Biotransformation of (*S*)-(+)-Linalool by *Aspergillus niger*: An Investigation of the Culture Conditions

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The biotransformation of (*S*)-(+)-linalool by different *Aspergillus niger* strains was studied, using submerged shaken liquid cultures. One strain, *A. niger* DSM 821, was able to convert the substrate to *cis*- and *trans*-furanoid linalool oxide (yield 30% and 5%, respectively) and *cis*- and *trans*-pyranoid linalool oxide (yield 14% and 1.5%, respectively). The main metabolites, *cis*-(2*S*,5*R*)-furanoid and *cis*-(3*S*,6*S*)-pyranoid linalool oxide, have a sweet, floral, creamy odor and are used in perfumery. The culture conditions involved, such as the composition of the broth and the type and concentration of cosolvent applied and possible adaptation to the substrate during inoculation, were investigated. It was found that (*S*)-(+)-linalool was converted much better than (*R*)-(−)-linalool and that no significant chemical conversion of the substrate occurred in control flasks at pH 3.5. Three cosolvents for improving the solubility of linalool in the culture broths were compared, namely MeOH, EtOH, and acetone. The highest bioconversion yields were obtained when the substrate was applied as a diluted solution in acetone. Screening of the fungi for their biotransformation capacity was performed by solid-phase microextraction.

Keywords: *Aspergillus niger; fungi; biotransformation; bioconversion; linalool; (S)-(+)-linalool; furanoid linalool oxides; pyranoid linalool oxides;* α*-terpineol; SPME*

INTRODUCTION

Together with the terpene alcohol linalool, linalool oxides are important constituents of oolong and black tea (*1*). Linalool oxides are also found in many essential oils and are characteristic of fruit aromas; they have an earthy-flowery, slightly bergamot-like odor (*2*). Linalool oxide is used in perfumery (e.g., for lavender notes) and for reconstitution of essential oils. Linalool oxide consists of four furanoid linalool oxides [5-(1-hydroxy-1-methylethyl)-2-methyl-2-vinyltetrahydrofuran] and four pyranoid linalool oxides [3-hydroxy-2,2,6-trimethyl-6-vinyltetrahydropyran]. The structures of the eight stereoisomers are shown in Figure 1.

The enantiomeric distribution of the linalool oxides can be used for authenticity control of essential oils. Literature data on the correct elution order of the four furanoid and four pyranoid linalool oxides on chiral columns have been very confusing and contradictory. Recently, the correct elution order for furanoid (*3*, *4*) and pyranoid (*5*) linalool oxides has been described and reviewed (*6*). From the analysis of the different stereoisomers of the linalool oxides by enantio-GC-olfactometry, it was shown that the isomers have different odor characteristics (*6*).

Although the microbial conversion of linalool by *Pseudomonas pseudomallei* (*7*) and *P. incognita* (*8*) and its degradation by a soil pseudomonad (*9*−*11*) was studied in the 1970s, the fungal conversion of linalool was only reported in the late 1980s. The fungus *Aspergillus niger*, isolated from garden soil, was able to convert linalyl acetate to linalool by hydrolysis and further to 8-hydroxylinalool by hydroxylation of the C-8 methyl group (*ω*-hydroxylation) (*12*, *13*). The bioconversion of linalool by *Botrytis cinerea*, a fungus of high interest in winemaking, was also described (*14*, *15*). More recently the microbial epoxidation of linalool and its further conversion to furanoid and pyranoid linalool oxides by the fungus *Diplodia gossypina* ATCC 10936 have been reported (*16*, *17*).

Previously we reported the biotransformation of (±)-linalool and (*R*)-(−)-linalool to furanoid and pyranoid linalool oxides by liquid cultures of *Aspergillus niger* (*18*). More detailed further studies on the bioconversion of (*S*)-(+)-linalool by *A. niger* DSM 821 and the influence of various culture conditions on the conversion capacity are now described.

MATERIALS AND METHODS

Microorganisms and Cultivation. More than 60 fungal strains were used in this study. They belonged to the following species: *Penicillium digitatum*, *P. italicum*, *P. roquefortii*, *P. chrysogenum*, *P. lividum*, *Aspergillus niger*, *A. versicolor*, *Botryodiplodia malorum*, *Rhizopus oryzae*, *Beauveria bassiana*, *Cunninghamella elegans*, *C. blakesleeana*, *Hyphozima roseoniger*, *Corynespora cassiicola*, *Chaetomium cochliodes*, and *Mortierella* sp. The fungi were either isolated from spoiled fruit or contaminated media or obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). After screening experiments, some fungal strains were selected for further study and identified as follows: ANA (*Aspergillus niger* DSM 821), AND (*A. niger* DSM 63263), and ANV (*A. niger* isolated from spoiled plant material, kindly provided by Vioryl S. A., Kifissia, Greece). The fungi were cultivated and conserved by periodic replications (every 2 weeks) on malt extract agar (MEA: malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2% − pH 5.4).

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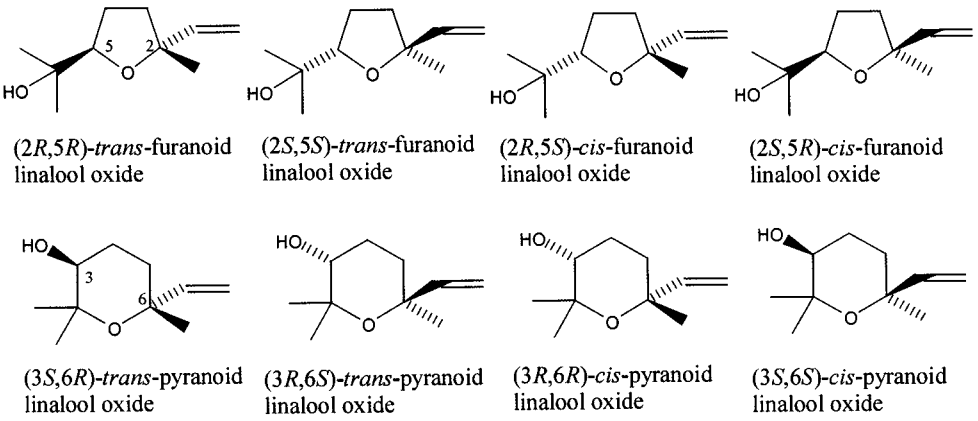

Figure 1. Structures of the eight stereoisomers of furanoid and pyranoid linalool oxides.

Screening of Sporulated Surface Cultures of Fungi by SPME. For screening, the fungi were cultivated as small sporulated surface cultures in 40 mL SPME-vials (Supelco Inc., Bellefonte, U.S.A.) (*19, 20*). The vials were filled with 10 mL of medium (MEA), autoclaved, inoculated with fresh spores, and then covered with cotton wool, and the cultures were incubated at 30 °C during 24 h and at room temperature during 48 h, after which complete growth and sporulation had taken place. To each sporulated surface culture, 5 μL of a solution of (±)-linalool (purum, 97%, Aldrich) in EtOH (10%) was sprayed. The vials were covered with PTFE-silicone septa and open top phenolic closures (Supelco) and stored at room temperature until the start of the headspace SPME-extraction. During extraction, the SPME fibers were exposed to the headspace of the cultures for 5, 15, or 30 min at 25 °C.

To select the best sampling parameters, 20 mL SPME-vials were filled with 10 mL of distilled water into which 10 μL of the same solution of (±)-linalool in EtOH (10%) or solutions containing linalool and furanoid linalool oxides (mixture of the four isomers) in different ratios were added. Different SPME fibers were used, namely 100 μm poly(dimethylsiloxane) (PDMS), 65 μm poly(dimethylsiloxane)/divinylbenzene, 50/30 μm divinylbenzene/Carboxen on poly(dimethylsiloxane), 65 μm Carbowax/divinylbenzene (CWDB), and 85 μm polyacrylate (PA) (Supelco Inc., Bellefonte, U.S.A.). Different sampling times (15, 30, and 60 min) and temperatures (20, 25, and 30 °C) were compared.

Control Experiments with Culture Broths at Different pH. To test the possible conversion of linalool due to acid conditions, a control experiment was run. Three 250 mL control flasks with YMPG broth (yeast extract 0.5%, malt extract 1%, bacteriological peptone 0.5%, glucose 1%) at different pH were used, namely without pH adjustment (YMPG medium pH 6.3), with adjustment to pH 5, and with adjustment to pH 3.5. The pH adjustment was done with 20% (v/v) HOAc. Substrate addition to the control media was performed by spraying 0.5 mL of a 10% (v/v) solution of the substrate in absolute EtOH. After 15 min and after 1 week, samples were taken and extracted with Et_2O. The samples were analyzed by GC/MS.

Biotransformation of Linalool by Submerged Liquid Cultures. The biotransformations were run during 8 days with the following *A. niger* strains: ANA, AND, and ANV. The fungi were cultivated in 500 mL conical flasks, filled with 100 mL of liquid medium (YMPG: malt extract 1%, glucose 1%, bacteriological peptone 0.5%, yeast extract 0.5%; MYB (*21*): malt extract 2%, glucose 1%, bacteriological peptone 1%, yeast extract 0.3%; MEB: malt extract 2%, glucose 2%, bacteriological peptone 0.1%) as described previously (*18, 22*). Inoculation was performed with spore suspensions. The test substrates [(*R*)-(−)-linalool, (*S*)-(+)-linalool, and (±)-linalool] were added as solutions in absolute EtOH, MeOH, or acetone. At different time intervals, 5 mL samples were taken and extracted with 2 × 2 mL Et_2O. After addition of 1 mL of a standard solution of 0.1% (v/v) *n*-decane in Et_2O, the samples were directly analyzed by GC/MS. Experiments were also run with control flasks, which contained sterile culture broth that was not inoculated and to which the substrate was added. The culture flasks were stirred at 150 rpm, at 24 °C.

Chemical Compounds. The substrates used for the biotransformation experiments were as follows: (*R*)-(−)-linalool (97%, Fluka, ee 99.3%), (*S*)-(+)-linalool (83.3%, ee 76.7%) (kindly provided by Aroma 2000 B.V., An Uden, The Netherlands), natural coriander oil (containing 69% linalool, mainly (*S*)-(+)-linalool, ee 75%, Aroma 2000 B.V.), and (±)-linalool (97%, Aldrich). As reference compounds, two standards of furanoid linalool oxides were used, namely a commercial mixture of the four isomers (97%, Fluka) and a nature identical mixture of the four isomers (99.3%, kindly provided by Aroma 2000 B.V.).

Analysis of the Samples with GC and GC-MS. GC-MS analyses were performed with a HP 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector − Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) Injector (Gerstel), and a HP5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 μm). Operating conditions were as follows: injector 250 °C; transfer line to MSD 250 °C; oven temperature: start 50 °C, programmed from 50 to 120 °C at 5 °C/min, from 120 to 200 °C at 20 °C/min, held 2 min; carrier gas (He) 1.0 mL/min; split 1/20; ionization: EI 70 eV; acquisition parameters: scanned *m/z*: 40−200 (5−15 min), 40−300 (>15 min). For the calculation of the Kováts Retention Indexes, a linear temperature program was used: from 60 to 160 °C at 3 °C/min, from 160 to 220 °C at 15 °C/min, held 5 min. Analysis of the SPME-extracts was carried out as described previously (*19*). GC-analyses were performed with a HP 6890 GC Plus, equipped with a split/splitless-injector and an FID-detector and an EC-5 column (30 m × 0.25 mm i.d.; coating thickness 0.25 μm). Operating conditions were as follows: injector 250 °C; detector 300 °C (makeup gas He 10 mL/min), oven temperature: start 50 °C, programmed from 50 to 120 °C at 5 °C/min, from 120 to 180 °C at 20 °C/min, held 2 min; carrier gas (He) 0.8 mL/min; split 1/10. Chiral GC-analyses were performed with the same GC, equipped with a Cydex-B chiral column (SGE: 50 m × 0.22 mm i.d.; coating thickness 0.25 μm). Isothermal conditions were used (95 °C) with a column head pressure of 30 psi.

Substances were identified by comparison of their mass spectra and retention indexes (Kováts Indexes) with those of reference substances (where possible) and by comparison with the NIST Mass Spectral Library (Version 1.6d, 1998).

Mass spectra and retention indexes of linalool, furanoid and pyranoid linalool oxides, and α-terpineol have been published previously (*18*).

RESULTS AND DISCUSSION

Control Experiments with Culture Broths at Different pH. The stability of the substrate (*S*)-(+)-linalool in acidic conditions was checked during a period of 1 week. It was found that the substrate did not

Table 1. Yield of *cis*- and *trans*-Furanoid Linalool Oxide and Substrate Recovery (%) after Bioconversion of (*S*)-(+)-Linalool by Liquid Cultures of *Aspergillus niger*—Samples 1, 3, and 4

	sample 1			sample 3			sample 4		
compound → strain[a] − medium ↓	*cis*-linalool oxide	*trans*-linalool oxide	linalool	*cis*-linalool oxide	*trans*-linalool oxide	linalool	*cis*-linalool oxide	*trans*-linalool oxide	linalool
ANA − YMPG	2.60	0.84	76.71	2.92	0.88	74.90	10.44	1.95	45.52
ANA − MEB	0.82	0.00	58.96	1.46	0.78	52.45	3.34	1.80	15.43
ANA − MYB	2.34	0.78	76.86	5.87	1.28	64.51	25.56	4.27	0.00
AND − YMPG	0.84	0.00	84.84	0.72	0.59	84.65	0.76	0.63	71.72
AND − MEB	0.00	0.00	83.46	0.53	0.54	74.97	0.53	0.54	55.63
AND − MYB	0.90	0.68	85.87	0.79	0.64	81.80	0.82	0.67	70.60
ANV − YMPG	0.65	0.00	87.71	0.59	0.54	83.97	0.62	0.56	70.20
ANV − MEB	0.00	0.00	52.50	0.67	0.55	37.93	0.71	0.61	7.91
ANV − MYB	0.00	0.00	87.49	0.60	0.55	85.52	0.64	0.58	69.76

[a] ANA = *Aspergillus niger* DSM 821 − AND = *A. niger* DSM 63263 − ANV = *A. niger* isolated from plant.

undergo any acid catalyzed conversion, even at pH 3.5. Only small traces of furanoid linalool oxides were recovered from the control media, in the same concentration as their initial concentration in the substrate (0.5−0.7%). After 1 week, at least 60% of the substrate was lost due to evaporation.

Screening of Fungi for Their Biotransformation Capacity with Solid-Phase Microextraction. More than 60 fungal strains, grown as small surface cultures in SPME vials, were screened for their ability to bioconvert the substrate (±)-linalool, using solid-phase microextraction (SPME) as the monitoring technique. Therefore, a method was developed to cultivate sporulated surface cultures of the fungi in small vials, and the SPME-parameters were optimized (*19, 22*). Different SPME-fibers, extraction times, and temperatures were compared. Extraction of volatiles (linalool and metabolites) was done by headspace SPME. It was found that the best SPME fiber for extraction of linalool and its metabolites furanoid linalool oxide and α-terpineol was 50/30 μm divinylbenzene/Carboxen on poly(dimethylsiloxane). The optimum adsorption time was 30 min. The best extraction temperature was 25 °C. A desorption temperature of 250 °C and desorption time of 2 min were sufficient to desorb all volatiles into the gas chromatographic inlet. Due to the relatively high volatility of linalool compared to the linalool oxides, the adsorption of linalool on the fiber was on the order of 20 times higher than the adsorption of linalool oxides when standard solutions of linalool and linalool oxides in equal concentration were extracted by headspace SPME. Therefore, SPME can only be used as a fast and qualitative screening technique for this conversion.

The most interesting conversion of linalool was the formation of furanoid linalool oxides by various *Aspergillus niger* strains.

Comparison of Different Strains and Media. In this experiment, three strains of *Aspergillus niger* were compared, namely *A. niger* DSM 821 (ANA), *A. niger* DSM 63263 (AND), and *A. niger* isolated from plant material (ANV). Inoculation was performed by 1 mL of freshly prepared spore suspensions of 1.0×10^7 spores/mL (ANA), 0.67×10^7 spores/mL (AND), and 0.77×10^7 spores/mL (ANV) (as counted with a Malassez cell) per 100 mL of culture medium. Also three culture broths were compared, namely YMPG, MYB, and MEB (see Materials and Methods). The first substrate addition took place 51 h after inoculation (250 μL of a solution of 20% (*S*)-(+)-linalool in MeOH, i.e. 50 μL of linalool). After 16 h, samples were taken and extracted, followed by a second substrate addition (250 μL of the same solution). Three more samples were taken, after 8, 24, and 96 h, respectively. The results with the yields of *cis*- and *trans*-furanoid linalool oxides, obtained from samples 1, 3, and 4, are shown in Table 1. From these data it is clear that only *A. niger* DSM 821 (ANA) was able to convert the substrate linalool to *cis*- (major product) and *trans*-furanoid linalool oxide (minor product). The best culture medium for this conversion was MYB. The amount of unconverted substrate recovered from the culture broth of the nonconverting strains was very high (up to 72%). On the other hand, the amount of unconverted substrate was zero in the culture broth of *A. niger* DSM 821 where the highest yield of *cis*-furanoid linalool oxide was obtained.

Comparison of Different Media and Comparison with Control Flasks. In this experiment the influence of the culture media (YMPG, MYB, and MEB were used) on the conversion of linalool was studied only with the strain *A. niger* DSM 821 (ANA) and the possible chemical conversion of the substrate in the absence of the fungus was also checked, using control flasks with the three media. The first substrate addition (250 μL of a 10% solution of (*S*)-(+)-linalool in MeOH = 25 μL) took place 42 h after inoculation (with 1 mL spore suspension of 1×10^6 spores/mL). After 24 h the first series of samples was taken and extracted, and a second substrate addition took place (500 μL of a 10% solution = 50 μL). The second and third series of samples were taken after 24 and 32 h, respectively. The last series of samples was taken 63 h after the third substrate addition (500 μL of the same solution), which was performed after the third sampling time. The results with the yields of *cis*- and *trans*-furanoid linalool oxides, and *cis*- and *trans*-pyranoid linalool oxides obtained from the last sample, are displayed in Table 2.

From these results it is clear that a slight chemical oxidation of (*S*)-(+)-linalool to furanoid linalool oxides occurred in the control flasks (up to 0.6%), as already shown previously for (*R*)-(−)-linalool (*18*). However, this conversion could also have taken place during storage of the substrate, since GC/MS analysis revealed that the substrate contained 0.7% *cis*- and 0.5% *trans*-furanoid linalool oxide. No pyranoid linalool oxides were detected in the control flasks, however. The conversion of (*S*)-(+)-linalool to *cis*-furanoid and pyranoid linalool oxide was carried out in good yields (17−23% and 10−13%, respectively). Some *trans*-furanoid (3−5%) and *trans*-pyranoid linalool oxide (1.3−1.8%) was also formed, mainly because the substrate (*S*)-(+)-linalool was only of 83.3% purity (ee 76.7% as checked with chiral GC). Unfortunately no pure (>99%) (*S*)-(+)-linalool is commercially available. The influence of the medium composition on the conversion yield was not so clear,

Table 2. Final Yield (%) of *cis*- and *trans*-Furanoid Linalool Oxide and *cis*- and *trans*-Pyranoid Linalool Oxide after Bioconversion of (*S*)-(+)-Linalool by Liquid Cultures of *Aspergillus niger* and Conversions in Control Flasks

culture/control	medium	*cis*-furanoid linalool oxide	*trans*-furanoid linalool oxide	*cis*-pyranoid linalool oxide	*trans*-pyranoid linalool oxide
ANA	YMPG	21.83 ± 1.75	3.65 ± 0.29	12.64 ± 1.03	1.57 ± 0.07
ANA	MEB	17.09 ± 0.63	4.80 ± 0.07	10.01 ± 0.44	1.77 ± 0.06
ANA	MYB	18.16 ± 0.37	3.06 ± 0.05	10.97 ± 0.24	1.36 ± 0.03
BLANCO	YMPG	0.56	0.58	0.00	0.00
BLANCO	MEB	0.54	0.56	0.00	0.00
BLANCO	MYB	0.60	0.62	0.00	0.00

Table 3. Final Yield of *cis*- and *trans*-Furanoid Linalool Oxide and *cis*- and *trans*-Pyranoid Linalool Oxide and Substrate Recovery (%) after Bioconversion of (*S*)-(+)-Linalool, (*R*)-(−)-Linalool, and (±)-Linalool by Liquid Cultures of *Aspergillus niger*

		furanoid linalool oxide			pyranoid linalool oxide			
medium	substrate	*cis*-linalool oxide	*trans*-linalool oxide	sum cis + trans	*cis*-linalool oxide	*trans*-linalool oxide	sum cis + trans	linalool recovery
YMPG	(*S*)-(+)-linalool	16.98	2.85	19.83	9.40	1.11	10.52	0.00
MYB	(*S*)-(+)-linalool	14.22	2.43	16.65	8.46	1.01	9.47	0.00
MYB	(*S*)-(+)-linalool	14.72	2.50	17.22	8.75	1.04	9.79	0.00
YMPG	(*R*)-(−)-linalool	0.75	3.64	4.39	0.00	1.20	1.20	49.96
MYB	(*R*)-(−)-linalool	0.92	3.05	3.97	0.00	1.00	1.00	46.77
MYB	(*R*)-(−)-linalool	0.95	3.11	4.06	0.00	1.01	1.01	48.34
YMPG	(±)-linalool	10.63	10.44	21.06	5.87	4.31	10.18	0.21
MYB	(±)-linalool	9.91	9.70	19.61	5.73	4.09	9.82	0.21
MYB	(±)-linalool	9.90	9.69	19.58	5.70	4.07	9.77	0.21

although the results obtained with YMPG were slightly better than with MEB and MYB. No significant difference could be seen between MEB and MYB. In the control flasks, more than 70% of the substrate was recovered unchanged, while in the culture flasks, all substrate was completely metabolized.

Influence of Medium Composition on the Fungal Growth and Biomass Production. To test the influence of the medium composition on the fungal growth and biomass production, six cultures of *A. niger* were grown in the three culture media, YMPG, MEB, and MYB in duplicate. Exactly 48 h after inoculation of the broths with 1 mL of a spore suspension (10^7 CFU/mL), the full grown cultures were harvested, filtered, washed, and dried for 4 h at 121 °C. The dry weight obtained in the media YMPG, MEB, and MYB was 0.56 ± 0.07, 0.51 ± 0.05, and 0.87 ± 0.08 g/100 mL, respectively. Hence the best medium for highest fungal growth (biomass production) was MYB. The two broths YMPG and MEB were not significantly different for fungal growth. Since the best medium for bioconversion is YMPG, there is no correlation between best fungal growth and best bioconversion yield.

Comparison of the Conversion of (*S*)-(+)-Linalool and (*R*)-(−)-Linalool. In this experiment the bioconversion of (*S*)-(+)-linalool was compared with the bioconversion of (*R*)-(−)-linalool and (±)-linalool, using *A. niger* DSM 821 and two media, YMPG and MYB. Inoculation was performed with 1 mL of a spore suspension of 1.1×10^6 CFU/mL. The first substrate addition was done 42 h after inoculation (250 μL of a 10% solution of linalool in MeOH). The first series of samples was taken 24 h after the first substrate addition, after which a new substrate was administered (500 μL of the same solution). After 24 and 32 h, respectively, the second and third series of samples were taken. The last substrate addition took place after the third sampling (500 μL of the same solution), and after 63 h the last series of samples was taken. The results obtained from the last series of samples are displayed in Table 3.

All samples were also analyzed by chiral GC. From these results the following conclusions could be drawn. The bioconversion of (*S*)-(+)-linalool yielded 14.2−17% (2*S*,5*R*)-*cis*-furanoid linalool oxide in its enantioselectively pure form (ee 100%) and 2.4−2.9% *trans*-furanoid linalool oxide (mainly the (2*R*,5*R*)-enantiomer produced from the (*R*)-(−)-linalool impurity, ee 78−79%) (see Figure 2). The bioconversion of (*R*)-(−)-linalool yielded 3.1−3.6% pure (2*R*,5*R*)-*trans*-furanoid linalool oxide (ee 100%) and 0.7−1% pure (2*R*,5*S*)-*cis*-furanoid linalool oxide. The bioconversion of (±)-linalool yielded 9.9−10.6% *cis*-furanoid linalool oxide (mainly (2*S*,5*R*)-enantiomer as conversion product of the (*S*)-linalool, ee 96%) and 9.7−10.4% (2*R*,5*R*)-*trans*-furanoid linalool oxide (ee 96%, obtained from the (*R*)-linalool).

From these data it is clear that the conversion of (*S*)-(+)-linalool took place with much higher yields than the conversion of (*R*)-(−)-linalool. It is interesting to note that the yields of *trans*-furanoid and *trans*-pyranoid linalool oxide from pure (*R*)-(−)-linalool were not much higher than the yields obtained from the (*R*)-(−)-linalool impurity of the (*S*)-(+)-linalool substrate solution (ee 77% pure as mentioned before) and that they were much lower than the yields obtained from (±)-linalool. It is believed that addition of pure (*R*)-(−)-linalool to the cultures causes inhibition. Indeed, a total substrate amount of 125 μL of (*R*)-(−)-linalool (i.e. 0.125% v/v) was added to the liquid cultures, whereas the maximum tolerated concentration of (*R*)-(−)-linalool for *Aspergillus niger* was shown to be 0.05% (v/v) (*23*). From Table 3 it can be seen that the final concentration of unconverted linalool in the cultures treated with (*S*)-(+)-linalool was zero, and in the cultures treated with (±)-linalool approximately 0.0003%, whereas in the cultures treated with (*R*)-(−)-linalool it was still 48−50% of the total amount of 125 μL applied, at the end of the experiment, corresponding with 61−63 μL/100 mL or 0.06% (v/v), which is an inhibitory concentration. When the total bioconversion yield is expressed as the sum of the *cis*- and *trans*-isomers of the linalool oxides, it can be concluded that the conversions of (*S*)-(+)-linalool and (±)-linalool are very similar. In both cases, the conversion yield was higher with YMPG broth than with MYB. From the commercial and application point of view

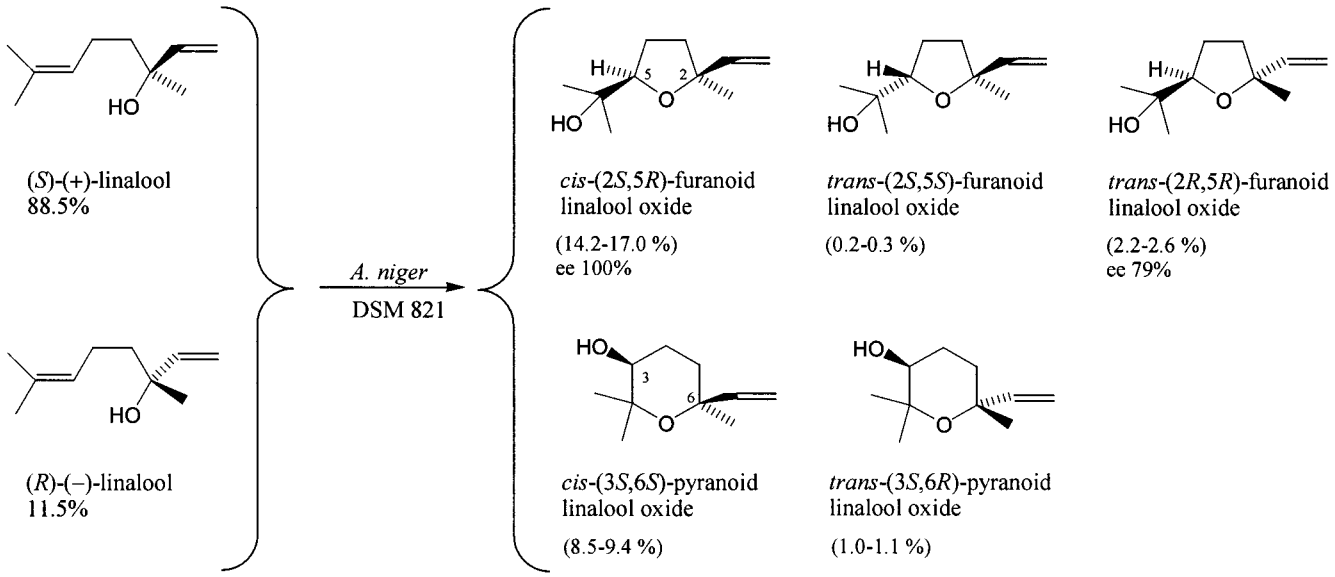

Figure 2. Bioconversion of a natural mixture of (*S*)-(+)-linalool (ee 77%) and (*R*)-(−)-linalool to furanoid and pyranoid linalool oxides by *Aspergillus niger* DSM 821.

Table 4. Final Yield (%) of *cis*- and *trans*-Furanoid and -Pyranoid Linalool Oxide after Bioconversion of (*S*)-(+)-Linalool by Liquid Cultures of *Aspergillus niger*—Influence of Culture Broth and Cosolvent Applied and Concentration of Substrate Solution Applied

medium	solvent	concn substrate solution	*cis*-furanoid linalool oxide	*trans*-furanoid linalool oxide	*cis*-pyranoid linalool oxide	*trans*-pyranoid linalool oxide
YMPG	acetone	5%	30.34 ± 0.59	5.08 ± 0.10	13.73 ± 0.41	1.54 ± 0.05
YMPG	acetone	10%	29.42 ± 0.56	4.88 ± 0.08	11.41 ± 0.06	1.31 ± 0.00
MYB	acetone	5%	26.68 ± 0.02	4.47 ± 0.02	14.11 ± 0.19	1.56 ± 0.02
YMPG	MeOH	5%	27.75 ± 1.12	4.66 ± 0.18	11.59 ± 0.28	1.41 ± 0.04
MYB	MeOH	5%	23.98	4.05	12.77	1.51

however, the conversion of (*S*)-(+)-linalool is preferable, since the obtained linalool oxides, *cis*-(2*S*,5*R*)-furanoid and *cis*-(3*S*,6*S*)-pyranoid linalool oxide exhibit a better aroma than their chiral stereoisomers. A scheme of this bioconversion is given in Figure 2.

Comparison of the Cosolvent Used, MeOH versus EtOH, and MeOH versus Acetone. In most of the previous experiments, MeOH was used as cosolvent to dissolve the water insoluble substrate in the culture medium, because MeOH was believed to be less toxic to the fungal cultures than EtOH due to its lower log *P* value (−0.76, respectively, −0.24) (*24*). In the following experiment a comparison was made between MeOH and EtOH as cosolvent. Two broths were used, YMPG and MYB, and all tests were run in duplicate. From the results (data not shown) it was clear that the conversion yields with the broth YMPG (22.59 ± 1.52% with MeOH and 20.28 ± 1.03% with EtOH as cosolvent) were now significantly higher than with the broth MYB (11.83 ± 2.63% with MeOH and 8.20 ± 0.32% with EtOH as cosolvent). It can also be concluded that the cosolvent MeOH was slightly better than EtOH, because higher yields were obtained (however not significant), but also lower amounts of unconverted substrate were recovered from the broths.

In a second experiment MeOH and acetone were compared as cosolvent, and also the effect of the concentration of the cosolvent on bioconversion activity was studied. Two culture broths were used, YMPG and MYB, and the substrate was administered in three different ways: as a 5% solution in acetone, as a 10% solution in acetone, or as a 5% solution in MeOH. Substrate addition was always performed in such a way that exactly the same amount of substrate was added to the cultures, e.g., 500 μL of the 5% solution or 250 μL of the 10% solution. The first substrate addition took place at the time of inoculation (12.5 μL) and the second after 41 h (25 μL). The first series of samples was taken 24 h after this substrate addition. After 10 h the following substrate addition was carried out (25 μL), and samples were taken after 19 h, after which the last substrate addition was performed (1 mL of the 5% solution or 500 μL of the 10% solution = 50 μL substrate). The last series of samples was taken 66 h after the last substrate addition. After this last substrate addition the final concentration of the cosolvent was approximately 2.2% and 1.1% in the cultures treated with solutions of 5% and 10%, respectively.

The results obtained from the last series of samples are displayed in Table 4. No substrate recovery was noticed in any of the samples. When the effect of the broth composition on bioconversion activity is studied for the cultures treated with the solutions of 5% in either acetone or MeOH, it can be concluded that the yields for furanoid linalool oxides were significantly higher with YMPG than with MYB. The yields of pyranoid linalool oxides were similar for both media. When the effect of the acetone concentration on the bioconversion capacity is studied for the YMPG broth, it can be seen that the yield of furanoid linalool oxides was not affected by the amount of cosolvent applied, whereas the yield of pyranoid linalool oxides was slightly higher when diluted substrate solutions (5%) were administered to the cultures. When the two cosolvents, acetone and MeOH, are compared, it can be concluded that the yields of both furanoid and pyranoid linalool oxides were

Table 5. Final pH of the Broths and Final Yield of *cis*- and *trans*-Furanoid and -Pyranoid Linalool Oxide and α-Terpineol (%) after Bioconversion of Coriander Oil by Liquid Cultures of *Aspergillus niger*—Values Are Average Values ± SD

	furanoid linalool oxide		pyranoid linalool oxide			
medium	*cis*-	*trans*-	*cis*-	*trans*-	α-terpineol	pH
BLANCO	0.20 ± 0.35	0.54 ± 0.05	0.00	0.00	6.94 ± 1.86	3.03 ± 0.08
YMPG	31.32 ± 1.60	4.87 ± 0.45	12.84 ± 3.42	1.31 ± 0.28	0.28 ± 0.06	5.65 ± 0.83
MEB	19.64 ± 5.69	6.47 ± 0.07	11.27 ± 3.34	2.40 ± 0.02	5.52 ± 2.65	2.85 ± 0.24
MYB	26.23 ± 2.08	4.10 ± 0.37	13.25 ± 1.67	1.41 ± 0.19	1.08 ± 0.52	5.77 ± 0.23

significantly higher when acetone was used as cosolvent. Overall, it can be concluded that the best bioconversion broth was YMPG, and the best cosolvent was acetone. The substrate should preferably be applied to the cultures dissolved in the cosolvent at a low concentration (5%) and a final cosolvent concentration of 2% does not inhibit the bioconversion capacity.

Conversion of (+)-Linalool, (−)-Linalool and (±)-Linalool in Acidified Water. As a final control, the possible conversion of linalool in acidified water (pH 2.3) was checked. To six flasks containing 50 mL of distilled water, acidified to pH 2.5 with HOAc, 25 μL of (+)-, (−)-, or (±)-linalool was added (each substrate was tested in duplicate). The conversion of the substrate was monitored during 11 days (data not shown). In the last sample, taken after 11 days of shaking the flasks, 2.16 ± 0.08 μL of α-terpineol and 25.02 ± 1.56 μL of unconverted linalool was noticed. From these results it can be concluded that linalool can be converted to α-terpineol with a molar yield of 13.42 ± 0.49% due to acid catalysis (pH 2.3). However, we have shown before that in less acidic conditions (pH 3.5), this conversion was not noticeable (*18*).

Bioconversion of Natural Coriander Oil by *Aspergillus niger*. In a final experiment the bioconversion of natural coriander oil (ee (*S*)-(+)-linalool 75%) by liquid cultures of *Aspergillus niger*, strain ANA was carried out. Three media, YMPG, MEB, and MYB (all in duplicate), and three controls (broth acidified to pH 4.5) were used. Substrate addition (500 μL of a 5% solution of coriander oil in absolute EtOH) took place 45 h after inoculation (1 mL of spore suspension of 10^6 CFU/mL). After 28 h the cultures were sampled, and again substrate (same amount) was administered to the cultures. One day later the last substrate addition took place (500 μL of a 10% solution of coriander oil in absolute EtOH), and the final sample was taken after 66 h. The results of the bioconversion yields obtained from the last series of samples and the final pH-values of the culture broths are displayed in Table 5. Again the best yields were obtained with YMPG-broth, and the main bioconversion products were *cis*-furanoid linalool oxide (average yield with broth YMPG 31.32%) and *cis*-pyranoid linalool oxide (12.84%). It is important to note that the pH in the YMPG-broth remained more or less neutral (pH 5.65 ± 0.83), and hence the conversion in this broth was purely biocatalytic, since in acidified broth (final pH ≈ 3) the chemical conversion of linalool to linalool oxides was negligible (<0.5%). It can be concluded that the bioconversion of natural coriander oil by liquid cultures of *Aspergillus niger* offers a very elegant way of producing natural *cis*-furanoid and -pyranoid linalool oxides.

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LITERATURE CITED

(1) Wang, D.; Ando, K.; Morita, K.; Kubota, K.; Kobayashi, A. Optical isomers of linalool and linalool oxides in tea aroma. *Biosci. Biotech. Biochem.* **1994**, *58*, 2050−2053.

(2) *Common fragrance and flavor materials. Preparation, properties and uses*, 2nd, revised ed.; Bauer, K., Garbe, D., Surburg, H., Eds.; VCH Publishers: New York, U.S.A., 1990.

(3) Askari, C.; Mosandl, A. Stereoisomeric flavor compounds. LII: Separation and structure elucidation of the furanoid linalool oxide stereoisomers using chirospecific capillary gas chromatography and nuclear magnetic resonance spectroscopy. *Phytochem. Anal.* **1991**, *2*, 211−214.

(4) Kreis, P.; Dietrich, A.; Mosandl, A. Elution order of the furanoid linalool oxides on common gas chromatographic phases and modified cyclodextrin phases. *J. Essent. Oil Res.* **1996**, *8*, 339−341.

(5) 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.

(6) 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.

(7) Mizutani, S.; Hayashi, T.; Ueda, H.; Tatsumi, C. Microbiological conversion of terpenes. IX. Conversion of linalool. *Nippon Nogei Kagaku Kaishi* **1971**, *45*, 368−373. Source: CA 76, 11863d.

(8) Rama Devi, J.; Bhattacharyya, P. K. Microbiological transformations of terpenes: Part XXIII − Fermentation of geraniol, nerol & limonene by a soil Pseudomonad, *Pseudomonas incognita* (linalool strain). *Indian J. Biochem. Biophys.* **1977**, *14*, 288−291.

(9) Madyastha, K. M.; Bhattacharyya, P. K.; Vaidyanathan, C. S. Metabolism of a monoterpene alcohol, linalool, by a soil pseudomonad. *Can. J. Microbiol.* **1977**, *23*, 230−239.

(10) Renganathan, V.; Madyastha, K. M. Linalyl acetate is metabolized by *Pseudomonas incognita* with the acetoxy group intact. *Appl. Environ. Microbiol.* **1983**, *45*, 6−15.

(11) Madyastha, K. M. Microbial transformations of acyclic monoterpenes. *Proc. Indian Acad. Sci. (Chem. Sci.)* **1984**, *93*, 677−686.

(12) Madyastha, K. M.; Krishna Murthy, N. S. R. Transformation of acetates of citronellol, geraniol, and linalool by *Aspergillus niger*: Regiospecific hydroxylation of citronellol by a cell-free system. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 324−329.

(13) Madyastha, K. M.; Krishna Murthy, N. S. R. Regiospecific hydroxylation of acyclic monoterpene alcohols by *Aspergillus niger*. *Tetrahedron Lett.* **1988**, *29*, 579−580.

(14) Rapp, A.; Mandery, H. Influence of *Botrytis cinerea* on the monoterpene fraction of wine aroma. In *Bioflavour '87. Analysis-Biochemistry-Biotechnology*; Schreier, P., Ed.; Walter de Gruyter & Co.: Berlin, 1988; pp 445−452.

(15) Bock, G.; Benda, I.; Schreier, P. Biotransformation of linalool by *Botrytis cinerea*. *J. Food Sci.* **1986**, *51*, 659−662.
(16) Kieslich, K.; Abraham, W.-R.; Stumpf, B.; Thede, B.; Washausen, P. Transformation of terpenoids. In *Progress in Essential Oil Research*; Brunke, E.-J., Ed.; Walter de Gruyter & Co.: Berlin, 1986; pp 367−394.
(17) Abraham, W.-R.; Stumpf, B.; Arfmann, H.-A. Chiral intermediates by microbial epoxidations. *J. Essent. Oil Res.* **1990**, *2*, 251−257.
(18) Demyttenaere, J. C. R.; Willemen, H. M. Biotransformation of linalool to furanoid and pyranoid linalool oxides by *Aspergillus niger*. *Phytochemistry* **1998**, *47*, 1029−1036.
(19) Demyttenaere, J. C. R.; Herrera, M. del C.; De Kimpe, N. Biotransformation of geraniol, nerol and citral by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. *Phytochemistry* **2000**, *55*, 363−373.
(20) Demyttenaere, J.; De Kimpe, N. Biotransformation of geraniol and nerol by sporulated surface cultures of *Aspergillus niger*. In *Frontiers of Flavour Science*; Schieberle, P., Engel, K.-H., Eds.; Proc. Ninth Weurman Flavour Research Symp., Freising, Germany, 22−25 June 1999; 2000; pp 400−404.
(21) Tan, Q.; Day, D. F.; Cadwallader, K. R. Bioconversion of (*R*)-(+)-limonene by *P. digitatum* (NRRL 1202). *Process Biochem*. **1998**, *33*, 29−37.
(22) Demyttenaere, J. C. R.; Van Belleghem, K.; De Kimpe, N. Biotransformation of (*R*)-(+)- and (*S*)-(−)-limonene by fungi and the use of solid-phase microextraction for screening. *Phytochemistry* **2001**, *57*, 199−208.
(23) Demyttenaere, J. C. R.; Willemen, H. M.; Herrera, M. del C.; Verhé, R. Antifungal properties of essential oil components. In *Progress in Essential Oil Research. Proceedings 28th ISEO (International Symposium on Essential Oils) Eskisehir, Turkey*; Baser, K. H. C., Kirimer, N., Eds.; Anadolou University Press: Eskisehir, Turkey, 1998; pp 183−200.
(24) Tan, Q.; Day, D. F. Organic cosolvent effects on the bioconversion of (*R*)-(+)-limonene to *R*-(+)-α-terpineol. *Process Biochem*. **1998**, *33*, 755−761.

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