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 \([1\text{-methyl}\cdot 4\text{-}(1\text{-methylene})\text{-cyclohexene}]\) is the most widespread terpene in nature and is formed by more than 300 plant species (van der Werf \textit{et al.}, 1999). \((4\text{R})\)-(+)\text{-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 \textit{et al.}, 2011). Nevertheless, the major contribution to citrus flavour is due to the minor oxygenated constituents rather than limonene (Ahmed \textit{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 \textit{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 \textit{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 mono-hydroxylated 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.

<table>
<thead>
<tr>
<th>Product</th>
<th>TSB Cultivation system</th>
<th>MB Cultivation system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perillyl alcohol (7)</td>
<td>31.8</td>
<td>27.9</td>
</tr>
<tr>
<td>trans-Carveol (1)</td>
<td>39.9</td>
<td>42.8</td>
</tr>
<tr>
<td>cis-Carveol (2)</td>
<td>27.9</td>
<td>31.8</td>
</tr>
<tr>
<td>Terpinen-4-ol (11)</td>
<td>27.9</td>
<td>4.9</td>
</tr>
<tr>
<td>α-Terpineol (12)</td>
<td>58.4</td>
<td>13.4</td>
</tr>
<tr>
<td>cis-β-Terpineol (13)</td>
<td>13.4</td>
<td>4.9</td>
</tr>
<tr>
<td>trans-β-Terpineol (14)</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>cis-p-Mentha-2,8-dien-1-ol (3)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>trans-p-Mentha-2,8-dien-1-ol (4)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Linalool (1S)</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Carvone (5)</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Dihydrocarvone (6)</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Propanoic acid (8)</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>Isobutanoic acid (9)</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>Isopentanoic acid (10)</td>
<td>26.4</td>
<td></td>
</tr>
</tbody>
</table>

TSB, tryptic soy broth; MB, malt broth.

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 10⁵ 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%), carvone (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 C₉*
to C$_{20}$-n-alkanes prepared with standards obtained from Fluka and Sigma-Aldrich 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.

| 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) |
| $\alpha$-Terpineol$^c$ | 1189            | 1193            | 136 [M$^+$ – H$_2$O] (15), 121 (19), 107 (3), 93 (31), 81 (21), 67 (14), 59 (100), 55 (13), 43 (59) |
| cis-$\beta$-Terpineol | 1144          | 1151            | 136 [M$^+$ – H$_2$O] (11), 121 (12), 107 (14), 93 (28), 79 (12), 71 (50), 55 (23), 43 (100) |
| trans-$\beta$-Terpineol | 1163          | 1160            | 136 [M$^+$ – H$_2$O] (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$_2$O] (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$^d$          | 88 [M$^+$] (4), 73 (20), 55 (4), 43 (100) |
| Isopentanoic acid$^c$ | –           | Nd$^d$          | 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$_2$Cl$_2$. The mycelia were washed several times with the same solvent. Organic phases were combined, then dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure at room temperature. Concentrated extracts were cleaned up on a silica gel column eluted with CH$_2$Cl$_2$ anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure at room temperature. Concentrated extracts were cleaned up on a silica gel column eluted with CH$_2$Cl$_2$, 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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