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Biotransformation of (–)β-Pinene by *Aspergillus niger* ATCC 9642

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Abstract

The main objective of this work was to investigate the biotransformations of (–)α-pinene, (–)β-pinene, and (+) limonene by *Aspergillus niger* ATCC 9642. The culture conditions involved—concentration of cosolvent (EtOH), substrate applied, and sequential addition of substrates—were investigated. Adaptation of the precultures with small amounts of substrate was also studied. The experiments were performed in conical flasks with liquid cultures. This strain of *A. niger* was able to convert only (–)β-pinene into α-terpineol. An optimum conversion of (–)β-pinene into α-terpineol of about 4% was obtained when the substrate was applied as a diluted solution in EtOH and sequential addition of substrate was used.

Index Entries: Biotransformation; β-pinene; α-terpineol; flavors; *Aspergillus niger.*

Introduction

Extensive research has been devoted to the biotechnological production of flavors and fragrances. Consumer demand for natural products has increased the production of "natural" flavors by biotransformation or bioconversion *(1)*. This trend has forced companies to direct their attention toward finding natural sources of flavors. However, presently, most flavor compounds are produced via chemical synthesis or by direct extraction from plants. Specifically, among all available flavor compounds, 84% are synthetically or artificially produced *(2)*.

Monoterpenes as substrates of microbial transformations have led to a great variety of oxyfunctionalized compounds. Allylic hydroxylation of monoterpene hydrocarbons is interesting because of the multiple bioac-

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tivities of many of the resulting aroma compounds *(3)*. Hydroxylation of monoterpene substrates has been exclusively achieved by the use of bacteria and filamentous fungi with no reference to the use of yeasts. The use of yeasts for hydroxylation purposes of other substrates has, in fact, seldom been considered, because these unicellular fungi have been employed for reduction reactions and as a possible source of lipases *(4)*. Terpenes and especially their oxygenated derivates are extensively used by the flavor and fragrance industries, where about 2.8×10^5 kg of monoterpenes is consumed each year, and this amount is increasing *(5)*. The production of flavors via a biotechnological route offers several advantages. One important attribute of microbial biocatalysis is the ability to synthesize products that can be labeled as natural, if derived from natural substrates, and added to foods without being considered as additives *(6)*. Another important attribute is the conversion of monoterpene precursors or intermediates into their more aggregated value products for the flavor and fragrance industries *(1,5)*.

Pinenes (α- and β-) are major components of turpentine, a byproduct of the pulp-making industry. About 90% of turpentine produced in the United States is derived from the pulp and paper industry via chemical or thermomechanical processes. The main components of turpentine from the southeastern United States are α-pinene (70%) and β-pinene (25%). In relation to the available turpentine oil, which amounts to about 13,761 metric t produced in the United States, 20–25% is used by the flavor and fragrance industries. Owing to the low yield at present in the microbiologic production of terpenes, the process cannot compete economically with the recovery of terpenes from essential oils. Recently, efforts to improve the conversion yield of pinenes and limonene into other compounds have been reported *(7)*. α-Pinene and β-pinene are also low-priced monoterpenes, commonly used as substrates for the chemical synthesis of nature-identical monoterpenoid odorants. These syntheses are often multistep and laborious *(8)*.

α-Terpineol ($C_{10}H_{18}O$) is the most important monocyclic monoterpene alcohol. The annual consumption of α-terpineol has been estimated at more than 13,000 kg, which places it among the top 30 commonly used flavor compounds *(9)*. From the terpene alcohols, α-terpineol is an important flavor and fragrance chemical because of its lilac odor *(1,5)*. α-Terpineol is widely distributed in nature and is one of the most commonly used perfume chemicals *(10)*. It is mainly produced chemically, starting from pinene or crude turpentine oil by acid hydration to terpine, followed by partial dehydration *(1,9)*. In this way, α-terpineol is commercially available at relatively low price. Therefore, a microbial process must ensure high yields of α-terpineol in order to be competitive *(1)*. Figure 1 presents the chemical structures of (–)β-pinene end α-terpineol.

The main difficulty related to the use of microbial transformations is associated with the low yield obtained in the reactions, and also to the necessity of specific laboratory equipment and training on microbial techniques.

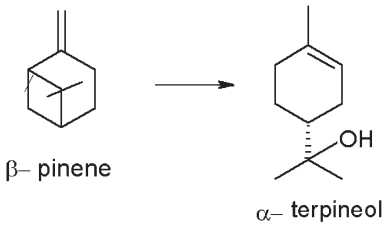

Fig. 1. Chemical structures of (–)β–pinene and α-terpineol.

Determination of the conditions for the overall biotransformation process enables conduction of experiments in a more useful and faster way, avoiding degradation and/or overmetabolization of a specific product *(11)*.

In this sense, the present work reports the bioconversion of (–)β-pinene into α-terpineol by the fungus *Aspergillus niger*. The addition of a diluted solution of substrate in ethanol, sequential addition of substrate, and adaptation of the precultures with the addition of small amounts of substrate are discussed.

Materials and Methods

Strain

A. niger ATCC 9642 was obtained from the culture collection of Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil.

Chemicals

(–)α-Pinene (98%) and (+)limonene (95%) were purchased from Aldrich and (–)β-pinene (97%) from Spectrum. All the substrates were used as received. All other chemicals or solvents were of commercial grade.

Cultivation

The growth medium (YM) was consisted of 3 g/L yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 10 g/L of glucose dissolved in distilled water. The pH was adjusted to 6.0 with 0.1 *M* NaOH prior to sterilization.

Spore Counting

Spore counting was performed by using a solid culture medium (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 10 g/L of glucose, and 15 g/L of agar [YMA] in distilled water) with different supplements. The supplements used were vitamin solution (100 g/L of nicotinic acid, 0.2 g/L of ρ-aminobenzoic acid, 0.2 mg/L of biotin, 50 mg/L of peridoxin, 100 mg/L of riboflavin in deionized water, aseptically filtered and stored at 4°C) and traces of $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$. The pH was adjusted to 5.0, 6.0, 7.0, and 8.0 (0.1 *M* NaOH and 25% HCl); thirty percent H_2O_2 (23 and 50 m*M*) was used for enrichment of the medium with O_2. This step

was performed in a germination chamber at 25°C for 7 d. The microorganisms were cultivated in inclined solid medium in an assay tube. Five milliliters of a Tween solution (1% [w/v]) was added to the tube, and cell counting was accomplished in a Carl Zeiss Standard 20 microscope. All experimental counts were carried out in duplicate.

Preparation of Inoculum

For preparation of inoculum, several loopfuls of cells stored on mineral medium agar (YMA) slants were aseptically transferred to test tubes (18 × 1.5 cm) containing 5 mL of mineral medium (YM). Four days after inoculation, the medium was transferred to flasks containing 50 mL of medium. The culture was aerobically incubated in a shaker (150 rpm) at 25°C for 3 d.

Cell Induction

For the experiments in which cells were induced, 50 µL of substrate was added to the precultures in six subsequent additions of 8.33 µL in 5 mL of medium in test tubes every 12 h, started 24 h after inoculation. This step was performed in a germination chamber. Twelve hours after the last addition of the substrate, the medium was transferred to flasks containing 50 mL of medium. The culture was aerobically incubated in a shaker (150 rpm) at 25°C for 3 d.

Biotransformation Experiments

Biotransfomations were started 3 d after inoculation by adding 500 µL of substrate directly into the culture flasks with 50 mL of medium. In some experiments, the substrate was added in five subsequent additions of 100 µL each 24 h.

The tested substrates were added as solutions in absolute ethanol (1 mL of substrate/mL of EtOH). All experiments were conducted in duplicate. Experiments were also run with control flasks, which contained sterile culture broth that was not inoculated. The flasks were closed with a glass stopper in order to avoid substrate and product evaporation.

Extraction of Products

At the end of the experiments, the cells were removed by filtration. The product recovery was performed by liquid-liquid extraction with 3 × 20 mL of ethyl acetate (Et_2O). The final solution was dried over anhydrous sodium sulfate.

Identification of Biotransformation Products

The reaction products were identified by gas chromatography/mass spectroscopy (GC/MS) (Shimadzu QP5050A), using a DB5 capillary column

(30 m × 0.25 mm × 25 mm). The column temperature was programmed at 50°C for 3 min, increased at 5°C/min at 130°C, and then increased at 15°C/min at 220°C by 5 min. Helium was the carrier gas, and the injection and detector temperatures were 250 and 300°C, respectively. One microliter of the dried solution was injected into the GC/MS system. The apparatus operated with a flow rate of 1 mL/min in an electronic impact mode of 70 eV and in split mode (split ratio of 1:10).

The compounds were identified by comparing the mass spectra with those from the Wiley library and by additional comparison of the GC retention time of standard compounds.

Results and Discussion

Table 1 presents the spore-counting results for all tested experimental conditions. It can be observed that maximum growth of the microorganism was obtained when a standard medium with the pH adjusted to 6.0 was used. The use of the vitamin solution did not affect the spore numbers when compared to standard medium. When using traces of Fe and Zn, the cell growth was affected negatively, with a decrease in the number of spores of 29 and 32%, respectively, in relation to the standard medium. The addition of H_2O_2 decreased the number of spores related to that obtained with the standard medium. Previous studies using recombinant *A. niger* indicated that oxidative stress caused by either gassing with O_2-enriched air or the addition of H_2O_2 in chemostat cultures could have a significant effect on heterologous protein synthesis, and that under enriched O_2 conditions this effect was mediated via a morphologic shift in response to a hyperoxidant state in the fungal hyphae *(12,13)*. Bai et al. *(14)*, using a recombinant *A. niger* in batch fermentation and chemostat cultures, found different effects on intracellular protein synthesis and secretion of recombinant hen egg white lysozyme with sparging of O_2-enriched air. For batch cultures, the O_2-enriched air (25 or 50%) did not increase the synthesis of heterologous proteins, but with the addition of 75% oxygen enrichment, a decrease in maximum hen egg white lysozyme concentration was observed. In the present work, under operation in batch mode, the addition of H_2O_2 produced a negative effect on the reaction conversion.

Sequential Addition of Substrate

In the experimental condition investigated, *A. niger* was able to convert only (–)β-pinene into α-terpineol. Figure 2 presents a typical chromatogram obtained in the biotransformation of (–)β-pinene to α-terpineol by *A. niger*.

The literature points out that one of the problems commonly found in the biotransformation of monoterpenes is the toxicity of these compounds for microorganisms *(5)*. The addition of substrate in high concentrations

Table 1
Number of Spores (*n*) of *A. niger* ATCC 9642 After 7 d of Growth Using YM Medium with Different Supplements

YMA standard medium:	$n/10^9$ [a]
YMA at pH 6.3 (standard)	2.5 ± 0.4
YMA at pH 5.0	2.3 ± 0.4
YMA at pH 6.0	3.6 ± 0.6
YMA at pH 7.0	1.6 ± 0.3
YMA at pH 8.0	1.8 ± 0.3
YMA with addition of a vitamin solution	2.2 ± 0.4
YMA with addition of traces of Fe	1.8 ± 0.3
YMA with addition of traces of Zn	0.8 ± 0.1
YMA with addition of 50 m*M* solution of 30% H_2O_2	1.9 ± 0.3
YMA with addition of 23 m*M* solution of 30% H_2O_2	1.7 ± 0.3

[a]Average value ± SD from duplicate counts performed.

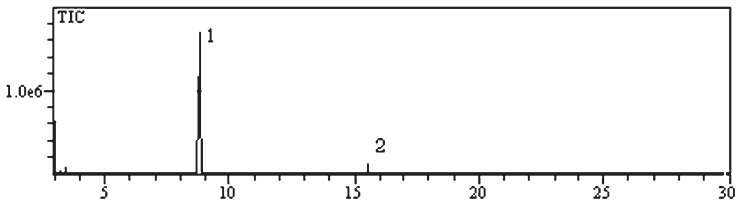

Fig. 2. Typical chromatogram obtained in biotransformation of (–)β-pinene to α-terpineol by *A. niger*.

can lead to inhibition. On the other hand, a significant amount of the added substrate is often lost owing to evaporation. To provide the fungal cells with appropriate amounts of substrate at each time, the effect of a sequential addition of the substrate was tested. Starting with 3-d-old growing cultures cultivated in 50 mL of YM medium, the biotransformation was performed by adding (–)β-pinene in two different ways: one (500 µL) or five subsequent additions (100 µL every 24 h). Table 2 presents the results obtained in this step. The best results were obtained when the substrate was partially added to the reaction medium and with no induced cells. Some works in the literature point out the substrate induction of cytochrome P-450 monooxygenases for several fungi, transforming polycyclic aromatic hydrocarbons or steroids *(3,5)*. The adaptation significantly increased the transformation activity of the fungus, but an improvement in the resistance to the toxic substrate was not observed, as shown by the decrease in dry matter after the addition of substrate. In this sense, the adaptation mechanism of the fungus seems to play a minor role in this fungus, and an induction of the terpene transforming enzymes might be the main effect *(3)*.

Table 2
Experimental Conversions Obtained in Biotransformation of (–)β-Pinene by *A. niger*

Substrate addition	Cell induction	Ethanol addition	Conversion (%)[a]
One step	With	With	0.20 ± 0.10
	Without	Without	1.80 ± 0.01
	Without	With	0.27 ± 0.05
	With	Without	0.49 ± 0.23
Five step	With	With	0.83 ± 0.48
	Without	Without	0.26 ± 0.01
	Without	With	3.68 ± 0.11
	With	Without	0.40 ± 0.31

[a]The conversion was obtained in relation to the normalized peak areas.

Influence of Cosolvent

To improve the availability of water-insoluble substrate for the biocatalyst, present in the water phase, a cosolvent (EtOH) was used. This water-miscible solvent is applied to increase the solubility of the substrate in the medium, which could decrease the mass transfer limitations, leading to a higher bioconversion rate.

Tan and Day (*9*) investigated the effect of organic cosolvents on the bioconversion of (*R*)-(+)-limonene into (*R*)-(+)-α-terpineol by *Penicillium digitatum*. They found that ethanol caused inhibition of the bioconversion at a concentration of 2% (v/v). Methanol showed a positive influence on the bioconversion, with an optimal concentration of 0.5% (v/v), but caused cytotoxic effects at concentrations ≥2%. The results obtained in the present work (Table 2) showed that EtOH had a positive effect on the bioconversion when applied in a concentration of 1% (v/v). When the addition of substrate was performed in ethanol solution in five steps using no inducted cells, 3.68% conversion in α-terpineol was obtained.

Conclusion

The results obtained in this work indicated that optimum growth of microorganism (*A. niger*) was obtained when using the standard medium (YMA) at pH 6.0. The addition of a vitamin solution presented similar results when compared to standard medium and, hence, did not influence the growth of microorganism. On the other hand, the addition of Fe, Zn, and H_2O_2 solutions seemed to inhibit cell growth.

The *A. niger* fungus was not able to biotransform (–)α-pinene and (+)limonene in the experimental conditions investigated. On the other hand, (–)β-pinene conversions of about 4% were achieved. Higher conversions of (–)β-pinene into α-terpineol catalyzed by *A. niger* were obtained when sequential addition of substrate was used. When the substrate was

applied as a diluted solution in EtOH, the best results were obtained. The induction did not affect the biotransformation process. The results obtained in the present work show that the addition of 1% ethanol had a positive effect on bioconversion.

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References

1. Adams, D., Demyttenaere, J. C. R., and Kimpe, N. (2003), *Food Chem.* **80,** 525–534.
2. Yoo, S. K., Day, D. F., and Cadwallader, K. R. (2001), *Process Biochem.* **36,** 925–932.
3. Onken, J. and Berger, R. G. (1999), *J. Biotechnol.* **69,** 163–168.
4. Van Rensburg, E., Moleleki, N., van der Walt, J. P., Botes, P. J., and van Dyk, M. S. (1997), *Biotechnol. Lett.* **19,** 779–782.
5. Tan, Q., Day, D. F., and Cadwallader, K. R. (1998), *Process Biochem.* **33,** 29–37.
6. Tecelão, C. S. R., Van Keulen, F., and da Fonseca, M. M. R. (2001), *J. Mol. Catal. B: Enzymat.* **11,** 719–724.
7. Yoo, S. K. and Day, D. F. (2002), *Process Biochem.* **37,** 739–745.
8. Van Dyk, M. S., Van Rensburg, E., and Moleleki, N. (1998), *Biotechnol. Lett.* **20,** 431–436.
9. Tan, Q. and Day, D. F. (1998), *Process Biochem.* **33,** 755–761.
10. Demyttenaere, J. C. R., Van Belleghem, K., and de Kimpe, N. (2001), *Phytochemistry* **37,** 199–208.
11. Takahashi, J. A., de Barroso, H. A., and de Oliveira, A. B. (2000), *Braz. J. Microbiol.* **31,** 10–21.
12. Kreiner, M., McNeil, B., and Harvey, L. M. (2003), *J. Biotechnol.* **100(3),** 251–260.
13. Wongwicharn, A., McNeil, B., and Harvey, L. M. (1999), *Biotechnol. and Bioeng.* **65,** 416–424.
14. Bai, Z., Harvey, L. M., White, S., and McNeil, B. (2004), *Enzyme Microb. Technol.* **34,** 83–86.