LINALOOL BIOTRANSFORMATION WITH FUNGI

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Abstract

Different fungal strains were screened for their ability to convert linalool into valuable flavour compounds. *Aspergillus niger* DSM 821, *Botrytis cinerea* 5901/02 and *Botrytis cinerea* 02/FBII/2.1 produced isomers of lilac aldehyde and lilac alcohol via 8-hydroxylinalool as postulated intermediate. Linalool oxides and 8-hydroxylinalool accumulated as major products during fungal linalool biotransformations. Furanoid *trans*-(2*R*, 5*S*) and *cis*-(2*S*, 5*R*) linalool oxide as well as pyranoid *trans*-(2*R*, 5*S*) and *cis*-(2*S*, 5*S*) were identified as main stereoisomers, formed by epoxidation of (±)-linalool via the postulated key intermediates (3*S*, 6*S*)-6,7-epoxylinalool and (3*R*, 6*S*)-6,7-epoxylinalool. *Corynespora cassiicola* DSM 62475 was shown for the first time to stereospecifically produce 357 mg/L linalool oxides from linalool in just three days, corresponding to a productivity of 120 mg/L*d and a molar conversion yield close to 100 %.

Introduction

Lilac aldehyde and lilac alcohol have been described as characteristic minor components of *Syringa vulgaris* L. flowers. Recently biogenetic studies with stable isotope labelled precursors have shown that *S. vulgaris* L. converts linalool into lilac aldehydes and lilac alcohols (1, 2). Due to the evidence of a plant biosynthetic pathway we hypothesized that there are also other biological systems capable of transforming linalool into the desired lilac aroma compounds. Microorganisms, especially fungi, have been shown to be very versatile biocatalysts for the production of a wide range of flavour and fragrance compounds from cheap natural precursors such as terpenoids (3). Therefore, this work aimed at screening different fungi for their capacity to transform linalool to valuable products such as the aforementioned lilac compounds or linalool oxides. This included also the analysis of the stereoisomeric distribution of furanoid and pyranoid linalool oxides.

Experimental

The experimental details described in detail in (4). In the following the main procedures will be summarised.

Microorganisms, culture media, chemicals. Botrytis cinerea 5901/2, 5909/1, 92/lic/1, 97/4, 99/16/3, 00/II10.1, 02/FB II/2.1, and P10 (BLWG, Veitshöchheim, Germany); *Aspergillus niger* ATCC 16404, DSM 821, *Corynespora cassiicola* DSM

62475, *Penicillium digitatum* DSM 62840, *P. italicum* DSM 62846 (DSMZ, Braunschweig, Germany); *Geotrichum candidum* (HEVs, Sion, Switzerland); P. digitatum NRRL 1202 (ARS culture collection, Illinois, USA). *Saccharomyces cerevisiae* Ceppo 20, Zymaflor VL1, Uvaferm 228, SIHA Riesling n° 7 (E. Begerow GmbH & Co., Langenlonsheim, Germany). The strains were grown on malt extract agar (MEA) and biotransformation experiments were performed in malt yeast broth (MYB). (±)-Linalool (> 97 % (v/v)), (−)-linalool (> 98.5 % (v/v)), 1-octanol (> 99.5 % (v/v)), cis- and trans-furanoid linalool oxide (> 97 % (v/v), mixture of isomers) and tert-butyl methyl ether (MTBE) (> 99.8 % (v/v)) were purchased from Fluka, Germany. Lilac alcohol and lilac aldehyde stereoisomers, 8-hydroxylinalool and standards of *cis*- and *trans*-furanoid and pyranoid linalool oxide isomers were synthesized as described in (4).

Biotransformations. Screening experiments and linalool toxicity assays were performed in 40 mL SPME vials filled with 15 mL MYB. Linalool biotransformations with the selected strains using a feed strategy were carried out for 12 d in 2L Erlenmeyer flasks filled with 500 mL MYB. After the cultivation period, the cultures were analyzed by SPME as well as by organic phase extraction to characterize the linalool bioconversion products and to quantify 8-hydroxylinalool and linalool oxides, the stereoisomeric distribution of which was also established. To test the potential of a non-biological formation of the target compounds a blank experiment was performed in 100 mL MYB medium. The cultivations conditions, the amount of substrate used and the feed strategy have been described in (4).

Analytical methods. For identification of linalool bioconversion products 15 mL liquid culture was analyzed by GC/MS using a SPME headspace extraction (4). Lilac aldehydes and alcohols were identified by comparing their mass spectra and retention indices with those of the references. Other compounds were identified by NIST mass spectral library V 2.0. The concentrations of linalool, cis- and trans-furanoid linalool oxide, cis- and trans-pyranoid linalool oxide and 8-hydroxylinalool in the liquid cultures were determined using 1-octanol as internal standard. The stereoisomeric distribution of linalool oxides was determined by enantioselective GC. The dry biomass was determined with an infrared moisture analyzer (Sartorius, Germany). Glucose was analyzed enzymatically (Yellow Spring Instrument, USA).

Results

Detection of characteristic mass spectrum fragments of lilac aldehyde and lilac alcohol. Nineteen fungal strains were screened for their ability to convert (±)-linalool into lilac aldehyde and lilac alcohol isomers. The fungal strains were cultivated on a 15 ml scale 14 days in linalool-supplemented (30 mg/L) malt yeast broth. The culture headspace SPME-GC/MS was the analytical method used to detect the three characteristic fragments m/z 111, 153 (lilac aldehyde) and 155 (lilac alcohol) within the time window of the chemically synthesized references compounds. The positive strains were *A. niger* ATCC 16404 and DSM 821, *B. cinerea* 5901/2 and 02/FBII/2.1, *S. cerevisiae* Zymaflor VL1 and Uvaferm 228, and *C. cassiicola* DSM 62475.

Determination of linalool toxicity. The positive strains were cultivated with twelve increasing linalool concentrations (0 mg/L to 1000 mg/L). In order to acquire the maximum non-growth-inhibiting concentration of linalool in the culture media, toxicity curves were determined. All strains tested tolerated linalool in the range of 50 g/L to 200 mg/L. In the case of B. cinerea 5901/2 a linalool concentration of 150 mg/L was identified as the maximum value at which growth was still unhampered (4).

Biotransformation of linalool and identification of lilac aldehyde and lilac alcohol isomers. In subsequent experiments the selected strains were grown on 500 mL scale with sequential feeding of linalool and glucose to avoid toxic effects by the substrate and to enhance product formation. SPME GC/MS analysis and comparison with a non-biological blank experiment confirmed that B. cinerea 5901/2, B. cinerea 02/FBII/2.1 and *A. niger* DSM 821 are clearly positive strains to produce detectable amounts of lilac aldehyde and lilac alcohol isomers, as shown in Figure 1 with the GC/MS analysis of *B. cinerea* 5901/2.

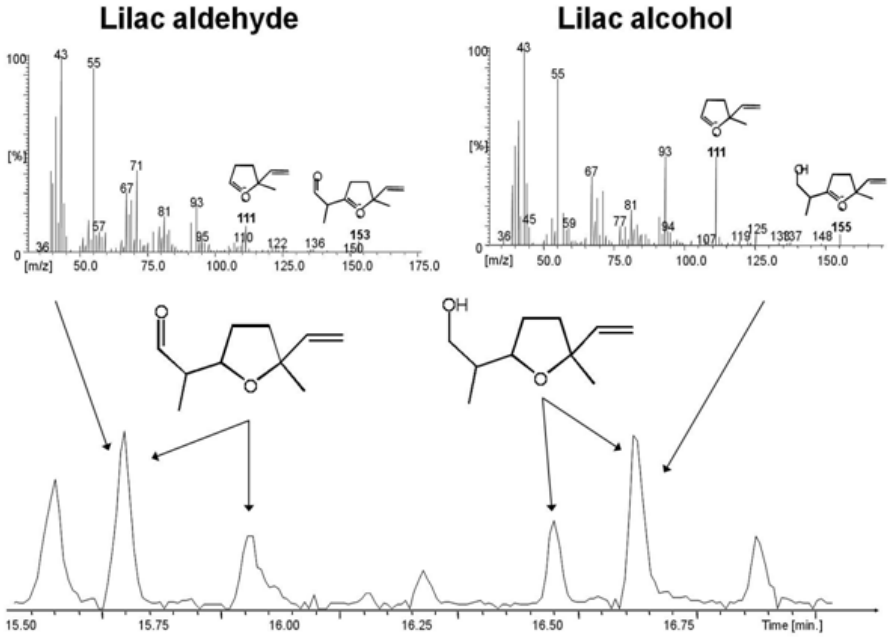

Figure 1. *Fragment ion chromatogram (m/z 111) showing two lilac aldehyde and two lilac alcohol isomers produced by B. cinerea 5901/2. Exemplary mass spectra of lilac alcohol and lilac aldehyde are given. (Adapted with permission from J. Agric. Food Chem. 2008, 56, 3287-96. Copyright 2008 ACS).*

Quantification of the major biotransformation products. Linalool oxide diastereoisomers and 8-hydroxylinalool turned out to be the major linalool biotransformation products of the aforementioned cultivation experiments. These compounds were quantified after fed-batch cultivation of A. niger ATCC 16404 and DSM 821, B. cinerea 5901/2 and 02/FBII/2.1, and C. cassiicola DSM 62475 to determine the molar conversion yield for the major products. Aspergillus niger DSM 821 (Fig. 2) converted almost 80 % of the substrate (323 mg/L) into a mixture of 252 mg/L of linalool oxides principally and 8-hydroxylinalool (37 mg/L) after 6 days of cultivation, while biotransformation of A. niger ATCC 16404 was less pronounced. B. cinerea 5901/2 converted 60 % of the given linalool (285 mg/L) into 167 mg/L 8-hydroxylinalool after 9 days cultivation (Fig. 2). However, B. cinerea 02/FBII/2.1 produced 116 mg/L of a mixture of linalool oxides and 8-hydroxylinalool with a conversion yield of 50 % from 230 mg/L linalool. With a conversion yield >96 % after only 3 days, corresponding to 357 mg/L of linalool oxides, C. cassiicola DSM 62475 (Fig. 2) turned out to be the most actively transforming strain. In contrast to B. cinerea 02/FBII/2.1 and A. niger ATCC 16404, this improved productivity can be explained by a faster growth of C. cassiicola DSM 62475, thereby leading to an enhanced biomass formation.

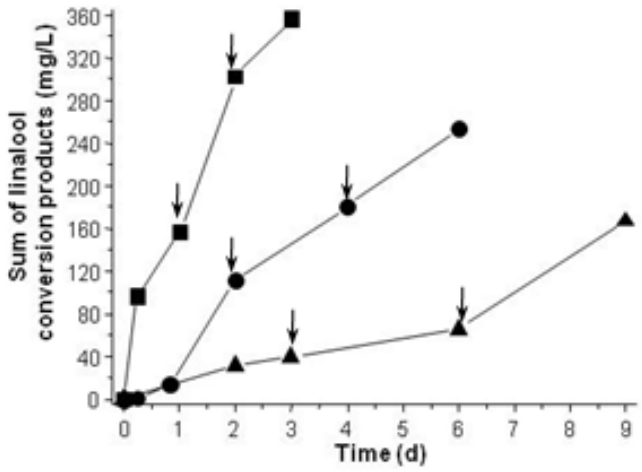

Figure 2. *Product formation kinetics of linalool conversion by A. niger DSM 821 (●), B. cinerea 5901/2 (▲) and C. cassiicola DSM 62475 (■) with sequential feeding of linalool and glucose as indicated by the arrows (4). (Reproduced with permission from J. Agric. Food Chem. 2008, 56, 3287-96. Copyright 2008 ACS)*

Stereoisomeric distribution of furanoid and pyranoid linalool oxides. The enantiomeric and diastereoisomeric distribution of linalool oxides produced by *A. niger* DSM 821, *B. cinerea* 02/FBII/2.1, and *C. cassiicola* DSM 62475 was analyzed by enantioselective GC (4). (Figure 3) shows that the stereoselective conversion of (R/S)-linalool by A. niger DSM 821*, B. cinerea* 02/FBII/2.1, and *C. cassiicola* DSM 62475 led to 5R-configured furanoid linalool oxides and 5S-configured pyranoid linalool oxides, both via 6S-configured epoxylinalool as the postulated intermediates, as previously described by Demyttenaere and Willemen (1998) (5).

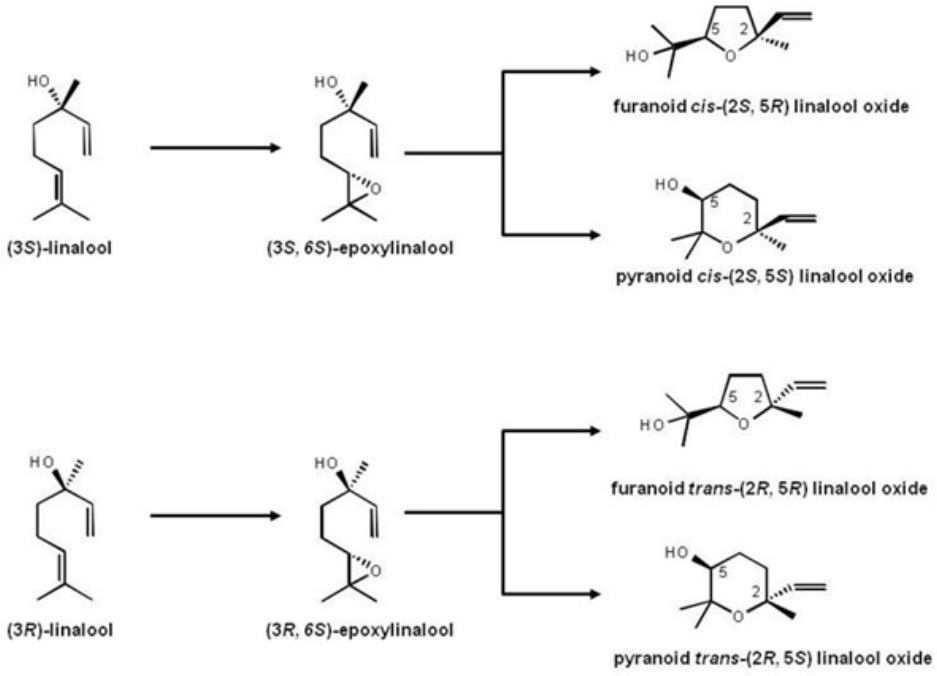

Figure 3. *Stereoisomeric distribution of furanoid and pyranoid linalool oxide from conversion of (R/S)-linalool by fungi. (Adapted with permission from J. Agric. Food Chem. 2008, 56, 3287-96. Copyright 2008 ACS)*

The production of lilac compounds from (±)-linalool by fungal-biotransformation, via 8-hydroxylinalool as postulated intermediate, as been demonstrated for the first time. Furthermore, the 6*S*-configurated epoxylinalool enantiomers are the postulated key intermediates for fungal production of linalool oxides. *C. cassiicola* DSM 62485 was identified as novel and highly efficient biocatalyst producing linalool oxides. Figure 4 summarises the linalool conversion pathways by fungi.

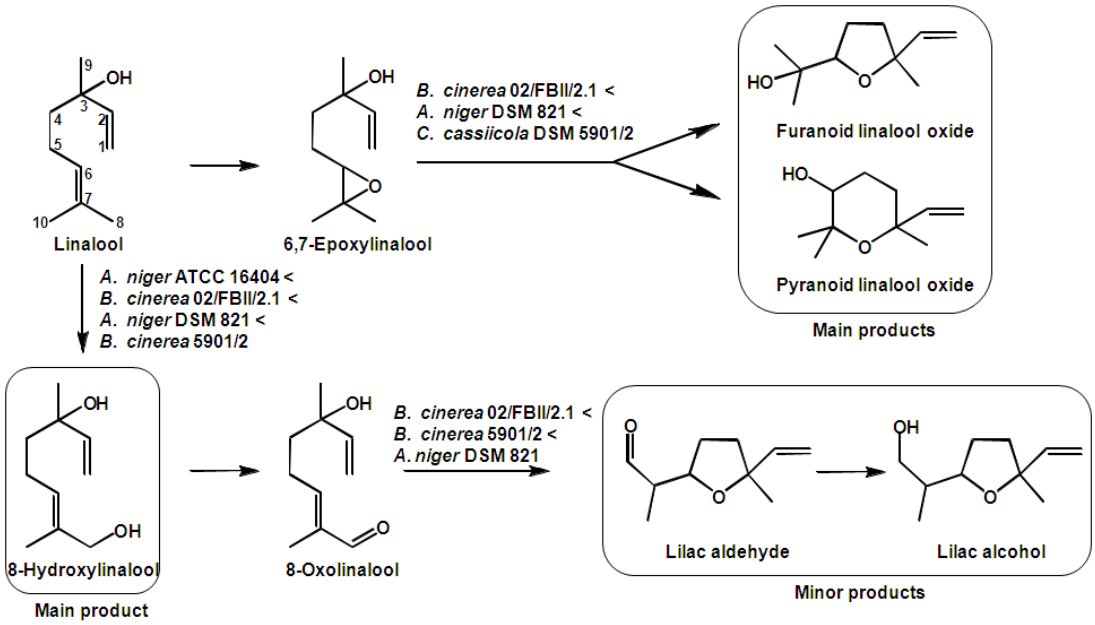

Figure 4. *Postulated conversion of linalool to main and minor products by fungi ordered in ascending catalytic activity (4). (Reproduced with permission from J. Agric. Food Chem. 2008, 56, 3287-96. Copyright 2008 ACS)*

References

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