Influence of Culture Conditions on the Biotransformation of (+)-Limonene by *Aspergillus niger*

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The influence of the cultivation system and of the culture medium on the biotransformation of (+)-limonene by a strain of *Aspergillus niger* was investigated. Biooxidation products were obtained in all conditions tested. Using a laboratory bioreactor, six terpenes were identified in every medium, predominantly terpineols and carveols, whereas terpinen-4-ol and perillyl alcohol were the only terpenes found when flasks were used for culture. Perillyl alcohol and carveols predominated when the medium was tryptic soy broth (TSB), whereas the formation of terpineols was clearly favoured in malt broth (MB). Thus, there was a marked influence of the culture conditions on the results of the biotransformation. Changes in the conditions led to variations both in the type and relative amount of products obtained.

Key words: Limonene, Fungal Biotransformation, *Aspergillus niger*

Introduction

Limonene [1-methyl-4-(1-methylethenyl)-cyclohexene] is the most widespread terpene in nature and is formed by more than 300 plant species (van der Werf *et al.*, 1999). (4*R*)-(+)-Limonene is the most widely distributed form, and it is the major constituent of citrus peel essential oils, in which it is usually found at contents between 90% and 96% (Badee *et al.*, 2011). Nevertheless, the major contribution to citrus flavour is due to the minor oxygenated constituents rather than limonene (Ahmed *et al.*, 1978). World production of citrus and its derivates (essential oils among others) increased significantly in the last decades [Food and Agriculture Organization of the United Nations (FAO), 2006]. The yield of (+)-limonene separated from cold-pressed citrus peel oil was estimated at 36,000 tons per year (Krings and Berger, 2010). Due to its low sensory activity, low water solubility, and tendency to autoxidize and polymerize, it is usually rectified from the oil and regarded as a processing waste. The essential citrus oils, from which some of the undesirable components (usually limonene) have been removed by high vacuum distillation, are called "folded" or "concentrated" oils (Schmidt, 2010). These properties, in conjunction with their high structural similarity to high-value oxyfunctionalized derivatives, turn limonene into an interesting starting material for microbial transformations.

The first reports on the biocatalytic conversion of limonene are from the 1960s (Dhavalikar and Bhattacharyya, 1966; Dhavalikar *et al.*, 1966). Since then, several investigations related to microorganisms, plant cells, enzymes, and microalgae capable of transforming limonene to many oxyfunctionalized derivatives have been carried out (Maróstica Jr. and Pastore, 2009). In the last decade, at least two reviews on limonene biotransformations were published (Duetz *et al.*, 2003; Maróstica Jr. and Pastore, 2009). Among microorganisms, several fungi have shown the ability to metabolize limonene into various derivatives (Erasto and Viljoen, 2008). The fungal biooxidation of limonene can occur at virtually all carbon atoms in the structure, the unactivated methylene carbon atom

C-5 being the only one for which no derivatization has been reported. Although not directly proven in all cases, all bioconversions of limonene by yeasts and fungi seem to be initiated by P-450 monooxygenases (Duetz *et al.*, 2003). The first successful fungal bioconversion was reported in the late sixties using *Cladosporium* species (Kraidman *et al.*, 1969). Since then, several developments in the use of fungal species as limonene-metabolizing agents have been reported.

The influence of nutritional and environmental parameters on the growth of microorganisms is well known, affecting the number of viable cells per unit volume (Roberts *et al.*, 1995). Variations in these parameters can lead to morphological changes in the fungus, concomitant with variations in its metabolism (Žnidaršič and Pavko, 2001; Calvo *et al.*, 2002). Therefore, variations in growth conditions could potentially lead to different biotransformation products. It is possible that, under different conditions, the same microorganism effects different biotransformations of a given substrate, either in the amount or type of products obtained. However, the literature on fungal limonene biotransformations rarely reports the influence of culture conditions. When this is the case, usually variations in the percentage of bioconversion are reported, but not variation in the type of products obtained. In this work we report the influence of the cultivation system and the culture medium on the biotransformation of limonene by a strain of *Aspergillus niger* previously isolated from contaminated orange peel.

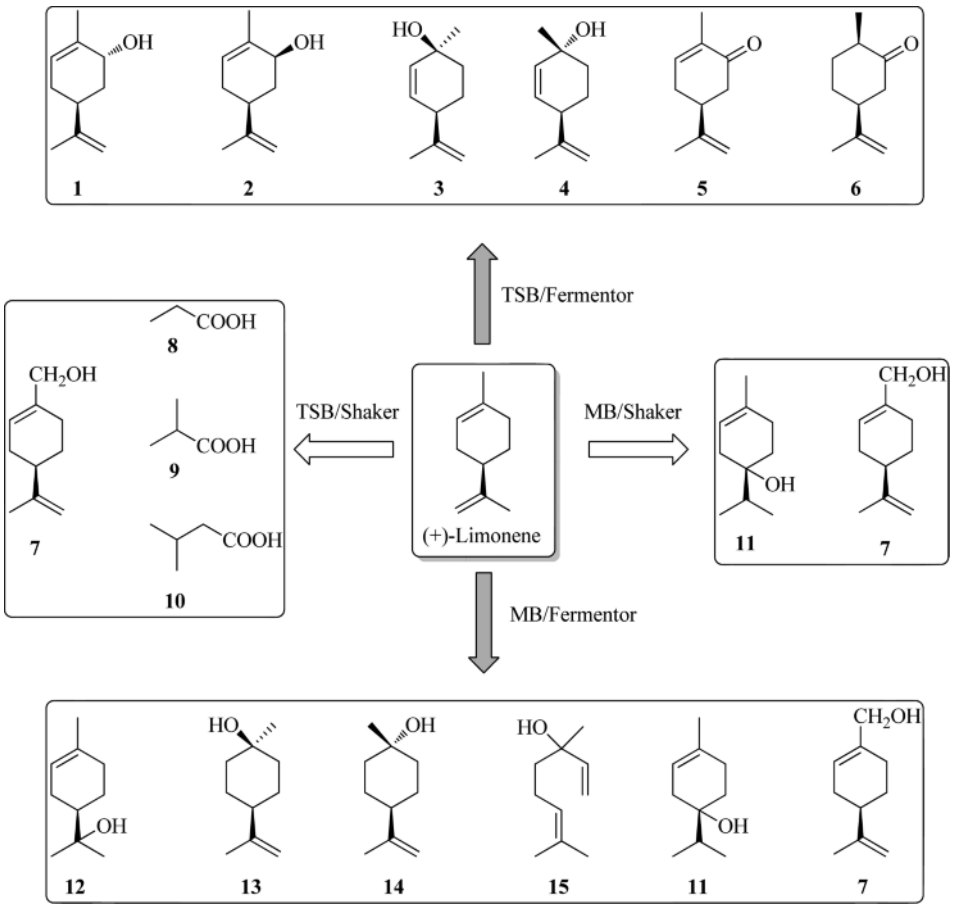
Results and Discussion

Biotransformation products were obtained in all conditions tested, and a total of 15 compounds were identified (Scheme 1). Although *A. niger* is the most frequently used fungal biocatalyst (Ward *et al.*, 2006), reports of successful bioconversions of (+)-limonene with this fungus are very scarce (Rama Devi and Bhattacharyya, 1978; Toniazzo *et al.*, 2006). Several authors found that either *A. niger* was not able to convert limonene (Demyttenaere *et al.*, 2001; Chatterjee and Bhattacharyya, 2001; Toniazzo *et al.*, 2005; Rozenbaum *et al.*, 2006; Rottava *et al.*, 2010) or yield was practically nil (Kaspera *et al.*, 2005; Divyashree *et al.*, 2006). In these reports, different culture media, substrate concentrations, and forms of the biocatalyst were used, but all studies, with the exception of that of Divyashree *et al.* (2006), were conducted with strains from culture collections. This is not the case for the results presented here, as the microorganism used was isolated from a natural environment rich in the substrate. This fact seems essential for the success of the biotransformation, since adaptation to a potentially toxic substrate is likely to have occurred, counteracting the membrane damage caused by limonene (Onken and Berger, 1999).

Table I presents the percentages of the bioconversion products, obtained by applying the method of normalization of areas to the chromatograms obtained by gas chromatography (GC) and correcting the values of areas by calculating relative response factors according to the model proposed by Tissot *et al.* (2012). In each trial we obtained more than one product, and monohydroxylated products comprised nearly 80 % of total products obtained. The accumulation of multiple compounds (*e. g.*, hydroxy limonene isomers) may be due to either the presence of multiple oxygenases with different regiospecificities or of a single enzyme displaying incomplete regiospecificity (Duetz *et al.*, 2003).

Fungi growing either in flasks in a shaker or in a fermentor gave different types and yields of biotransformation products. As a rule, biotransformation in flasks resulted in fewer products. Terpinen-4-ol [in malt broth (MB)] and perillyl alcohol [both in MB and tryptic soy broth (TSB)] were the only terpenes found in this system. We also obtained short-chain organic acids when working with flasks and TSB, which may have resulted from the catabolic use of monoterpene by the microorganism, a fact that had been previously reported (Menéndez *et al.*, 2002) and was confirmed in the present work. When the reaction was carried out in a fermentor, six terpenes were identified in each medium, tertiary alcohols predominating in MB (over 80 % of the products were terpineols) and secondary alcohols in TSB (60 % were carveols). A striking influence of the nature of the system on the outcome of the biotransformation is the high production of terpinen-4-ol in MB in a shaker compared with its production in a fermentor in the same growth medium. Interestingly, terpinen-4-ol is a rarely reported metabolite of limonene biotransformation (Bowen, 1975; Menéndez *et al.*, 2000; Kaspera *et al.*, 2005) which, in this particular combination of reaction conditions, represented almost 50 % of the transformation products.

Cultures in flasks in a shaker or in a fermentor, respectively, differ in the aeration and stirring conditions that influence the fungal growth and, therefore, enzyme production and specificity. In this context, qualitative changes in the biotransformation profile could be expected, but studies using different types of biore-



Scheme 1. Biotransformation products of (+)-limonene: *trans*-carveol (**1**), *cis*-carveol (**2**), *cis*-*p*-mentha-2,8-dien-1-ol (**3**), *trans*-*p*-mentha-2,8-dien-1-ol (**4**), carvone (**5**), dihydrocarvone (**6**), perillyl alcohol (**7**), propanoic acid (**8**), isobutanoic acid (**9**), isopentanoic acid (**10**), terpinen-4-ol (**11**), α-terpineol (**12**), *cis*-β-terpineol (**13**), *trans*-β-terpineol (**14**), linalool (**15**). Shaker and fermentor correspond to the cultivation system, while TSB (tryptic soy broth) and MB (malt broth) are the culture media.

actors for fungal bioconversion of limonene reported only quantitative, rather than qualitative, changes in the products (Tan and Day, 1998; Kaspera *et al.*, 2005; Pescheck *et al.*, 2009).

The culture medium also played a role in limonene biotransformation, as significant differences between products formed in MB and TSB were observed. The allylic hydroxylation of the methyl (perillyl alcohol) and methylene groups (carveols) predominated in TSB, while the formation of terpineols (stoichiometrically equivalent to the addition of water to limonene) was clearly favoured in MB. The regioselectivity of terpineol production also changed with the cultivation system, as pointed out above. According to the literature, mainly quantitative variations in the biotransformation products of limonene have been reported for different fungal growth media (Adams *et al.*, 2003; Bicas *et al.*, 2008; Badee *et al.*, 2011). A more recent report noted that the culture medium can affect both specificity and product concentration in the biotransformation of limonene with a strain of *Penicillium digitatum* (Prieto *et al.*, 2011).

The quantitative variation in biotransformation products reported in the literature (Tan and Day, 1998; Adams *et al.*, 2003; Kaspera *et al.*, 2005; Bicas *et al.*, 2008; Pescheck *et al.*, 2009; Badee *et al.*, 2011) can be explained by the influence of nutritional and environmental parameters, which may affect both the number of viable cells, and the amount and activity of the relevant enzymes catalyzing the biotransforma-

Table I. Percentages of bioconversion products under the different culture conditions.

Product	Cultivation system			
	TSB		MB	
	Fermentor	Shaker	Fermentor	Shaker
Perillyl alcohol (**7**)	–	19.0	5.0	39.9
trans-Carveol (**1**)	31.8	–	–	–
cis-Carveol (**2**)	27.9	–	–	–
Terpinen-4-ol (**11**)	–	–	4.9	42.8
α-Terpineol (**12**)	–	–	58.4	–
cis-β-Terpineol (**13**)	–	–	13.4	–
trans-β-Terpineol (**14**)	–	–	4.9	–
cis-*p*-Mentha-2,8-dien-1-ol (**3**)	8.0	–	–	–
trans-*p*-Mentha-2,8-dien-1-ol (**4**)	8.0	–	–	–
Linalool (**15**)	–	–	7.3	–
Carvone (**5**)	2.5	–	–	–
Dihydrocarvone (**6**)	2.7	–	–	–
Propanoic acid (**8**)	–	15.2	–	–
Isobutanoic acid (**9**)	–	37.7	–	–
Isopentanoic acid (**10**)	–	26.4	–	–

TSB, tryptic soy broth; MB, malt broth.

tion (Roberts *et al.*, 1995). Moreover, Cornelissen *et al.* (2011) have recently shown that, irrespective of the kinetics of the respective enzyme, the efficacy of a cell-based biocatalytic reaction is largely determined by the physiology of the used microbial cells. In the present work, higher yields of biomass were obtained in MB, both in shaken flasks and in the biofermentor. In this medium, a dry weight of 13.2 mg/mL was obtained in flasks vs. 7.2 mg/mL in the fermentor, as compared to 7.4 and 5.3 mg/mL, respectively, in TSB. However, visually the pellets grown in MB were smaller than those grown in TSB.

Qualitative changes in the products obtained can be attributed to several causes. Limonene is a small molecule with groups of similar electronic properties, which can be biooxidized at different carbon atoms. On the other hand, as mentioned by Žnidaršič and Pavko (2001), a dynamic relationship exists between environmental conditions and the growth pattern of filamentous fungi in submerged cultures. Distinct cultivation conditions could result in different morphological and physico-chemical characteristics of fungal hyphal elements. This results in cellular differentiation in terms of biochemical specialization and structural changes, which could lead to a variety of specific metabolic patterns. This is clear in the production of secondary metabolites by fungi (Calvo *et al.*, 2002), and also appears important in biotransformation processes carried out by such microorganisms, according to the results obtained in the present work.

In conclusion, there is a clear influence of the culture conditions, *i. e.* both the nutrient medium and the geometry of the culture system, on the results of the biotransformation. Changes in these bioprocess parameters led to variations both in the type and relative amount of the products obtained.

Experimental

Microorganism and inoculum

An *Aspergillus niger* strain was isolated from orange peels and maintained as previously reported (Menéndez *et al.*, 2002). For identification of this strain, a culture was grown on Czapek yeast extract agar (CYA) at 5 °C, 25 °C, and 37 °C, and malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) at 25 °C. All plates were incubated for 7 d. Fungal identification was done according to Pitt and Hocking (1999). A spore suspension in sterile normal saline was used as inoculum, reaching a final concentration of 10^5 spores/mL in the respective culture medium. The suspension was prepared from a fresh culture after 72 h of growth.

Chemicals

R-(+)-Limonene (~99%) was purchased from Fluka (Buchs, Switzerland). α-Terpineol (95%), carveol (97%, mixture of *cis*- and *trans*-isomers), perillyl alcohol (96%), carvone (96%), and linalool (97%), as well as propanoic, isobutanoic, and isopentanoic acids, respectively, were obtained from Sigma-Aldrich (St. Louis, MO, USA). A mixture of C_9

to C_{20} *n*-alkanes prepared with standards obtained from Fluka and Sigma-Adrich was used for determination of Kováts retention indices. Technical grade dichloromethane was distilled prior to use. Merck (Darmstadt, Germany) 60 silica gel (230 – 400 mesh) was used for sample clean-up.

Cultivation system and culture media

Biotransformation experiments were carried out in two systems: (i) 1-L conical flasks in an orbital shaker (IOC400.XX2.C; Sanyo, Tokyo, Japan), and (ii) a 3-L fermentor (BIOFLO III batch/continuous fermentor; New Brunswick Scientific, Enfield, CT, USA).

The culture media used were tryptic soy broth (TSB) (DIFCO, Detroit, MI, USA) and malt broth (MB) prepared as a 3% solution of malt extract (Amresco, Solon, OH, USA) in distilled water.

Biotransformation assays

A total of four biotransformation experiments were carried out. Two of these were conducted in the fermentor with both TSB and MB as culture media, under the following conditions: agitation, 150 rpm; volume, 1.5 L; aeration rate, 0.5 vvm. The other two trials were conducted in flasks in a shaker with the same culture media (0.2 L), at 100 rpm. All incubations were for 5 d at 28 °C. (+)-Limonene was added once after 48 h of incubation such as to obtain a content of 0.5% (v/v) in the culture media. All experiments were conducted in duplicate. Two negative controls were

Table II. Kováts retentions indices (KRI) and MS data of identified biotransformation products.

Compound	KRI[a]	KRI[b]	MS: m/z (rel. int.)
Perillyl alcohol[c]	1295	1294	152 [M^+] (5), 134 (10), 121 (38), 109 (18), 93 (48), 79 (79), 68 (88), 55 (57), 41 (100)
cis-Carveol[c]	1229	1229	152 [M^+] (1), 134 (37), 119 (24), 109 (51), 91 (25), 84 (83), 69 (43), 55 (68), 41 (100)
trans-Carveol[c]	1217	1216	152 [M^+] (6), 137 (8), 134 (4), 119 (14), 109 (100), 91 (26), 84 (91), 69 (33), 55 (56), 41 (94)
Carvone[c]	1243	1240	150 [M^+] (1), 135 (3), 122 (2), 108 (30), 93 (31), 82 (100), 54 (68), 41 (39)
cis-Dihydrocarvone	1193	1184	152 [M^+] (10), 137 (10), 121 (12), 109 (29), 95 (56), 81 (37), 67 (93), 55 (47), 41 (100)
Terpinen-4-ol	1177	1181	154 [M^+] (6), 136 (7), 111 (33), 93 (33), 86 (16), 71 (100), 55 (33), 43 (93)
α-Terpineol[c]	1189	1193	136 [$M^+ - H_2O$] (15), 121 (19), 107 (3), 93 (31), 81 (21), 67 (14), 59 (100), 55 (13), 43 (59)
cis-β-Terpineol	1144	1151	136 [$M^+ - H_2O$] (11), 121 (12), 107 (14), 93 (28), 79 (12), 71 (50), 55 (23), 43 (100)
trans-β-Terpineol	1163	1160	136 [$M^+ - H_2O$] (15), 121 (12), 107 (16), 93 (19), 79 (13), 71 (41), 55 (21), 43 (100)
trans-*p*-Mentha-2,8-dien-1-ol	1123	1119	152 [M^+] (2), 137 (14), 134 (6), 121 (19), 109 (30), 94 (37), 91 (20), 79 (39), 67 (17), 55 (17), 43 (100)
cis-*p*-Mentha-2,8-dien-1-ol	1138	1134	152 [M^+] (1), 137 (18), 134 (30), 119 (17), 109 (34), 91 (19), 79 (35), 67 (17), 55 (18), 43 (100)
Linalool[c]	1097	1106	136 [$M^+ - H_2O$] (3), 121 (6), 93 (31), 80 (16), 71 (57), 55 (56), 43 (100)
Propanoic acid[c]	–	Nd[d]	74 [M^+] (72), 57 (29), 45 (100), 44 (18)
Isobutanoic acid[c]	–	Nd	88 [M^+] (4), 73 (20), 55 (4), 43 (100)
Isopentanoic acid[c]	–	Nd	87 (21), 74 (61), 60 (84), 57 (37), 41 (100)

[a] KRI reported by Adams (2007) for an SE52 stationary phase.
[b] Experimental KRI obtained in the conditions mentioned above.
[c] Products identified by comparison of retention times with those of standard compounds.
[d] Nd, not determined.

performed, one using (+)-limonene in culture medium (without inoculum), and in the other the medium was inoculated with the fungus but no substrate was added. For determination of biomass production, the four trials were repeated under the same conditions. Biomass yields were determined after filtration and drying to constant weight.

Extraction and identification of bioconversion products

The liquid medium was separated from the biomass by filtration and then was extracted with CH_2Cl_2. The mycelia were washed several times with the same solvent. Organic phases were combined, then dried over anhydrous Na_2SO_4, and concentrated under reduced pressure at room temperature. Concentrated extracts were cleaned up on a silica gel column eluted with CH_2Cl_2, as optimized earlier in the laboratory.

The bioconversion products were identified by comparing their Kováts retention indices (KRI) with those reported in the literature (Adams, 2007), and their GC retention times with those of standard compounds whenever it was possible (Table II). Comparison of fragmentation patterns in the mass spectra with those stored in the GC-MS databases (McLafferty and Stauffer, 1991; Adams, 2007) was also performed.

Analysis conditions

High-resolution GC (HRGC) analyses were performed on a Shimadzu (Kyoto, Japan) GC14B instrument equipped with an FID and EZ Chrom integration software for data processing. An SE-52 fused silica capillary column (30 m × 0.32 mm i.d., 0.40 – 0.45 μm film thickness) was used. The temperature program was as follows: 60 °C for 8 min; 60 – 210 °C at 3 °C/min. The GC conditions were as follows: injector temperature, 280 °C; detector temperature, 290 °C; carrier gas, N_2 at 49 kPa; split ratio, 1:50.

HRGC-MS was carried out using a Shimadzu QP 5500 instrument under the conditions described above, with: carrier gas, He; ionization voltage, 70 eV; temperature interface, 250 °C; acquisition mass range, m/z 40 – 400.

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Adams R. P. (2007), Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy, 4th ed. Allured Publishing, Carol Stream, IL, USA.

Adams A., Demyttenaere J. C. R., and De Kimpe N. (2003), Biotranformation of (*R*)-(+)- and (*S*)-(–)-limonene to α-terpineol by *Penicillium digitatum* – Investigation of the culture conditions. Food Chem. **80**, 525 – 534.

Ahmed E. M., Dennison R. A., Dougherty R. H., and Shaw P. E. (1978), Flavor and odor thresholds in water of selected orange juice components. J. Agric. Food Chem. **26**, 187 – 191.

Badee A. Z. M., Helmy S. A., and Morsy N. F. S. (2011), Utilisation of orange peel in the production of α-terpineol by *Penicillium digitatum* (NRRL 1202). Food Chem. **126**, 849 – 854.

Bicas J. L., Barros F. F. C., Wagner R., Godoy H. T., and Pastore G. M. (2008), Optimization of *R*-(+)-α-terpineol production by the biotransformation of *R*-(+)-limonene. J. Ind. Microbiol. Biotechnol. **35**, 1061 – 1070.

Bowen E. R. (1975), Potential by-products from microbial transformations of D-limonene. P. Fl. St. Hortic. Soc. **88**, 304 – 308.

Calvo A. M., Wilson R. A., Bok J. W., and Keller N. P. (2002), Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66**, 447 – 459.

Chatterjee T. and Bhattacharyya D. K. (2001), Biotransformation of limonene by *Pseudomonas putida*. Appl. Microbiol. Biotechnol. **55**, 541 – 546.

Cornelissen S., Liu L., Deshmukh A. T., Schmid A., and Bühler B. (2011), Cell physiology rather than enzyme kinetics can determine the efficiency of cytochrome P450-catalyzed C–H-oxyfunctionalization. J. Ind. Microbiol. Biotechnol. **38**, 1359 – 1370.

Demyttenaere J. C. R., Van Belleghem K., and De Kimpe N. (2001), Biotransformation of (*R*)-(+)- and (*S*)-(–)-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry **57**, 199 – 208.

Dhavalikar R. S. and Bhattacharyya P. K. (1966), Microbiological transformations of terpenes: Part VIII – Fermentation of limonene by a soil Pseudomonad. Indian J. Biochem. **3**, 144 – 157.

Dhavalikar R. S., Rangachari P. N., and Bhattacharyya P. K. (1966), Microbiological transformations of terpenes: Part IX – Pathways of degradation of limonene in a soil Pseudomonad. Indian J. Biochem. **3**, 158 – 164.

Divyashree M. S., George J., and Agrawal R. (2006), Biotransformation of terpenic substrates by resting cells of

Aspergillus niger and *Pseudomonas putida* isolates. J. Food Sci. Technol. **43**, 73 – 76.

Duetz W. A., Bouwmeester H., van Beilen J. B., and Witholt B. (2003), Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Appl. Microbiol. Biotechnol. **61**, 269 – 277.

Erasto P. and Viljoen A. M. (2008), Limonene – a review: Biosynthetic, ecological and pharmacological relevance. Nat. Prod. Commun. **3**, 1193 – 1202.

Food and Agriculture Organization of the United Nations (2006), Citrus: Statistics – fresh and processed citrus fruit. http://www.fao.org/es/ESC/esce/escr/citrus/citruse.htm.

Kaspera R., Krings U., Pescheck M., Sell D., Schrader J., and Berger R. G. (2005), Regio- and stereoselective fungal oxyfunctionalisation of limonenes. Z. Naturforsch. **60c**, 459 – 466.

Kraidman G., Mukherjee B. B., and Hill I. D. (1969), Conversion of D-limonene into an optically active isomer of α-terpineol by a *Cladosporium* species. Bacteriol. Proc. **69**, 63 – 67.

Krings U. and Berger R. T. (2010), Terpene bioconversion – How does its future look? Nat. Prod. Commun. **5**, 1507 – 1522.

Maróstica Jr. M. R. and Pastore G. M. (2009), Limonene and its oxyfunctionalized compounds: Biotransformation by microorganisms and their role as functional bioactive compounds. Food Sci. Biotechnol. **18**, 833 – 841.

McLafferty F. W. and Stauffer D. B. (1991), The Wiley/NBS Registry of Mass Spectral Data, 5th ed. Wiley, New York, USA.

Menéndez P., Rossini C., O'Neill S., Soubes M., Heinzen H., and Moyna P. (2000), Biotransformation of *R*-(+)-limonene by *Pseudomonas aeruginosa* and *Streptococcus faecalis*. An. Asoc. Quim. Argent. **88**, 79 – 82.

Menéndez P., García C., Rodríguez P., Moyna P., and Heinzen H. (2002), Enzymatic systems involved in D-limonene bio-oxydation. Braz. Arch. Biol. Technol. **45**, 111 – 114.

Onken J. and Berger R. G. (1999), Effects of *R*-(+)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus*. J. Biotechnol. **69**, 163 – 168.

Pescheck M., Mirata M. A., Brauer B., Krings U., Berger R. G., and Schrader J. (2009), Improved monoterpene biotransformation with *Penicillium* sp. by use of a closed gas loop bioreactor. J. Ind. Microbiol. Biotechnol. **36**, 827 – 836.

Pitt J. A. and Hocking A. D. (1999), Fungi and Food Spoilage, 2nd ed. Aspen Publisher Inc., Gaithersburg, MD, USA.

Prieto G. A., Perea J. A., and Ortiz C. C. (2011), Microbial biotransformation of (*R*)-(+)-limonene by *Penicillium digitatum* DSM 62840 for producing (*R*)-(+)-terpineol. Vitae **18**, 163 – 172.

Rama Devi J. and Bhattacharyya P. K. (1978), Molecular rearrangements in the microbiological transformations of terpenes and the chemical logic of microbial processes. J. Indian Chem. Soc. **55**, 1131 – 1137.

Roberts S. M., Turner N. J., Willets A. J., and Turner M. K. (1995), Introduction to Biocatalysis Using Enzymes and Micro-Organisms. Cambridge University Press, Cambridge, UK.

Rottava I., Toniazzo G., Cortina P. F., Martello E., Grando C. E., Lerin L. A., and Oestreicher E. G. (2010), Screening of microorganisms for bioconversion of (−)-β-pinene and *R*-(+)-limonene to α-terpineol. LWT – Food Sci. Technol. **43**, 1128 – 1131.

Rozenbaum H. F., Patitucci M. L., Antunes O. A. C., and Pereira Jr. N. (2006), Production of aromas and fragrances through microbial oxidation of monoterpenes. Braz. J. Chem. Eng. **23**, 273 – 279.

Schmidt E. (2010), Production of essential oils. In: Handbook of Essential Oils: Science, Technology, and Applications (Baser K. H. C. and Buchbauer G., eds.). CRC Press, Taylor & Francis Group, Boca Raton, FL, USA.

Tan Q. and Day D. F. (1998), Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*. Appl. Microbiol. Biotechnol. **49**, 96 – 101.

Tissot E., Rochat S., Debonneville C., and Chaintreau A. (2012), Rapid GC-FID quantification technique without authentic samples using predicted response factors. Flavour Fragr. J. **27**, 290 – 296.

Toniazzo G., De Oliveira D., Dariva C., Oestreicher E. G., and Antunes O. A. C. (2005), Biotransformation of (–)-β-pinene by *Aspergillus niger* ATCC 9642. Appl. Biochem. Biotechnol. **123**, 837 – 844.

Toniazzo G., Lerin L., De Oliveira D., Dariva C., Cansian R. L., Padilha F. F., and Antunes O. A. C. (2006), Microorganism screening for limonene bioconversion and correlation with RAPD markers. Appl. Biochem. Biotechnol. **132**, 1023 – 1033.

van der Werf M. J., Swarts H. J., and de Bont J. A. M. (1999), *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. Appl. Environ. Microbiol. **65**, 2092 – 2102.

Ward O. P., Qin W. M., Dhanjoon J., Ye J., and Singh A. (2006), Physiology and biotechnology of *Aspergillus*. Adv. Appl. Microbiol. **58**, 1 – 75.

Žnidaršič P. and Pavko A. (2001), The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. Food Technol. Biotechnol. **39**, 237 – 252.