $R^2 = 0.83$ is a perfectly acceptable value for biological systems [30]. Consequently, it is possible to define a statistically valid model ($P < 0.05$), given by Eq. 1:

$$\alpha\text{T Area}(\times 10^6) = 17.37 + 6.18T - 2.44T^2 + 2.73A - 1.93A^2 - 3.40L^2 \quad (1)$$

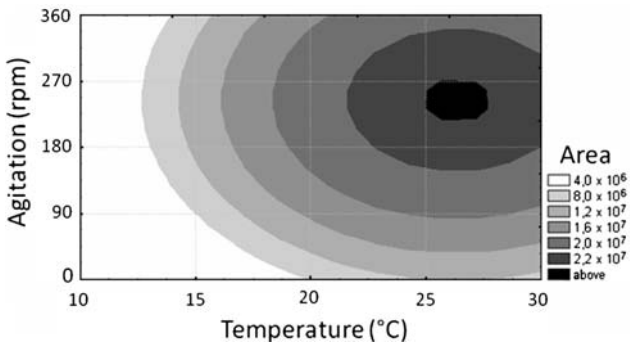


**Fig. 1** Contour plot of the *R*-(+)-α-terpineol area after 72 h-biotransformation as a function of agitation and temperature. Limonene concentration fixed at 0.5% (v/m)

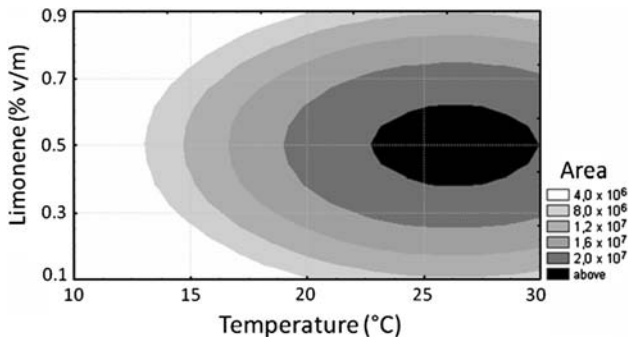


**Fig. 2** Contour plot of the *R*-(+)-α-terpineol area after 72 h-biotransformation as a function of limonene concentration and temperature. Agitation fixed at 180 rpm

where the αT Area, *T*, *A* and *L* are, respectively, the *R*-(+)-α-terpineol peak area in the GC-FID, temperature, agitation and limonene concentration in codified values (Table 4).

This equation is graphically represented by the agitation × temperature (Fig. 1), limonene × temperature (Fig. 2) and limonene × agitation (Fig. 3) contour curves. The profile of the response surfaces obtained was ideal, since all the figures presented the predicted optimal regions comprised inside the levels studied. It can be seen that a strict control of the biotransformation conditions was not necessary, which simplifies the process even more. As previously cited, the medium pH could vary from 5.2 to 8.2 without any significant changes in yield. An analysis of Eq. 1 showed that the temperature and agitation, for their part, could oscillate from 23 to 29.5 °C and from 180 to 310 rpm, respectively, maintaining the production above 95% of the maximal value, when the limonene concentration remained fixed at 0.5% (v/m). In a non-agitated process, the maximal *R*-(+)-α-terpineol production reached 1 g $L^{-1}$. The optimal conditions could be determined using mathematical methods (equation derivation). In this case, the values obtained were: temperature = +1.27 (codified level) ≈ 26 °C, agitation = +0.71 (codified level) ≈ 240 rpm and limonene concentration = 0 (codified level) = 0.5% (v/m), with a predicted *R*-(+)-α-terpineol area of 22.25 × $10^6$, equivalent to 2.44 g of *R*-(+)-α-terpineol per liter of medium [$\alpha T_{g\,L^{-1}} = (\alpha T_{GC-FID\,area} + 1.57 \times 10^6)/9.76 \times 10^6$; $R^2 = 0.992$]. Due to practical reasons, it was decided to use 30 °C/270 rpm and 0.5% substrate as standard, keeping the production of approximately 2.4 g $L^{-1}$.

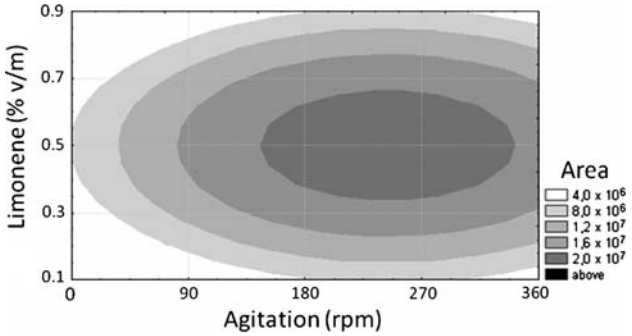


**Fig. 3** Contour plot of the *R*-(+)-α-terpineol area after 72 h-biotransformation as a function of limonene concentration and agitation. Temperature fixed at 20 °C

## Conclusions

For the first time a variable screening (Plackett–Burman methodology) followed by a central composite design was described to optimize the main parameters involved in the biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol. The effect of a biosurfactant on this bioprocess was an additional original report. These optimization techniques were very useful for a full understanding of the process. According to the contour plots, the best conditions for the highest *R*-(+)-α-terpineol recovery were: 72 h-reaction in pure distilled water as the culture medium, temperature between 24 and 28 °C, agitation of 200 to 310 rpm, *R*-(+)-limonene concentration of 0.5% (v/m) and an inoculum/culture medium ratio of 0.25 (m/m). This is a simple and low cost process with relative high *R*-(+)-α-terpineol production (up to 2.44 g $L^{-1}$), representing the first step in an industrial process development. The following stage of this research should be work on the inoculum (immobilization, freeze drying, membrane permeabilization), use biphasic systems and a scale up and product recovery study. In the future, a possible genetic manipulation of the microorganism should be considered.

**Acknowledgments** The authors are truly grateful for the valuable suggestions from Prof. Maria Isabel Rodrigues (DEA/FEA–Unicamp, Brazil) and acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for their financial support.

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