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Fungal Biotransformation of (±)-Linalool

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The biotransformation of (±)-linalool was investigated by screening 19 fungi. Product accumulation was enhanced by substrate feeding and, for the first time, lilac aldehydes and lilac alcohols were identified as fungal biotransformation byproduct using SPME-GC-MS headspace analysis. *Aspergillus niger* DSM 821, *Botrytis cinerea* 5901/02, and *B. cinerea* 02/FBII/2.1 produced different isomers of lilac aldehyde and lilac alcohol from linalool via 8-hydroxylinalool as postulated intermediate. Linalool oxides and 8-hydroxylinalool were the major products of fungal (±)-linalool biotransformations. Furanoid *trans*-(2*R*,5*R*)- and *cis*-(2*S*,5*R*)-linalool oxide as well as pyranoid *trans*-(2*R*,5*S*)- and *cis*-(2*S*, 5*S*)-linalool oxide were identified as the main stereoisomers with (3*S*,6*S*)-6,7-epoxylinalool and (3*R*,6*S*)-6,7-epoxylinalool as postulated key intermediates of fungal (±)-linalool oxyfunctionalization, respectively. With a conversion yield close to 100% and a productivity of 120 mg/L·day linalool oxides, *Corynespora cassiicola* DSM 62485 was identified as a novel highly stereoselective linalool transforming biocatalyst showing the highest productivity reported so far.

KEYWORDS: Biotransformation; fungi; lilac aldehyde; lilac alcohol; linalool; 8-hydroxylinalool; 6,7-epoxylinalool; linalool oxide; SPME

INTRODUCTION

Lilac aldehydes and lilac alcohols have been described as characteristic monoterpenoids in *Syringa vulgaris* L. (Oleraceae) flowers positively influencing the lilac odor quality (*1*). Lilac-type fragrance compounds are in high demand by the perfume industry because there are no natural lilac flower oils or concentrates commercially available and synthetic fragrance compounds are used to imitate the desired odor. Lilac aldehydes and lilac alcohols are very powerful fragrance compounds due to their exceptionally low odor thresholds of about 0.2–0.4 and 2–4 ng, respectively (*2*). Recently, the biogenetic pathway in *S. vulgaris* was elucidated using ^{2}H- and ^{18}O-labeled precursors (*3, 4*). It was shown that the formation of the genuine lilac aldehydes and alcohols proceeds via linalool (the *S*-enantiomer) as key intermediate. By the same approach the elucidation of the chirality and biosynthesis of lilac compounds in *Actinidia arguta* flowers have delivered comparable conclusions (*5*). This raised the question of whether other biological systems, namely, microorganisms, were able to convert linalool into the desired fragrance compounds as well. Among microorganisms fungi especially are well-known for their versatility in monoterpene biotransformation, which has been well documented during the past decades (*6–9*). The motivation for these research activities was not only to better understand the biochemical pathways of microbial terpene transformation and degradation but also to find alternative synthetic routes toward highly valuable industrially relevant flavor and fragrance compounds from cheap natural precursors. Linalool occurs as one of its enantiomers in many essential oils, for example, 80–85% (−)-linalool in *Cinnamomum camphora* oil or 60–70% (+)-linalool in coriander oil (*10*), thus representing a precursor that is abundantly available in nature.

Aspergillus niger DSM 821 and ATCC 9142 were shown to convert (±)-linalool into a mixture of *cis*- and *trans*-furanoid linalool oxide and *cis*- and *trans*-pyranoid linalool oxide (*8, 11*). Linalool oxide isomers are also valuable aroma compounds constituting the flavor of tea and being used in the perfume industry for lavender notes (*12, 13*).

Another research group found 8-hydroxylinalool as the main linalool derivative after biotransformation of linalyl acetate by *A. niger* (*14*). The biotransformation of linalool by the plant pathogenic fungus *Botrytis cinerea*, which is responsible for the noble rot of wine grapes, also yielded 90% 8-hydroxylinalool as the main metabolite, whereas linalool oxides were only minor products (*15*). *Corynespora cassiicola* DSM 62475 turned out to be a very efficient and selective monoterpene transforming microorganism, for example, producing pure (1*S*,2*S*,4*R*)-*p*-menth-8-ene-1,2-diol from (+)-limonene in high concentrations

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(*10*); surprisingly, its use for linalool biotransformation has not been reported so far. Besides *Aspergillus*, *Botrytis*, and *Corynespora*, many other genera, such as *Saccharomyces*, *Penicillium*, and *Geotrichum*, are also known to include strains efficient in monoterpene biotransformation (*8, 11, 16–18*).

Although the biotransformation of linalool has been extensively studied in the past, neither lilac aldehyde nor lilac alcohol formation has been reported so far.

In this work we systematically investigated whether certain fungi, most of them preselected from the literature due to their reported versatility to metabolize monoterpenes, are capable of converting linalool into the desired lilac aldehydes and alcohols. In a first screening run 19 strains were tested with a low linalool concentration to avoid any toxic effects of the precursor. Seven potentially positive strains were identified on the basis of headspace solid-phase microextraction and GC-MS analysis (SPME-GC-MS) after batch cultivation. Of these selected strains the linalool tolerance thresholds and the glucose/linalool consumption kinetics were determined to enhance biomass and product formation during subsequent feed-batch cultivation. For three strains, SPME-GC-MS analysis revealed the unprecedented formation of lilac aldehydes and lilac alcohols as metabolic byproducts of fungal linalool biotransformation. The target products were identified by comparing their mass spectra and retention indices with those of chemically synthesized reference substances. Moreover, linalool oxides and 8-hydroxylinalool turned out to be the major biotransformation products, and analysis of their stereochemistry revealed high selectivities of the biocatalytic reactions. The feed-batch strategy led to linalool biotransformations with the highest product concentrations reported so far.

MATERIALS AND METHODS

Microorganisms and Strain Maintenance. *B. cinerea* 5901/2, 5909/1, 92/lic/1, 97/4, 99/16/3, 00/II10.1, 02/FBII/2.1, and P10 were kindly provided by LWG, Bayerische Landesanstalt für Weinbau and Gartenbau, Veitshöchheim, Germany. *A. niger* ATCC 16404, DSM 821, *C. cassiicola* DSM 62475, *Penicillium digitatum* DSM 62840, and *Penicillium italicum* DSM 62846 were purchased from DSMZ, Braunschweig, Germany. *P. digitatum* NRRL 1202 was obtained from ARS Culture Collection, Illinois, USA, *Geotrichum candidum* was obtained from HEVs, Hochschule Wallis, Sitten, Switzerland. *Saccharomyces cerevisiae* Ceppo 20, Zymaflor VL1, Uvaferm 228, and SIHA Riesling 7 were a gift from E. Begerow GmbH & Co., Langenlonsheim, Germany. The strains were grown on agar plates with malt extract agar (MEA) consisting in w/v of malt extract 3%, soy peptone 0.3%, and agar 1.7%, adjusted to pH 5.6 (*10*). Filamentous fungi were grown at 25 °C and yeasts at 30 °C.

Chemicals. (±)-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, Ulm, Germany. Lilac alcohol and lilac aldehyde were prepared as previously described (*2*) and were used as a mixture of stereoisomers [0.01% (v/v) in MTBE]. 8-Hydroxylinalool was synthesized from (±)-linalool according to the literature (*19*) and was used as 0.02% (v/v) in MTBE. Standards of *cis*- and *trans*-furanoid and pyranoid linalool oxide isomers [used as 0.01% (v/v) in MTBE] were prepared as described elsewhere (*13, 20*).

Determination of Linalool Toxicity. To determine the toxicity of linalool, each of the seven fungi selected by the screening was cultivated in small liquid cultures with increasing concentrations of linalool. For each strain, 12 40 mL SPME vials were filled with 15 mL of MYB medium (in w/v, malt extract 3%, glucose 1%, peptone 1%, and yeast extract 0.3%; pH 6.4), autoclaved, and inoculated with 500 μL of a spore suspension consisting of (in w/v) 0.85% NaCl, 1% peptone, 0.1% Tween 80, and approximately 2.5×10^7 spores in distilled water; yeast cultures of the same volume were inoculated by transferring cells from an agar plate with a loop. The increasing concentrations of linalool in each vial, adjusted with a solution of 0.3% (w/v) (±)-linalool in EtOH, were (in mg/L) 0, 25, 50, 75, 100, 150, 200, 300, 400, 500, 600, and 1000. The vials were covered with cotton stoppers and incubated for 4 days at 220 rpm and 25 °C (higher fungi) or 30 °C (yeasts). To determine the concentration-dependent toxicity of linalool, the final dry biomass was analyzed for each vial.

Screening of 19 Fungi in Small-Scale Vials. The screening experiments were performed in 40 mL SPME vials filled with 15 mL of MYB medium, pH 6.4, as described above. After autoclaving and inoculation, 150 μL of 0.3% (w/v) (±)-linalool (in ethanol) were added (30 mg/L), the vials were covered with cotton stoppers and the cultures were incubated for 14 days at 220 rpm and 25 °C (filamentous fungi) or 30 °C (yeasts).

Biotransformation with Selected Strains in Erlenmeyer Flasks: Batch Mode. To study the biotransformation kinetics, the selected strains were cultivated in 2000 mL Erlenmeyer flasks for 12 days at 130 rpm and 25 °C (filamentous fungi) or 30 °C (yeasts). The flasks were filled with 450 mL of MYB medium. Inoculation was done with 50 mL of a preculture grown in MYB medium with 500 μL of spore suspension for the filamentous fungi and by loop for the yeast cultures. At the beginning of the biotransformations, the precursor was added according to the maximum concentration tolerated by the respective strain identified by the toxicity determination experiments. (±)-Linalool was added as ethanolic solution [3% (w/v)]. Concentrations of linalool, glucose, and dry biomass were determined every day from 5 mL samples aseptically withdrawn from the culture. The pH values were measured at the beginning and at the end of the experiments.

Biotransformation with Selected Strains in Erlenmeyer Flasks: Feed-Batch Mode. The experimental setup and the analytics were similar to those of the batch mode. To improve the final product concentrations a feed-batch approach was chosen by feeding additional linalool and glucose at intervals according to the strain-specific consumption rates determined in the preceding batch-mode studies. Glucose was dosed as a 1.1 kg/L aqueous solution. Dependent on the metabolic activities of the strains, cultivations lasted 3−9 days. Product analysis was done by headspace SPME-GC-MS after transferring 15 mL into 40 mL SPME vials at the end of cultivation.

Solid-Phase Microextraction Method. For SPME analysis, the pH of a sample was adjusted to 4.0 with 1 M HCl if necessary, and 25% of NaCl (3.75 g in 15 mL) was added. The SPME vials were covered with PTFE silicone and "open top phenolics closures" (Supelco) and incubated at 40 °C and 400 rpm until headspace SPME using a 75 μm CAR-PDMS (carboxene/polydimethylsiloxane) coated fiber and a manual holder (Supelco, Germany) was started. Extraction was carried out for 20 min at 40 °C and 400 rpm. For GC-MS analysis a desorption time of 5 min in a GC injector at 250 °C was used.

Analysis of the Sample with GC-MS and Enantioselective GC. GC-MS analyses were performed with a GC-17 A Shimadzu gas chromatograph, equipped with a VB-5 Valcobond column (30 m × 0.25 mm i.d.; coating thickness = 0.25 μm) and a QP5050 Shimadzu mass spectrometer (quadrupole type). The working conditions were as follows: injector, 250 °C; detector, 280 °C; oven temperature, start at 40 °C, hold for 7 min, programmed from 40 to 280 °C at 10 °C/min, hold for 2 min; carrier gas flow (He), 1.1 mL/min; injection mode, splitless for SPME samples and 1/30 for liquid samples (injection volume = 2 μL); EI, 70 eV; acquisition parameters, scanned *m/z*, 35–250 (10–25 min). Enantioselective GC analysis was performed with a GC-17 A Shimadzu gas chromatograph with FID analyzer, equipped with a Chiraldex B-DM chiral column (30 m × 0.32 mm i.d.; coating thickness = 0.12 μm). The working conditions were as follows: injector, 250 °C; detector, 280 °C; oven temperature, isothermal for 30 min at 95 °C; carrier gas flow (He), 1.1 mL/min; injection mode, split 1/20 for liquid samples (injection volume = 1 μL). Relevant substances were identified by comparison of their mass spectra and retention indexes (Kovats indices) (*11, 13, 16, 20–23*) with those of reference substances (when possible) and by comparison with MS library data (NIST mass spectral library V 2.0). Retention indices (for VB-5 Valcobond column) were as follows: *trans*-furanoid linalool oxide, 1082; *cis*-furanoid linalool oxide, 1096; linalool, 1115; lilac aldehyde isomer a, 1150; lilac aldehyde isomer b, 1159; lilac aldehyde isomer c, 1173; *trans*-pyranoid

linalool oxide, 1188; *cis*-pyranoid linalool oxide, 1195; lilac alcohol isomer d, 1213; lilac alcohol isomer e, 1230; lilac alcohol isomer f, 1247; 10-hydroxylinalool, 1385; 8-hydroxylinalool, 1399. Retention indices (for Chiraldex B-DM chiral column) were as follows: furanoid linalool oxides, *trans*-(2*R*,5*R*), 1149; *trans*-(2*S*,5*S*), 1160; *cis*-(2*R*,5*S*), 1163; *cis*-(2*S*,5*R*), 1169; *R*-(−)-linalool, 1209; *S*-(+)-linalool, 1216; pyranoid linalool oxides, *trans*-(2*S*,5*R*), 1271; *trans*-(2*R*,5*S*), 1284; *cis*-(2*S*,5*S*), 1290; *cis*-(2*R*,5*R*), 1293. Response factors in relation to 1-octanol were as follows: *cis*- and *trans*-furanoid linalool oxide, 1.27; *cis*- and *trans*-pyranoid linalool oxide, 1.32; (±)-linalool, 1.19; 8-hydroxylinalool, 2.80.

Mass Spectra. *trans*-Furanoid linalool oxide *m/z* (relative intensity): 41 (34), 43 (71), 55 (38), 59 (100), 67 (24), 68 (25), 81 (12), 93 (21), 94 (27), 111 (13), 137 (3), 155 (2). *cis*-Furanoid linalool oxide *m/z* (relative intensity): 41 (35), 43 (63), 55 (43), 59 (100), 67 (26), 68 (31), 81 (15), 93 (28), 94 (39), 111 (19), 137 (3), 155 (3). *trans*-Pyranoid linalool oxide *m/z* (relative intensity): 41 (51), 43 (88), 53 (24), 55 (38), 57 (15), 59 (99), 67 (70), 68 (100), 79 (27), 94 (66), 137 (1), 155 (2). *cis*-Pyranoid linalool oxide *m/z* (relative intensity): 41 (34), 43 (64), 53 (16), 55 (24), 57 (10), 59 (89), 67 (54), 68 (100), 79 (20), 94 (53), 137 (2), 155 (3). Linalool *m/z* (relative intensity): 41 (97), 43 (100), 53 (15), 55 (64), 67 (20), 69 (38), 71 (89), 80 (31), 93 (55), 121 (12), 136 (5), 154 (<1, M^+). Lilac aldehyde isomer a *m/z* (relative intensity): 41 (63), 43 (99), 55 (100), 67 (40), 69 (28), 71 (33), 81 (16), 91 (9), 93 (29), 111 (23), 125 (3), 153 (6). Lilac aldehyde isomer b *m/z* (relative intensity): 41 (58), 43 (99), 55 (100), 67 (35), 69 (24) 71 (37), 81 (17), 91 (8), 93 (28), 111 (20), 125 (2), 153 (10). Lilac aldehyde isomer c *m/z* (relative intensity): 41 (47), 43 (69), 55 (100), 67 (27), 69 (20), 71 (39), 81 (13), 91 (6), 93 (24), 111 (15), 125 (4), 153 (7). Lilac alcohol isomer d *m/z* (relative intensity): 41 (58), 43 (100), 55 (90), 67 (40), 69 (23), 71 (28), 81 (15), 91 (11), 93 (56), 111 (69), 125 (5), 155 (6). Lilac alcohol isomer e *m/z* (relative intensity): 41 (53), 43 (100), 55 (98), 67 (45), 69 (24), 71 (29), 81 (20), 91 (9), 93 (52), 111 (58), 125 (3), 155 (7). Lilac alcohol isomer f *m/z* (relative intensity): 41 (53), 43 (85), 55 (100), 67 (40), 69 (22), 71 (28), 81 (17), 91 (9), 93 (53), 111 (55), 125 (2), 155 (8). 10-Hydroxylinalool *m/z* (relative intensity): 41 (36), 43 (100), 53 (10), 55 (32), 67 (40), 68 (19), 71 (60), 79 (12), 81 (9), 93 (7), 137 (3), 152 (1). 8-Hydroxylinalool *m/z* (relative intensity): 41 (34), 43 (100), 53 (10), 55 (29), 67 (40), 68 (18), 71 (47), 79 (11), 81 (9), 93 (8), 137 (3), 152 (<1).

Sample Preparation. To determine the concentrations of linalool, *cis*- and *trans*-furanoid linalool oxide, *cis*- and *trans*-pyranoid linalool oxide, and 8-hydroxylinalool, 2 mL of liquid culture was filtered through a 0.45 μm/25 mm nylon filter (Macherey-Nagel) and extracted with 2 mL of *tert*-butyl methyl ether (MTBE) prior to GC-MS. 1-Octanol was used as internal standard for quantification. To determine the enantiomeric distribution of linalool oxides, samples were analyzed via enantioselective GC instead of GC-MS. All measurements were done in triplicate.

Target Compound Identification. For the initial screening of 19 fungi, the occurrence of one fragment ion (*m/z* 153 for lilac aldehyde or *m/z* 155 for lilac alcohol or *m/z* 111 for lilac aldehyde/alcohol) in a time window of ±0.5 min to the retention times of the chemically synthesized standards was used as the criterion for transferring a strain into the second, more detailed, screening run. For this second screening, the target compounds, lilac aldehyde and lilac alcohol, were identified by comparison with chemically synthesized standards on the basis of their mass spectra and retention indexes. Selected ion monitoring (SIM) using the following masses was applied: lilac aldehyde, *m/z* 153 (120×); lilac alcohol, *m/z* 155 (120×); and lilac aldehyde/alcohol, *m/z* 111 (30×).

Dry Biomass and Glucose Determination. The dry biomass was determined gravimetrically using an infrared moisture analyzer (MA 100, Sartorius, Germany) by filtering 2 mL of homogenized liquid culture through a dried, preweighed 0.45 μm/45 mm cellulose acetate membrane filter (Schleicher & Schuell, Germany). The concentration of glucose was determined enzymatically (YSI 2700 Biochemistry Analyzer, Yellow Springs Instruments, Yellow Springs, OH).

Chemical and Biological Control Experiments. The chemical stability of the substrate (±)-linalool was verified under cultivation conditions during a period of 9 days without inoculum and with an initial linalool concentration of 200 mg/L. The pH of the MYB broth was adjusted to pH 5 with HOAc. The concentration of the substrate was analyzed every 2 days and finally after 9 days. To exclude de novo biosynthesis of the target compounds, the selected strains were cultivated under biotransformation conditions but without linalool.

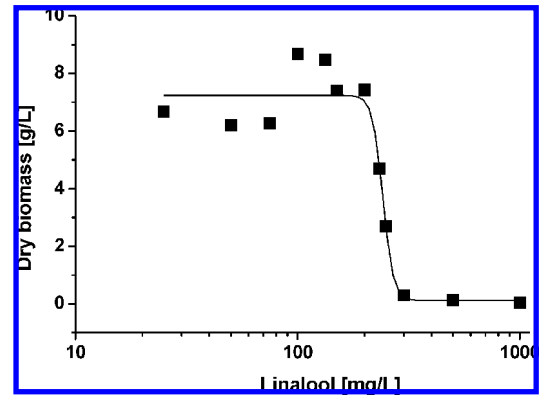

Figure 1. Linalool concentration−toxicity curve for *B. cinerea* 5901/2. The next lowest concentration above the threshold concentration, above which a sharp decrease in cell growth occurred, was chosen as maximum precursor concentration, here 150 mg/L. The other strains showed essentially the same toxicity profiles but varied concerning the maximum concentration values, which are listed in **Table 1**.

RESULTS AND DISCUSSION

Initial Screening of Fungi, Batch Cultivation. Nineteen fungal strains were screened for their capability to convert linalool particularly into lilac aldehyde and lilac alcohol using SPME-GC-MS as the monitoring technique. The strains were grown as 15 mL liquid cultures in 40 mL SPME vials enabling direct probing after 14 days of cultivation. The addition of 25% (w/v) NaCl increased the analytical sensitivity by a factor of 10 due to the enhanced volatility of the target compounds (data not shown). Potentially lilac aldehyde/alcohol-positive strains were selected by searching time windows of ±0.5 min around the retention times of the reference substances for the occurrence of at least one of the characteristic fragmentation ions *m/z* 155 (alcohol), *m/z* 153 (aldehyde), and *m/z* 111 (alcohol and aldehyde). Due to the fact that only a relatively low concentration of the toxic precursor was chosen for this orienting screening (30 mg/L), only low product concentrations near the detection limit occurred, and the SPME-GC-MS analyses were evaluated as indicative results: even those strains not giving unambiguously positive results were chosen for a second screening run under improved cultivation conditions. The seven potentially positive strains were *A. niger* ATCC 16404, *A. niger* DSM 821, *B. cinerea* 5901/2, *B. cinerea* 02/FBII/2.1, *C. cassiicola* DSM 62475, *S. cerevisiae* Zymalor VL1, and *S. cerevisiae* Uvaferm 228. To verify the preliminary results, we aimed at developing optimized feed-batch cultivation in Erlenmeyer flasks sequentially providing additional precursor and glucose while avoiding toxic precursor concentrations. By this means the final concentrations of linalool biotransformation products were to raised, thus enabling us to identify not only the major biotransformation products but also the lilac compounds as metabolic byproduct.

Linalool Toxicity. Strain-specific linalool concentration−toxicity profiles were determined by measuring the final cell dry weight after incubation of the fungi for 4 days in the presence of increasing concentrations of linalool. **Figure 1** exemplarily illustrates the toxic effects of linalool on *B. cinerea* 5901/2. Due to an observed sharp decrease in viability at a

Table 1. Feeding Strategies for Improved Biomass/Product Formation during Cultivation of the Preselected Strains under Feed-Batch Conditions

	feeding strategy[a]			
strain	start concn of linalool[b] (mg/L)	Δt feed (h)	feed linalool (mg/L)	feed glucose (g/L)
A. niger ATCC 16404	100	72	50	5
A. niger DSM 821	150	48	100	8
B. cinerea 5901/2	150	72	80	2
B. cinerea 02/FB II/ 2.1	100	24	80	0
C. cassiicola DSM 62475	150	24	100	5
S. cerevisiae Zymaflor VL1	200	48	0	10
S. cerevisiae Uvaferm 228	50	72	0	10

[a] The feed-batch experiments were carried out in a MYB medium over 12 days. The start concentration of glucose was 10 g/L. Feeding intervals were derived from the studies of the glucose and precursor consumption kinetics, cf. **Figure 2**. [b] Derived from the toxicity studies, cf. **Figure 1**.

certain threshold concentration, the next lower concentration tested was chosen as the maximum precursor concentration for the following feed-batch cultivation experiments to ensure a reasonable precursor supply while avoiding any toxic effects. 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 by the presence of the monoterpene. The other strains showed essentially the same toxicity profiles but varied with regard to the maximum precursor concentration values, which are listed in **Table 1**. The toxic effects of monoterpenoids are preferentially attributed to their intercalation into the cell membrane, thereby affecting its physiological function, rather than by specific interactions with defined targets (*24*). This general mechanism may explain why all threshold concentrations found in our studies were of the same order of magnitude independent of the fungal strain investigated. The low threshold concentrations are in good agreement with the log *P* concept (logarithm of the partition coefficient of an organic compound in the two-phase system 1-octanol/water) whereby lipophilic compounds with a log *P* between 1 and 5 are usually toxic to microorganisms (*25*). The log *P* of linalool can be calculated to be approximately 2.8 (http://www.logp.com), which is lower than those of monoterpene hydrocarbons due to the hydrophilic character introduced into the molecule by the alcohol group. On the basis of an empirical equation (*26*), the linalool concentration in the cell membrane, which corresponds to an aqueous solution of 1 mM linalool (154 mg/L), can be estimated to be about 120 mM (approximately 18 g/L). This illustrates the enormous linalool accumulation in the membrane and makes its detrimental effect on the cell physiology understandable.

Glucose and Precursor Consumption Kinetics. The glucose and linalool consumption kinetics were investigated under batch conditions to define a specific protocol for a subsequent feed-batch cultivation for each of the seven preselected strains. **Figure 2** exemplarily illustrates the results with *B. cinerea* 5901/2 and *C. cassiicola* DSM 62475, indicating that glucose and precursor consumption kinetics are highly strain-dependent. *B. cinerea* 5901/2 quickly consumed the linalool initially added to the culture. After 4 days of cultivation in Erlenmeyer flasks, almost 85% of the linalool was metabolized, followed by a less pronounced decline resulting in almost complete linalool consumption after 9 days. The amount of linalool that evaporated or was chemically degraded during the same time was ≤3.2%, as determined by a control experiment (data not shown). The consumption of glucose did not begin until 2 days after inoculation; obviously other C sources from the complex

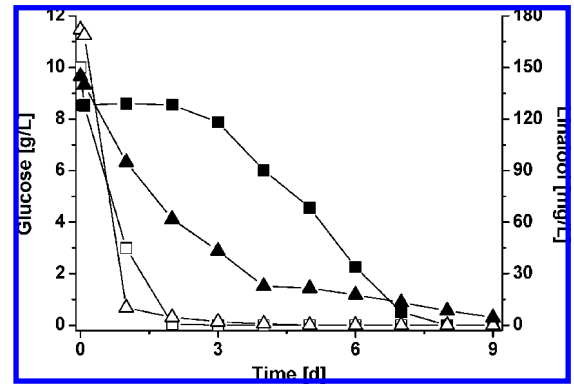

Figure 2. Linalool and glucose consumption kinetics: glucose (■) and linalool (▲) consumption by *B. cinerea* 5901/2; glucose (□) and linalool (△) consumption by *C. cassiicola* DSM 62475.

medium were favored for initial biomass growth. Total glucose depletion occurred after 8 days. In contrast, *C. cassiicola* DSM 62475 showed fast glucose catabolism starting immediately after inoculation and leading to total consumption in just 2 days (**Figure 2**). This was accompanied by stringent linalool consumption resulting in almost total depletion after about 1 day. *B. cinerea* 02/FBII/2.1 and *A. niger* ATCC 16404 showed glucose consumption profiles comparable to that of *B. cinerea* 5901/2, whereas the linalool consumption kinetics of *C. cassiicola* DSM 62475 was 2-fold faster than those of *A. niger* DSM 821 and *A. niger* ATCC 16404. *B. cinerea* 02/FBII/2.1 depleted linalool at a rate comparable with that of *C. cassiicola* DSM 62475 (data not shown). Both yeast strains tested also quickly consumed the glucose within 1 day, but after 6 days *S. cerevisiae* Uvaferm 228 had consumed only about half of the 50 mg/L linalool added, whereas *S. cerevisiae* Zymaflor VL1 had metabolized about 110 mg/L of the 200 mg/L added (data not shown). These data clearly indicate that glucose and precursor feeding strategies have to be carefully adapted strain-specifically to ensure maximum product formation under feed-batch conditions. The strain-specific feeding parameters derived from these batch studies are summarized in **Table 1**.

Screening of Selected Fungi, Feed-Batch Cultivation. On the basis of the data obtained during the toxicity and glucose/linalool consumption studies (cf. **Table 1**) the selected microorganisms were grown with strain-specific glucose and linalool feedings. By this means extended biomass growth and thus higher biotransformation product concentrations were targeted to facilitate the identification of the metabolites, particularly the lilac fragrance compounds, by headspace SPME-GC-MS analysis. **Table 2** exemplarily illustrates the relative contribution to the total peak area in percent of the biotransformation products obtained from linalool as well as other fungal metabolites and nonconverted linalool. The chemical control test was carried out without inoculum under otherwise identical conditions. Among the seven strains tested *A. niger* DSM 821, *B. cinerea* 5901/02, and *B. cinerea* 02/FII/2.1 were undoubtedly able to convert linalool into minor quantities of lilac aldehyde isomers and lilac alcohol isomers, whereas the other strains did not produce detectable amounts of the target compounds (**Table 2**). The identification of the lilac compounds is described in the next chapter. The furanoid *cis*/*trans*-linalool oxide stereoisomers and the pyranoid *cis*/*trans*-linalool oxide stereoisomers were the major products of linalool biotransformation by *A. niger* DSM 821, *B. cinerea* 02/FB II/2.1, and *C. cassiicola* DSM 62475. The capacity of *A. niger* and *B. cinerea* to oxidize linalool to linalool oxide stereoisomers as well as to stereoselectively form the 8-hydroxylinalool diastereoisomer has already

Table 2. Relative Contribution (Percent) of Linalool Biotransformation Products, Nonconverted Linalool, and Other Fungal Metabolites to the Total Peak Area after GC-MS Analysis of Headspace SPME Extracts of Liquid Cultures[a]

		A. niger		*B. cinerea*		*S. cerevisiae*		*C. cassiicola*	
no.	compound	AN1	AN2	BC1	BC2	SC1	SC2	CC1	control
1	methylheptenone	2.9	<0.1	nd	nd	nd	nd	5.4	nd
2	β-myrcene	7.2	nd	2.9	3.0	6.1	4.9	nd	7.1
3	methyheptenol	nd	nd	nd	nd	nd	nd	0.28	nd
4	2,6-dimethyl-1,6-octadiene	nd	0.46	nd	nd	nd	nd	nd	nd
5	limonene	0.93	nd	0.83	2.6	4.1	2.0	nd	1.7
6	ocimene	0.22	nd	1.2	nd	nd	0.46	nd	0.91
7	*trans*-linalool oxide furanoid	1.3	32	0.67	18	nd	0.25	44	nd
8	*cis*-linalool oxide furanoid	1.1	26	0.87	23	0.19	0.36	34	0.20
9	(±)-linalool	70	23	80	48	72	67	7.7	76
10	2-phenylethanol	2.0	2.7	3.0	0.16	6.6	15	0.87	nd
11	dihydrolinalool	5.6	0.65	7.3	0.34	8.8	6.6	0.70	10
12	lilac aldehyde (isomer a)	nd	0.12	nd	nd	nd	nd	nd	nd
13	lilac aldehyde (isomer b)	nd	<0.1	0.11	<0.1	nd	nd	nd	nd
14	lilac aldehyde (isomer c)	nd	<0.1	<0.1	nd	nd	nd	nd	nd
15	dihydrocarvone	<0.1	nd	nd	nd	nd	nd	nd	nd
16	*trans*-linalool oxide pyranoid	0.68	4.8	0.25	1.0	<0.1	nd	1.8	nd
17	*cis*-linalool oxide pyranoid	<0.1	8.5	0.10	3.6	nd	nd	4.8	0.31
18	α-terpineol	4.5	0.40	0.30	0.14	0.24	0.37	nd	<0.1
19	lilac alcohol (isomer d)	nd	<0.1	<0.1	nd	nd	nd	nd	nd
20	lilac alcohol (isomer e)	nd	nd	<0.1	<0.1	nd	nd	nd	nd
21	lilac alcohol (isomer f)	nd	<0.1	nd	nd	nd	nd	nd	nd
22	1-*p*-menthen-9-al	0.33	0.29	<0.1	nd	nd	nd	nd	nd
23	nerol	0.32	nd	<0.1	nd	nd	nd	nd	0.34
24	citronellol	nd	nd	nd	nd	0.24	0.21	nd	1.2
25	geraniol	0.33	nd	0.13	nd	0.27	nd	nd	0.87
26	citral	<0.1	nd	nd	nd	0.10	nd	nd	nd
27	geranylacetone	<0.1	<0.1	0.17	nd	<0.1	nd	nd	nd
28	dihydrocarveol	<0.1	<0.1	nd	nd	nd	nd	nd	nd
29	10-hydroxylinalool	<0.1	nd	<0.1	nd	nd	nd	nd	nd
30	8-hydroxylinalool	0.44	0.10	0.73	0.30	nd	nd	nd	nd
31	other fungal metabolites	1.7	0.45	0.19	nd	0.47	1.3	nd	nd
32	SPME artifact	0.53	0.55	0.84	0.22	0.48	0.65	0.31	0.65

[a] AN1, *Aspergillus niger* ATCC 16404; AN2, *A. niger* DSM 821; BC1, *Botrytis cinerea* 5901/2; BC2, *B. cinerea* 02/FB II/2.1; SC1, *Saccharomyces cerevisiae* Zymaflor VL1; SC2, *S. cerevisiae* Uvaferm 228; CC1, *Corynespora cassiicola* DSM 62475; control, chemical control experiment; nd, not detected.

been reported (*9, 11, 14, 27*). However, with this study we demonstrate for the first time that *C. cassiicola* DSM 62475, too, is capable of producing linalool oxides from linalool; furthermore, this proceeds in a highly selective and efficient way (more details are given under Quantification of the Major Biotransformation Products). The presence of methylheptenone and methylheptenol in cultures of *A. niger* ATCC 16404, *A. niger* DSM 821, and *C. cassiicola* DSM 62475 most probably resulted from the biotransformation of citronellol, geraniol, and nerol, which were found as impurities in the substrate (*17, 28*). Small amounts of α-terpineol in some cultivation broths resulted from either chemical conversion of linalool and citronellol (*15, 28*) or biotransformation by *A. niger* and *S. cerevisiae* (*11, 18*). The chemical conversion of (±)-linalool (purity ≥ 95%) in acidified MYB liquid broth (pH 5.0) was checked by SPME-GC-MS (see **Table 2**, control). After 9 days of shaking >95% of the precursor, linalool was recovered in the control experiment, as determined by organic solvent extraction with MTBE and 1-octanol as internal standard, indicating low chemical reactivity under the biotransformation conditions. Several compounds, such as dihydrolinalool, β-myrcene, limonene, ocimene, linalool oxide, α-terpineol, nerol, citronellol, geraniol, and citral were found in the chemical control experiments (and also as impurities of the precursor), indicating nonbiological conversion reactions (*11, 15*). Nevertheless, the analytical data (**Table 2**) clearly indicate that chemical linalool oxide formation was negligible compared to the microbial transformation (cf. **Table 3**). Neither lilac aldehyde, lilac alcohol, nor 8-hydroxylinalool was detected in the chemical control test.

Identification of the Target Compounds Lilac Aldehyde and Lilac Alcohol. Figure 3 exemplarily illustrates the product peak identification after biotransformations with *B. cinerea* 5901/02 and *A. niger* DSM 821 based on fragment ion chromatograms. The mixture of standard compounds contained all four diastereoisomers of lilac aldehyde and lilac alcohol. For each compound only three peaks were obtained after nonchiral gas chromatographic separation due to a coelution of two diastereoisomers (**Figure 3C**). By comparing the fragment ion chromatograms and corresponding mass spectra at least two isomers of both lilac aldehyde and lilac alcohol were detectable at the positions indicated in the *B. cinerea* chromatogram (**Figure 3A**) and at least three isomers of each compound in the *A. niger* chromatogram (**Figure 3B**). The differences between the retention times of the biologically produced lilac aldehydes and alcohols and the standards were ≤0.08 min. Besides, the authentication of the biological diastereoisomers of lilac aldehyde and lilac alcohol was verified by comparing the retention indices with those of the analogous synthetic reference substances. The mass spectra of the target compounds produced, exemplarily illustrated in **Figure 4A**,**B**, by one lilac aldehyde and one lilac alcohol diastereoisomer showed the characteristic fragmentation pattern and high agreement with the reference compounds given in **Figure 4C**,**D**. The target compounds accumulated as only minor components in the medium (**Table 2**). In the biological control experiment under the same conditions (with inoculum but without linalool) no lilac compound was found.

Table 3. Final Concentrations and Conversion Yields of Furanoid *cis*- and *trans*-Linalool Oxide, Pyranoid *cis*- and *trans*-Linalool Oxide, and 8-Hydroxylinalool after Feed-Batch Biotransformation of (±)-Linalool by Liquid Cultures of *A. niger* ATCC 16404 (AN1) (after 9 Days of Cultivation), *A. niger* DSM 821 (AN2) (after 6 Days of Cultivation), *B. cinerea* 5901/2 (BC1) (after 9 Days fo Cultivation), *B. cinerea* 02/FB II/2.1 (BC2) (after 3 Days of Cultivation), and *C. cassiicola* DSM 62475 (CC1) (after 3 Days of Cultivation)[a]

	A. niger				*B. cinerea*				*C. cassiicola*	
	AN1		AN2		BC1		BC2		CC1	
compound	concn (mg/L)	yield (%)	concn (mg/L)	yield (%)	concn (mg/L)	yield (%)	concn (mg/L)	yield (%)	concn (mg/L)	yield (%)
furanoid *trans*-linalool oxide	nd	nd	77.0 ± 0.9	24.0 ± 2.7	nd	nd	30.7 ± 0.4	13.4 ± 1.5	157 ± 3	42.1 ± 5.6
furanoid *cis*-linalool oxide	9.3 ± 0.2	5.3 ± 0.7	76.1 ± 1.8	23.7 ± 2.9	nd	nd	26.3 ± 0.6	11.5 ± 1.4	148 ± 5	39.5 ± 6.4
pyranoid *trans*-linalool oxide	nd	nd	22.4 ± 0.3	7.0 ± 0.8	nd	nd	5.7 ± 0.1	2.5 ± 0.3	18.7 ± 0.4	5.0 ± 0.7
pyranoid *cis*-linalool oxide	nd	nd	33.4 ± 0.5	10.4 ± 1.2	nd	nd	14.0 ± 0.2	6.1 ± 0.7	33.1 ± 0.5	8.9 ± 1.1
8-hydroxylinalool	37.2 ± 1.1	21.1 ± 2.5	45.7 ± 1.4	14.2 ± 1.9	167 ± 5	59.9 ± 7.7	39.3 ± 1.2	17.2 ± 2.3	nd	nd
(±)-linalool (recovered %)	56.3 ± 9.3		1.9 ± 0.4		21.9 ± 3.5		12.8 ± 2.7		1.4 ± 0.3	

[a] Analyses were done in triplicate with 1-octanol as internal standard. The molar conversion yields were calculated from the total amount of linalool added to the culture. The residual linalool not converted by the fungi is given as (±)-linalool (recovered in percent).

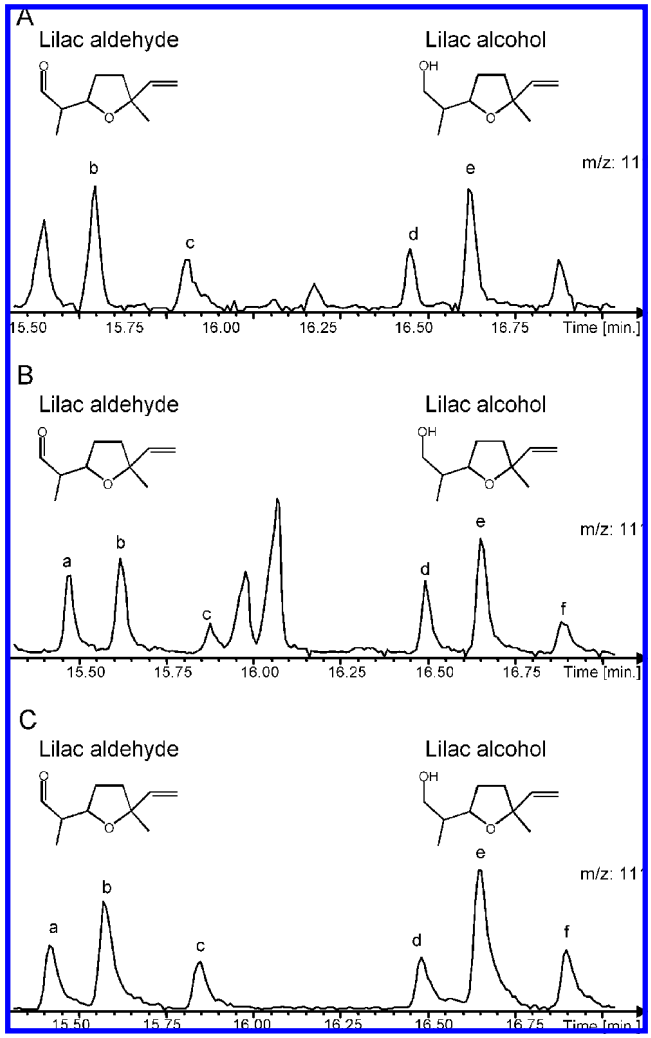

Figure 3. Fragment ion chromatograms (*m*/*z* 111) after SPME-GC-MS analysis of the medium after 9 days of feed-batch cultivation (cf. **Table 1**): (**A**) *B. cinerea* 5901/2; (**B**) *A. niger* DSM 821; (**C**) chemically synthesized lilac aldehyde and lilac alcohol reference substances.

Quantification of the Major Biotransformation Products. To determine the molar conversion yields for the major products, linalool oxide diastereoisomers, 8-hydroxylinalool, and the recovered linalool were quantified after feed-batch cultivation of *A. niger* ATCC 16404, *A. niger* DSM 821, *B. cinerea* 5901/2, *B. cinerea* 02/FB II/2.1, and *C. cassiicola* DSM 62475 (**Table 3**). The elution order of the linalool oxide diastereoisomers on the nonchiral column was furanoid *trans*-, *cis*- and pyranoid *trans*-, *cis*-. This was determined by injection of the enantiopure standards on a nonchiral column (VB-5, 5% phenyl/95% dimethylsiloxane). *A. niger* DSM 821 (AN2) converted almost 80% of the administered substrate into 153 mg/L furanoid *cis*- and *trans*-linalool oxides, 56 mg/L pyranoid *cis*- and *trans*-linalool oxides, and 46 mg/L 8-hydroxylinalool after 6 days of feed-batch cultivation (**Table 3**). We chose a specific glucose and linalool feeding to avoid any inhibitory effect by excessive substrate addition. This may be the main reason we were able to at least duplicate the conversion yield by *A. niger* DSM 821 compared to the literature (*16*). Biotransformation by *A. niger* ATCC 16404 (AN1) was significantly less pronounced (despite comparable biomass growth). *B. cinerea* 5901/2 (BC1) converted about 60% of the dosed linalool to 8-hydroxylinalool as the main product (**Table 3**), whereas linalool oxide isomers and 10-hydroxylinalool were detected by SPME analysis as minor products (cf. **Table 2**). This is in accordance with the observations by Bock et al., who found hydroxylated linalools and linalool oxides among an array of metabolites of *B. cinerea* 5901/2, whereas linalool was predominately metabolized to 8-hydroxylinalool (>90%) (*15*). Interestingly, *B. cinerea* 02/FB II/2.1 (BC2) produced a greater variety of metabolites with 39 mg/L 8-hydroxylinalool, 57 mg/L furanoid *cis*- and *trans*-linalool oxides, and 20 mg/L pyranoid *cis*- and *trans*-linalool oxides. Rapp and Mandry also identified a comparable mixture of ω-hydroxylated and oxidized compounds after biotransformation of linalool by a self-isolated *Botrytis* strain (*27*). In addition, 10-hydroxylinalool was also found as a byproduct of linalool biotransformation in minor concentrations by both groups (*15, 27*). Surprisingly, *C. cassiicola* DSM 62475 (CC1), which had not yet been reported to be active toward linalool, turned out to be the most actively transforming strain: it consumed >96% of the 340 mg/L (±)-linalool added and produced 357 mg/L linalool oxide isomers in just 3 days of feed-batch cultivation corresponding to a molar conversion yield close to 100% when compared with the amount of linalool consumed. The product formation occurred in a highly stereoselective way as described in the next section. Neither 8-hydroxylinalool nor other significant byproducts were detected (**Table 3**). Due to the fact that for all strains almost equivalent diastereoisomeric ratios between *cis*- and *trans*-linalool occurred, the substrate epoxidation does obviously not depend on the (±)-linalool configuration at the C-3 position (*29*). **Figure 5** exemplarily illustrates the kinetics of main product formation by *C. cassiicola* DSM 62475, *A. niger* DSM 821, and *B. cinerea* 5901/2. The productivity of *C. cassiicola* DSM 62475, expressed as the sum of linalool conversion products accumulated during the first 3 days, was 120 mg/L·day and, thus, almost 3 times

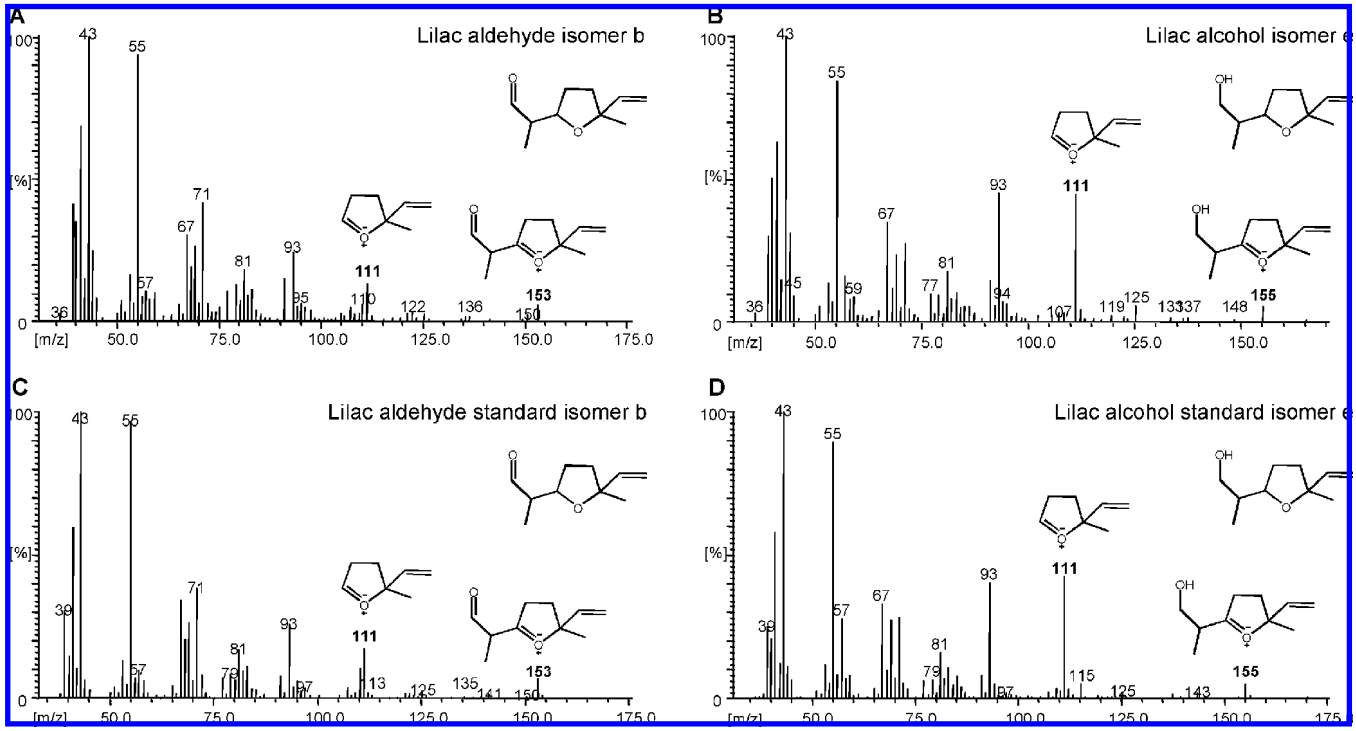

Figure 4. SPME-GC-MS pattern of target molecules lilac aldehyde and lilac alcohol: (**A**, **B**) Examples of target isomers produced by *B. cinerea* 5901/2 [lilac aldehyde at t_r = 15.692 min (peak b in **Figure 3A**); lilac alcohol at t_r = 16.667 min (peak e in **Figure 3A**)]; (**C**, **D**) mass spectra of the chemically synthesized reference compounds [lilac aldehyde at t_r = 15.642 min (peak b in **Figure 3C**); lilac alcohol at t_r = 16.662 min (peak e in **Figure 3C**)].

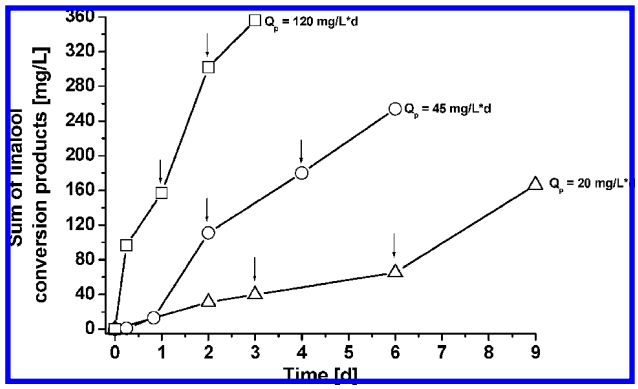

Figure 5. Formation kinetics of the main linalool biotransformation products of *C. cassiicola* DSM 62475 (sum of linalool oxide diastereoisomers) (□), *A. niger* DSM 821 (sum of linalool oxide diastereoisomers and 8-hydroxylinalool) (○), and *B. cinerea* 5901/2 (8-hydroxylinalool) (△). The arrows indicate time points of supplementation with linalool and glucose. Q_p = average productivity.

higher than that of *A. niger* DSM 821. This observation can be explained by a faster growth: at the end of the cultivation *C. cassiicola* DSM 62475 had consumed >88% of the added glucose (17 g/L) and built up a cell dry weight of 8.8 g/L; the pH of the broth was 5.4. In contrast, *A. niger* DSM 821 consumed 8.6 g/L glucose accompanied by a cell dry weight formation of 4.5 g/L and a pH of 4.3 at the end of the cultivation period (data not shown). Compared with *A. niger* and *C. cassiicola*, the productivity of *B. cinerea* remained low (20 mg/L·day, **Figure 5**). For all strains, even higher product concentrations seemed to be achievable because stationary growth phases had not yet been reached. Compared to other microbial linalool biotransformations (*11, 15, 16, 27, 29*), *C. cassiicola* DSM 62485 investigated in the present study showed a remarkable capability of producing linalool oxides in high conversion yields and with high selectivities. Submerged cultivation of *C. cassiicola* on a bioreactor scale may thus be an interesting option for the technical production of natural linalool oxides, which are of interest to the fragrance industry as lavender notes (*30*).

Analysis of the Stereoisomeric Distribution of Furanoid and Pyranoid Linalool Oxides by Enantioselective GC. To analyze the enantiomeric and diastereoisomeric distribution of linalool oxides produced by *A. niger* DSM 821 (AN2), *B. cinerea* 02/FB II/2.1 (BC2), and *C. cassiicola* DSM 62475 (CC1), the elution order on a DM-β-CD [(2,6-di-*O*-methyl)-β-cyclodextrin chiral stationary phase] column was determined by co-injection of enantiopure and racemic standards. The elution order of furanoid linalool oxides was *trans*-(2*R*,5*R*), *trans*-(2*S*,5*S*), *cis*-(2*R*,5*S*), *cis*-(2*S*,5*R*), as exemplarily illustrated in **Figure 6** for the analysis of the organic fraction of the *A. niger* DSM 821 culture. For pyranoid linalool oxides, the *trans*-(2*R*,5*S*) and *cis*-(2*S*,5*S*) diastereoiomers coeluted on this chiral phase (**Figure 6**, pyranoid linalool oxide, peaks a, b + c, d); nevertheless, the correct elution order, which is *trans*-(2*S*,5*R*), *trans*-(2*R*,5*S*), *cis*-(2*S*,5*S*), *cis*-(2*R*,5*R*), was solved by separate injection of enantiopure standards. The stereoisomeric distribution of the coeluted isomers could be calculated from the concentrations of the *cis*/*trans* diastereoisomers, which had been determined beforehand (cf. **Table 3**). The same elution order of linalool oxide stereoisomers was found with PME-β-CD (permethyl-β-cyclodextrine) and PET-β-CD (perethyl-β-cyclodextrine) as chiral stationary phases (*11, 31*). The pyranoid *trans*-(2*R*,5*S*) and *cis*-(2*S*,5*S*) linalool oxide enantiomers were presumably not separable due to a lower apolarity of DM-β-CD chiral stationary phase compared to PME-β-CD and PET-β-CD chiral stationary phases. It has been demonstrated that the cyclization of linalool into the furanoid and pyranoid linalool oxides can proceed by two distinct pathways. The diastereoisomers of 6,7-epoxylinalool, which are formed by epoxidation of linalool at C6−C7 position, have been isolated from *Carica papaya* fruit and proposed as biogenetic precursors for the

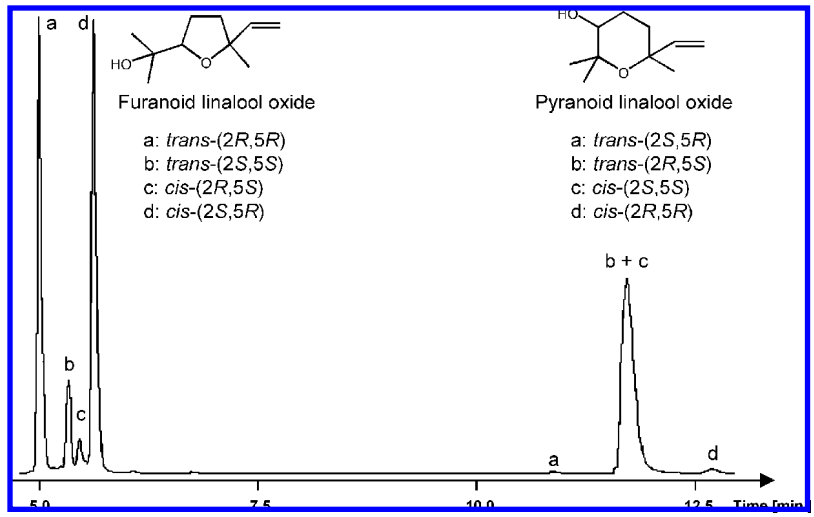

Figure 6. Enantioselective GC analysis of the furanoid and pyranoid linalool oxide stereoisomers from the organic fraction of *A. niger* DSM 821 (AN2) culture after biotransformation of linalool.

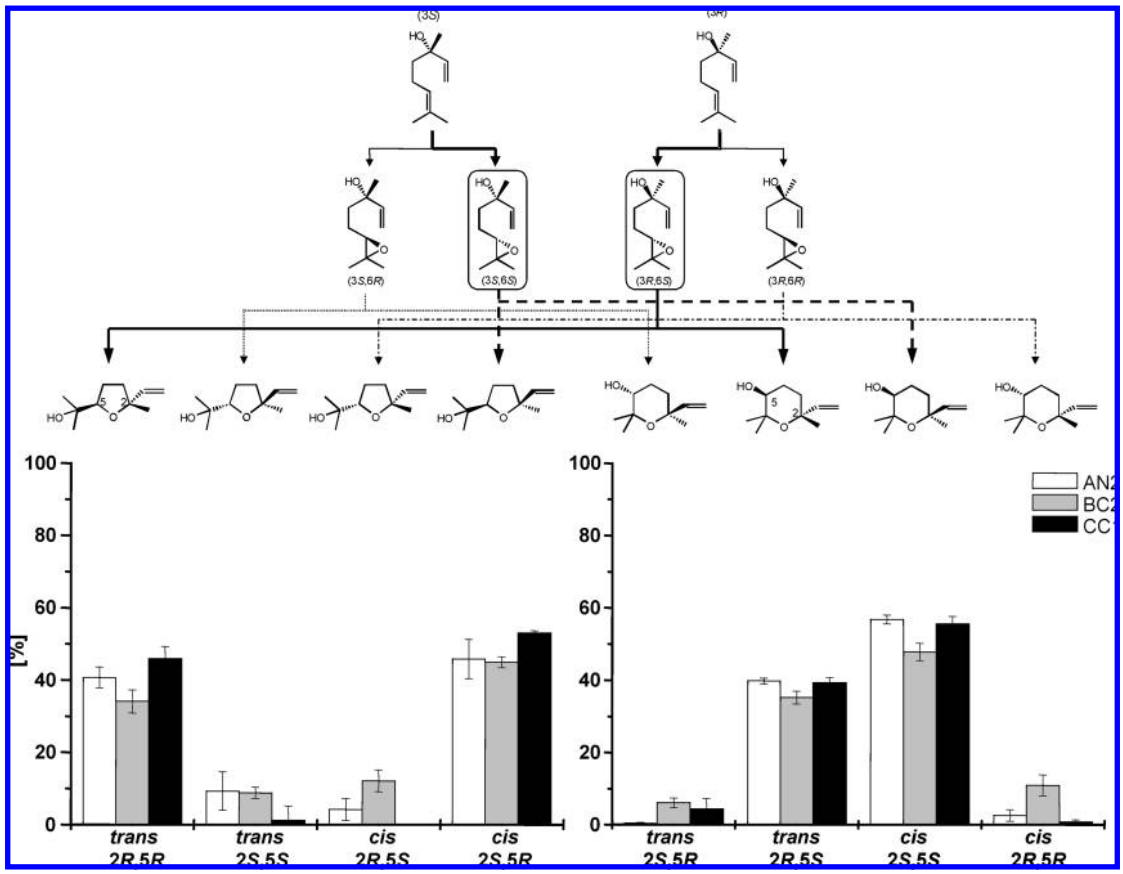

Figure 7. Stereoisomeric distribution in percentage of furanoid and pyranoid linalool oxides after analysis of organic solvent extracts of the feed-batch biotransformation broths of *A. niger* DSM 821 (AN2), *B. cinerea* 02/FB II/2.1 (BC2), and *C. cassiicola* DSM 62475 (CC1). One hundred percent furanoid linalool oxides corresponds to a total product concentration of 153, 57, and 305 mg/L for AN2, BC2, and CC1, respectively. One hundred percent pyranoid linalool oxides corresponds to a total product concentration of 56, 20, and 52 mg/L for AN2, BC2, and CC1, respectively. Above each series of columns the corresponding linalool oxide structure is depicted with its postulated biosynthetic pathway from (3*R*)- and (3*S*)-linalool via the corresponding 6,7-epoxylinalool. The pathways predominantly followed by the fungi investigated are highlighted in bold. The key intermediates, the 6*S*-configured 6,7-epoxylinalools, are framed. Mean values of duplicate measurements, indicated by error bars, are given.

formation of furanoid and pyranoid linalool oxides (*32*). In juices of Muscat grapes, it was shown that there was an in vitro acid-catalyzed formation of 3,7-dimethyloct-1-ene-3,6,7-triol from 6,7-epoxylinalool, which led to furanoid linalool oxides at pH <3.5 and/or upon heat treatment (*33*). More recently, Luan et al. proved that both mechanisms are responsible for the formation of linalool oxides in intact grape berries of *Vitis vinifera* L. cv. Morio Muscat by in vivo feeding experiments using mixed labeled d_2,^{18}O-linalool (*34, 35*). **Figure 7** illustrates the stereoisomeric distribution of the linalool oxides produced during biotransformation of a racemic mixture of (*R*)-(−)-linalool and (*S*)-(+)-linalool by *A. niger* DSM 821, *B. cinerea* 02/FB II/2.1, and *C. cassiicola* DSM 62475. The diastereomers furanoid *trans*-(2*R*,5*R*) and *cis*-(2*S*,5*R*) linalool oxide (from 37

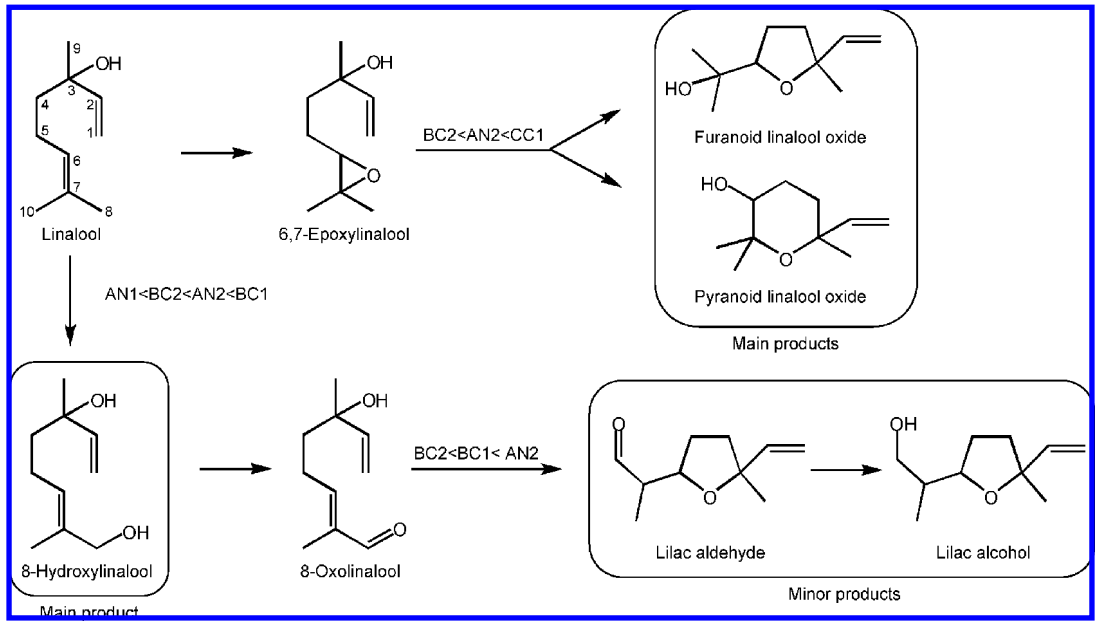

Figure 8. Postulated biotransformation of linalool to furanoid, pyranoid linalool oxide, and 8-hydroxylinalool as main products and lilac aldehyde and lilac alcohol as minor products by *A. niger* ATCC 16404 (AN1), *A. niger* DSM 821 (AN2), *B. cinerea* 5901/02 (BC1), *B. cinerea* 02/FBII/2.1 (BC2), and *C. cassiicola* DSM 62475 (CC1). For each biotransformation pathway the strains are classified in ascending order of catalytic activity.

to 55% per isomer) and pyranoid *trans*-(2*R*,5*S*) and *cis*-(2*S*,5S) linalool oxide (from 35 to 60% per isomer) occurred as main products and were roughly found as mixtures of equivalent concentrations. The absolute configuration of these compounds suggested that they had been stereoselectively formed via 6*S*-configured 6,7-epoxylinalool, as highlighted in **Figure 7**, which occurred as a pair of diastereomers due to the fact that their synthesis was independent of the C-3 configuration of (±)-linalool. Therefore, it can be postulated that (3*S*,6*S*)- and (3*R*,6*S*)-6,7-epoxylinalool are the dominating key intermediates in these microbial (±)-linalool transformations, because the minor furanoid and pyranoid linalool oxides isomers did not exceed 12% of the respective total product concentration. Moreover, a chemical linalool oxide formation can be excluded because a pH of ≤3.5, necessary for an acid-catalyzed reaction (*11*), was not given. A comparable biosynthetic pathway was postulated for *D. gossypina* ATCC 10936 (*29*). To our knowledge, this is also the first report on the microbial formation of lilac aldehyde and lilac alcohol. Previously, biogenetic investigation in *S. vulgaris* using deuterium-labeled precursors has shown that lilac aldehydes and lilac alcohols are synthesized from linalool via 8-hydroxylinalool and 8-oxolinalool (*3, 4*). More recently, Matich et al. have elucidated an analogous pathway leading to lilac compounds in *Actinidia arguta* flowers by the same approach (*5*). Because we also found 8-hydroxylinalool as the main linalool metabolite in the cultures where lilac compounds were detected, the same reaction sequence as in plants may be postulated for the biotransformation of linalool to lilac compounds in fungi (**Figure 8**). However, a rough estimation by comparing the peak areas of the products with those of the standards revealed that not more than 100−200 μg/L lilac compounds had been produced. It is not surprising that during previous investigations of linalool biotransformations neither lilac alcohol nor lilac aldehyde was found (*8, 11, 15*), although they are structurally very similar to furanoid linalool oxide, because these molecules were only produced at very low concentrations under conventional conditions, as illustrated by our initial investigations. Not until we carried out a direct comparison based on retention times and mass spectra with chemically synthesized reference compounds and applied an optimized, strain-specific feed-batch cultivation strategy were we able to undoubtedly identify the desired target compounds as minor biotransformation products. **Figure 8** summarizes the pathways discussed in this paper leading to the major products, linalool oxides and 8-hydroxylinalool, and the lilac compounds as novel metabolic byproduct found in fungal biotransformations of linalool. The low concentration of the latter compounds is most probably the result of an inherent low expression and/or (side) activity of the respective enzymes involved. The concentrations of the lilac compounds are obviously too low to make a molecular biological or process engineering approach a realistic option for obtaining economically relevant product concentrations. Nevertheless, the discovery of a biosynthetic pathway to lilac compounds from linalool in fungi, such as *Botrytis*, will surely complement our knowledge of aroma formation mechanisms during winemaking, where the role of fungal biotransformation products from linalool, such as linalool oxide and 8-hydroxylinalool, have already been described (*16, 17*).

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LITERATURE CITED

(1) Wakayama, S.; Namba, S.; Hosoi, K.; Ohno, M. The synthesis and absolute configurations of lilac alcohols, new naturally occurring odours ingredients of lilac flower. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 3183–3187.

(2) Kreck, M.; Mosandl, A. Synthesis, structure elucidation, and olfactometric analysis of lilac aldehyde and lilac alcohol stereoisomers. *J. Agric. Food Chem.* **2003**, *51* (2), 2722–2726.

(3) Kreck, M.; Püschel, S.; Wüst, M.; Mosandl, A. Biogenetic studies in *Syringa vulgaris* L.: synthesis and biotransformation of deuterium-labeled precursors into lilac aldehydes and lilac alcohols. *J. Agric. Food Chem.* **2003**, *51* (2), 463–469.

(4) Burkhardt, D.; Mosandl, A. Biogenetic studies in *Syringa vulgaris* L.: biotransformation of ^{18}O (2*H*)-labeled precursors into lilac aldehydes and lilac alcohols. *J. Agric. Food Chem.* **2003**, *51*, 7391–7395.

(5) Matich, A. J.; Bunn, B. J.; Comeskey, D. J.; Hunt, M. B.; Rowan, D. D. Chirality and biosynthesis of lilac compounds in *Actinidia arguta* flowers. *Phytochemistry* **2007**, *68*, 1746–1751.
(6) Krasnobajew, V. Terpenoids. In *Biotechnology*; Rehm, H.-J., Reed, G., Eds.; Verlag Chemie: Weinheim, Germany, 1984; Vol. 6a, pp 97–125.
(7) Kieslich, K.; Abraham, W.-R.; Stumpf, B.; Thede, B.; Washausen, P. Transformations of terpenoids. In *Progress in Essential Oil Research*; Brunke, H. J., Ed.; de Gruyter: Berlin, Germany, 1986; 367−394.
(8) Demyttenaere, J. C. R. Biotransformation of terpenoids by microorganisms. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, The Netherlands, 2001; Vol. 25, pp 125–178.
(9) Schrader, J.; Berger, R. G. Biotechnological production of terpenoid flavor and fragrance compounds. In *Biotechnology*, 2nd ed.;Rehm, H.-J., Reed, G., Eds.; Wiley-VCH: Weinheim, Germany, 2001; Vol. 10, pp 377–383.
(10) Bauer, K.; Garbe, D.; Surburg, H. *Common Fragrance and Flavor Materials*, 2nd ed.; Wiley-VCH: Weinheim, Germany, 2001; p 28.
(11) Demyttenaere, J. C. R.; Willemen, H. M. Biotransformation of linalool to furanoid and pyranoid linalool oxides by *Aspergillus niger*. *Phytochemystry* **1998**, *47*, 1029–1036.
(12) Wüst, M.; Mosandl, A. Important chiral monoterpenoid ethers in flavours and essential oils - enantioselective analysis and biogenesis. *Eur. Food Res. Technol.* **1999**, *209*, 3–11.
(13) Weinert, B.; Wüst, M.; Mosandl, A.; Hanssum, H. Stereoisomeric flavour compounds. LXXVIII. Separation and structure elucidation of the pyranoid linalool oxide stereoisomers using common gas chromatographic phases, modified cyclodextrin phases and nuclear magnetic resonance spectroscopy. *Phytochem. Anal.* **1998**, *9*, 10–13.
(14) Madyastha, K. M.; Murthy, N. S. R. K. Regiospecific hydroxylation of acyclic monoterpene alcohols by *Aspergillus niger*. *Tetrahedron Lett.* **1988**, *99* (5), 579–580.
(15) Bock, G.; Benda, I.; Schreier, P. Biotransformation of linalool by *Botrytis cinerea*. *J. Food Sci.* **1986**, *51*, 659–662.
(16) Demyttenaere, J. C. R.; Adams, A.; Vanoverschelde, J.; De Kimpe, N. Biotransformation of (*S*)-(+)-linalool by *Aspergillus niger*: an investigation of the culture conditions. *J. Agric. Food Chem.* **2001**, *49*, 5895–5901.
(17) Demyttenaere, J. C. R.; del Carmen Herrera, M.; De Kimpe, N. Biotransformation of geraniol, nerol and citral by sporulated surface culture of *Aspergillus niger* and *Penicillium* sp. *Phytochemistry* **2000**, *55*, 363–373.
(18) King, A. J.; Dickinson, J. R. Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Res.* **2003**, *3*, 53–62.
(19) Behr, D.; Wahlberg, I.; Nishida, T.; Enzell, C. R. (2*E*,6*S*)-2,6-dimethyl-2,7-octadiene-1,6-diol, a new monoterpenoid from Greek tobacco. *Acta Chem. Scand.* **1978**, *B32*, 228–229.
(20) Askari, C.; Hener, U.; Schmarr, H.-G.; Rapp, A.; Mosandl, A. Stereodifferentiation of some chiral monoterpenes using multidimensional gas chromatography. *Fresenius' J. Anal. Chem.* **1991**, *340*, 768–772.
(21) Ettre, L. S. Das Retentionsindex-System, seine Anwendung zur Identifizierung von Substranzen und Charakterisierung von flüssigen Phasen. *Chromatographia* **1973**, *6* (12), 525–532.
(22) Saroglou, V.; Dorizas, N.; Kypriotakis, Z.; Skaltsa, H. D. Analysis of the essential oil composition of eight *Anthemis* species from Greece. *J. Chromatogr., A* **2006**, *1104*, 313–322.
(23) Alissandrakis, E.; Tarantilis, P. A.; Paschalis, C. H.; Polissiou, M. Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic−mass spectrometric analysis. *Food Chem.* **2007**, *100*, 396–404.
(24) Sikkema, J.; de Bont, J. A. M.; Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **1995**, *59* (2), 201–202.
(25) Werf van der, M. J.; de Bont, J. A. M.; Leak, D. J. Opportunities in microbial biotransformation of monoterpenes. In *Biotechnology of Aroma Compounds*; Advances in Biochemical Engineering/Biotechnology 55; Scheper, T., Berger, R. G., Eds.; Springer: Berlin. Germany, 1997; pp 147–177.
(26) Sikkema, J.; de Bont, J. A. M.; Poolman, B. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **1994**, *269* (11), 8022–8028.
(27) Rapp, A.; Mandrey, H. Influence of *Botrytis cinerea* on the monoterpene fraction of wine aroma. In *Bioflavour '87*; Schreier, P., Ed.; de Gruyter: Berlin, Germany, 1988; pp 445–452.
(28) Demyttenaere, J. C. R.; Vanoverschelde, J.; De Kimpe, N. Biotransformation of (*R*)-(+)- and (*S*)-(−)-citronellol by *Aspergillus* sp. and *Penicillium* sp., and the use of solid-phase microextraction for screening. *J. Chromatogr., A* **2004**, *1027*, 137–146.
(29) Abraham, W.-R.; Stumpf, B.; Arfmann, H.-A. Chiral intermediates by microbial epoxidations. *J. Essent. Oil Res.* **1990**, *2*, 251–257.
(30) Schrader, J. Microbial flavour production. In *Flavours and Fragrances*; Berger, R. G., Ed.; Springer-Verlag: New York, 2007; pp 507–574.
(31) Mosandl, A. Enantioselective and isotope analysis−key steps to flavour authentication. In *Flavours and Fragrances*; Berger, R. G., Ed.; Springer-Verlag: New York, 2007; pp 379–408.
(32) Winterhalter, P.; Katzenberger, D.; Schreier, P. 6,7-Epoxy-linalool and related oxygenated terpenoids from *Carica papaya* fruit. *Phytochemistry* **1986**, *25*, 1347–1350.
(33) Williams, P. J.; Strauss, C. R.; Wilson, B. Hydroxylated linalool derivatives as precursors of volatiles monoterpenes of muscat grape. *J. Agric. Food Chem.* **1980**, *28*, 766–761.
(34) Luan, F.; Mosandl, A.; Degenhardt, A.; Gubesch, M.; Wüst, M. Metabolism of linalool and substrate analogs in grape berry mesocarp of *Vitis vinifera* L. cv. Morio Muscat: demonstration of stereoselective oxygenation and glycosylation. *Anal. Chim. Acta* **2006**, *563*, 353–364.
(35) Luan, F.; Mosandl, A.; Gubesch, M.; Wüst, M. Enantioselective analysis of monoterpenes in different grape varieties during berry ripening using stir bar sorptive extraction- and solid phase extraction−enantioselective multidimensional gas chromatography−mass spectroscopy. *J. Chromatogr., A* **2006**, *1112*, 369–374.

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