Biotransformation of Citral by *Botrytis cinerea*

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Biotransformation of citral (1) was studied with four strains of *Botrytis cinerea* using grape must (A), a synthetic medium (B) and mixtures of A and B. Whereas in A complete metabolization of 1 was achieved in B, neral (2) and geranial (3) were found to be one of the predominant volatile bioconversion products; in minor amounts (E,Z)-2,6-dimethyl-2,6-dien-1,8-diol (4) and (E,E)-2,6-dimethyl-2,6-dien-1,8-diol (5) and 2-methyl-2-hepten-6-one-1-ol (6), 2-methyl-2-hepten-6-ol (7), 2-methyl-2-hepten-6-one-1-ol (8) and 2-methyl-γ-butyrolactone (9) were identified. Using small amounts of A in B (1:700 to 5:700) low yields of 2 and 3 were obtained, whereas the quantities of 4, 5, 6 and 8 increased. Quantitatively, the results were strongly dependent on the strains used. The bioconversion products were all identified by capillary gas chromatography (HRGC) and coupled HRGC techniques, i.e., mass spectrometry (HRGC-MS) and Fourier transform infrared spectroscopy (HRGC-FIR).

**Introduction**

In the past, *Botrytis cinerea* has been recognized to be responsible for the degradation of terpenes observed in wines made from botrytized grapes [1, 2]. Recently, in bioconversion studies performed with linalool [3, 4] and citronellol [5] the first structural elucidation of biotransformation products formed by *B. cinerea* from terpene alcohols was achieved. In these investigations ω-hydroxylation has been found to be one of the predominant metabolic steps. Additionally, C1 oxidation and, in part, hydrogenation of double bonds have been observed. Continuing our work on microbial transformation of terpenes, we also studied citral (1), which consists of the geometric isomers neral and geranial [6]. This α,β-unsaturated terpene aldehyde is highly important in perfumery and aroma compositions such as citrus flavours as well as starting material in the synthesis of ionones [7].

**Materials and Methods**

**Botrytis cinerea** strains

The *B. cinerea* strains 5899/4, 5901/2, 5882/1 and 5909/1, used in this study, were obtained from the collection of the Bayerische Landesanstalt für Weinbau und Gartenbau, Würzburg. From the original cultures, a part was transferred to malt agar slants and incubated at 25 °C for 7 days.

**Media and incubation conditions**

a. Grape must. The sugar and acid content of the grape must (cultivar, Müller-Thurgau) used were adjusted to 200 g/l and 8.5 g/l (pH 3.5), respectively.

b. Synthetic medium. The medium contained (per l): NaNO₃ 3 g; K₂HPO₄ 1 g; MgSO₄·7H₂O 0.5 g; KCl 0.5 g and FeSO₄ 0.01 g. The pH was adjusted to 3.5 using 1 N HCl.

c. Synthetic medium/grape must. Mixtures of 700:1 to 700:5 were used.

The medium (a, b, c) (700 ml) was filled into 1-l Erlenmeyer flasks and sterilized (30 min at 110 °C). After addition of 50 mg/l citral (1) (in 1 ml ethanol) each flask was inoculated with a pure *B. cinerea* strain and incubated at 25 °C for 2 weeks. The mycelium was removed by filtration and the solutions
analyzed by capillary gas chromatography (HRGC), capillary gas chromatography-mass spectrometry (HRGC-MS) and capillary gas chromatography-Fourier transform infrared spectroscopy (HRGC-FTIR) after extractive sample preparation. In the same manner, blank tests without *B. cinerea* incubation and without 1 were carried out.

**Isolation of biotransformation products**

After addition of internal standards (2-methyl-1-pentanol for the neutral and 2,2-dimethylpentanoic acid for the acidic fraction; both 0.50 mg/l) to the filtrate of the above-mentioned untreated and botrytized media solvent extraction was carried out using a pentane-dichloromethane mixture (2:1) [8]. Acids were removed from the extracts by separation with 5% NaHCO₃ solution (3 x 50 ml). The organic phase containing the neutral biotransformation products was carefully concentrated to 1 ml using a Vigreux column (45 °C) for subsequent HRGC, HRGC-MS and HRGC-FTIR analysis. The alkaline aqueous phase (acidic fraction) was acidified to pH 1–2 using 5 n HCl, extracted with diethyl ether (3 x 50 ml), methylated with CH₂N₂, and analyzed in the same manner as the neutral fraction.

**Capillary gas chromatography (HRGC)**

Instrument: Carlo Erba Fractovap 4160 fitted with a flame ionization detector (FID) and an air-cooled on-column injector. Column: J & W DB-Wax (30 m x 0.32 mm i.d.; d.f. = 0.25 μm) fused silica capillary, connected with a 2 m uncoated fused silica precolumn as “retention gap”. On-column injection was used. The temperature program was isothermal for 3 min at 50 °C, then 50 °C to 240 °C at 5 °C/min. The flow rates for the carrier gas were 2 ml/min for He, for the make-up gas 30 ml/min N₂ as well as for the detector gases 30 ml/min H₂ and 300 ml/min air, respectively. The detector temperature was kept at 220 °C. Volumes of 0.5 μl were injected.

Results of qualitative analyses were verified by comparison of HRGC retention (Rₜ), mass spectral and vapour phase FTIR data with those of authentic reference substances. Quantitative determinations were carried out by standard controlled calculations using a Hewlett Packard 3388 A laboratory data system without consideration of extraction yields (calibration factors for all compounds, F = 1.00).

**Capillary gas chromatography-mass spectrometry (HRGC-MS)**

Instrument: Finnigan MAT 44 quadrupole mass spectrometer coupled by an open-split interface with a Varian Aerograph 1440 equipped with a water-cooled on-column injector. A J & W DB-Wax (30 m x 0.32 mm i.d., d.f. = 0.25 μm) fused silica capillary column connected to a 2 m uncoated piece of fused silica capillary column as “retention gap” was used. The conditions were as follows: temperature, isothermal for 5 min at 60 °C and then from 60 °C to 240 °C at 5 °C/min; carrier gas flow rate, 2.5 ml/min He; temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mA; injection volumes, 0.5 μl.

**Capillary gas chromatography-FTIR spectroscopy (HRGC-FTIR)**

Instrument: Nicolet 20 SXB interfaced by a DANI 6500 gas chromatograph equipped with FID. A J & W DB-Wax (30 m x 0.32 mm i.d., d.f. = 0.25 μm) fused silica capillary column was used. Total sample injection mode using PTV (40 °C–240 °C, 0.1 min) was performed. The temperature program was 3 min isothermal at 50 °C and the from 50 °C to 250 °C at 4 °C/min. Light pipe and transfer line were held at 250 °C. He (2.5 ml/min) was employed as carrier gas. Vapour phase FTIR spectra were recorded from 400–4000 cm⁻¹ with a resolution of 8 cm⁻¹. Injection volumes, 0.5 μl.

**Reference compounds**

(E,Z)- (4) and (E,E)-2,6-dimethylocta-2,6-dien-1,8-diol (5): Syntheses were accomplished by SeO₂ oxidation of 2 and 3, respectively, according to Behr et al. [9]. 4: MS (m/z, %): 43 (100), 68 (59), 84 (47), 55 (19), 93 (17), 121 (8), 137 (7), 134 (4). FTIR (vapour phase, v, cm⁻¹): 3658, 2931, 2873, 1666, 1450, 1386, 1190, 1008. Rₜ: 2600. 5: MS (m/z, %): 43 (100), 68 (53), 84 (18), 55 (16), 121 (5), 137 (3), 134 (2). FTIR (vapour phase, v, cm⁻¹): 3657, 2931, 2873, 1666, 1449, 1386, 1202, 1007. Rₜ: 2648.

All the other reference compounds were available from our own laboratory collection of flavour substances.

**Results and Discussion**

In grape must (A), synthetic medium (B) as well as mixtures of A and B, citral (1) was completely
metabolized by the four *Botrytis cinerea* strains used. Whereas in A volatile bioconversion products could not be observed, in B and A/B mixtures the compounds 2–9 shown in Fig. 1 were identified after extractive sample preparation in the fermentation media by capillary gas chromatography (HRGC), capillary gas chromatography-mass spectrometry (HRGC-MS) and capillary gas chromatography-Fourier transform infrared spectroscopy (HRGC-FTIR). In the acidic fractions of the extracts of fermentation media, biotransformation products of 1 were not detectable. C<sub>1</sub> oxidation has been previously found in bioconversion studies of 1 using *Pseudomonas convexa* [10]. Geranic acid and other acids have been also detected as biotransformation products from 1 by *Pseudomonas aeruginosa* [11].

As shown from Table I, in B predominant conversion of 1 to nerol (2) and geraniol (3) occurred; in minor amounts (E,Z)- (4) and (E,E)-2,6-dimethylocta-2,6-dien-1,8-diol (5), 2-methyl-2-hepten-6-one (6), 2-methyl-2-hepten-6-ol (7), 2-methyl-2-hepten-6-one-1-ol (8) and 2-methyl-γ-butyrolactone (9) were determined. Using small amounts of A in B (1:700 to 5:700) low yields of 2 and 3 were observed, whereas

Table I. Yields (mg/l) and distribution (%) of bioconversion products formed from citral (1) by *Botrytis cinerea* strains in a synthetic medium (B) and a 1:700 mixture of grape must/synthetic medium (A/B).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Yield [mg/l]</th>
<th>Percentage of yield for compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>5899/4</td>
<td>B</td>
<td>12.7</td>
<td>32 38 4 10 12 4 tr tr</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>1.8</td>
<td>4 10 38 32 1 1 10 4</td>
</tr>
<tr>
<td>5901/2</td>
<td>B</td>
<td>21.5</td>
<td>31 59 1 1 6 1 &lt;1 tr</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>5.9</td>
<td>16 1 9 3 56 5 10 1</td>
</tr>
<tr>
<td>5882/1</td>
<td>B</td>
<td>29.4</td>
<td>40 54 – – 6 &lt;1 – –</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>4.2</td>
<td>18 1 7 2 58 4 10 1</td>
</tr>
<tr>
<td>5909/1</td>
<td>B</td>
<td>19.7</td>
<td>30 59 1 2 6 2 tr –</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>5.3</td>
<td>16 1 14 5 46 5 13 1</td>
</tr>
</tbody>
</table>

tr = traces.
the production of 4, 5, 6 and 8 increased. Quantitatively, the findings were strongly dependent on the strains used. The results represented in Table I were obtained employing an 1:700 A/B mixture. In order to explain the effect caused by addition of grape must to medium B, a relation between fungal growth — reduced in A/B in comparison to A and quite different in B (1 as sole carbon source) — and induction of enzymes catalyzing the bioconversion of 1 could be considered. However, this effect was not further studied.

Corresponding to our bioconversion studies with cinnamic aldehyde leading to the formation of cinnamic alcohol by \textit{Botrytis cinerea} [12], in the experiments using 1 also reduction of the aldehyde function was found as predominant metabolic step. Recently, transformation of E-2-hexenal to E-2-hexenol and 1-hexanol by \textit{B. cinerea} has also been described [13]. From 2 and 3 two main pathways can be discussed to elucidate the formation of other bioconversion products formed from 1 by \textit{B. cinerea}. As schematically shown in Fig. 2, \(\omega\)-hydroxylation led to 4 and 5. In plants, \(\omega\)-hydroxylation of 2 and 3 catalyzed by cytochrome P-450-dependent monooxygenase has been reported [14, 15]. Furthermore, a hypothetic pathway as discussed earlier for bacteria by Seubert and coworkers [16, 17] can be considered leading to 6, from which reduction led to 7 and \(\omega\)-hydroxylation to 8.

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