J Ind Microbiol Biotechnol (2012) 39:1761–1769
DOI 10.1007/s10295-012-1181-2

BIOCATALYSIS

Integrated bioprocess for the stereospecific production of linalool oxides from linalool with *Corynespora cassiicola* DSM 62475

Sebastian Bormann · Maria M. W. Etschmann · Marco-Antonio Mirata · Jens Schrader

Received: 21 May 2012 / Accepted: 19 July 2012 / Published online: 18 August 2012

Abstract Linalool oxides are of interest to the flavour industry because of their lavender notes. *Corynespora cassiicola* DSM 62475 has been identified recently as a production organism because of high stereoselectivity and promising productivities [Mirata et al. (2008) J Agric Food Chem 56(9):3287–3296]. In this work, the stereochemistry of this biotransformation was further investigated. Predominantly (2R)-configured linalool oxide enantiomers were produced from (R)-(−)-linalool. Comparative investigations with racemic linalool suggest that predominantly (2S)-configured derivatives can be expected by using (S)-(+)-configured substrate. Substrate and product inhibited growth even at low concentrations (200 mg l^{-1}). To avoid toxic effects and supply sufficient substrates, a substrate feeding product removal (SFPR) system based on hydrophobic adsorbers was established. Applying SFPR, productivity on the shake flask scale was increased from 80 to 490 mg l^{-1} day^{-1}. Process optimisation increased productivity to 920 mg l^{-1} day^{-1} in a bioreactor with an overall product concentration of 4.600 mg l^{-1} linalool oxides.

Keywords Biotransformation · Linalool · Linalool oxide · Substrate feeding product removal · Hydrophobic adsorber

S. Bormann · M. M. W. Etschmann (✉) · M.-A. Mirata · J. Schrader
Biochemical Engineering, DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany
e-mail: etschmann@dechema.de

Introduction

Linalool oxides—cyclic ether derivatives of linalool—are a good example of the importance of the concept of terpene oxyfunctionalisation [3]. Because of their distinctive smell, which is dependent on the stereoform, they are interesting products for the fragrance industry and have been investigated since the beginning of the twentieth century [18]. They are especially useful for the production of lavender notes and for the recreation of certain essential oils [27, 31]. There are two structural isomers, furanoid and pyranoid linalool oxide, both of which posses two stereocenters (Fig. 1). This makes up for a total of eight isomers.

Stereochemistry can have a significant impact on the olfactorial properties of chiral molecules. According to Wüst and Mosandl [31], the olfactorial reception of linalool oxides depends solely on the configuration of the stereocenter at the C2-position. While (2R)-configured linalool oxides have an earthy, slightly leafy smell, (2S)-configured molecules are perceived as floral, creamy and sweet. Odour reception is equal for both furanoid and pyranoid linalool oxides. Thus, production of pure (2R)- or (2S)- configured products is desired, since they have distinctly different smells.

The postulated microbial biosynthetic pathway suggests that pure (2R)- or (2S)- configured products can be produced from enantiomerically pure (S)-(+)- or (R)-(−)-linalool, respectively (Fig. 1). Fortunately, linalool is available from several natural sources, often with high enantiomeric excess. The (S)-(+)-form can be found in coriander oil (*Coriandrum sativum*) with an enantiomeric excess of 60–70 %. Linalool from lavender (*Lavandula angustifolia*) has an even higher enantiomeric excess of more than 98 % (R)-(−) [1, 2, 27]. This is why natural (R)-(−)-linalool (>80 %) is available for as little as 90 US$ kg^{-1} (Sigma–Aldrich online catalogue Germany,

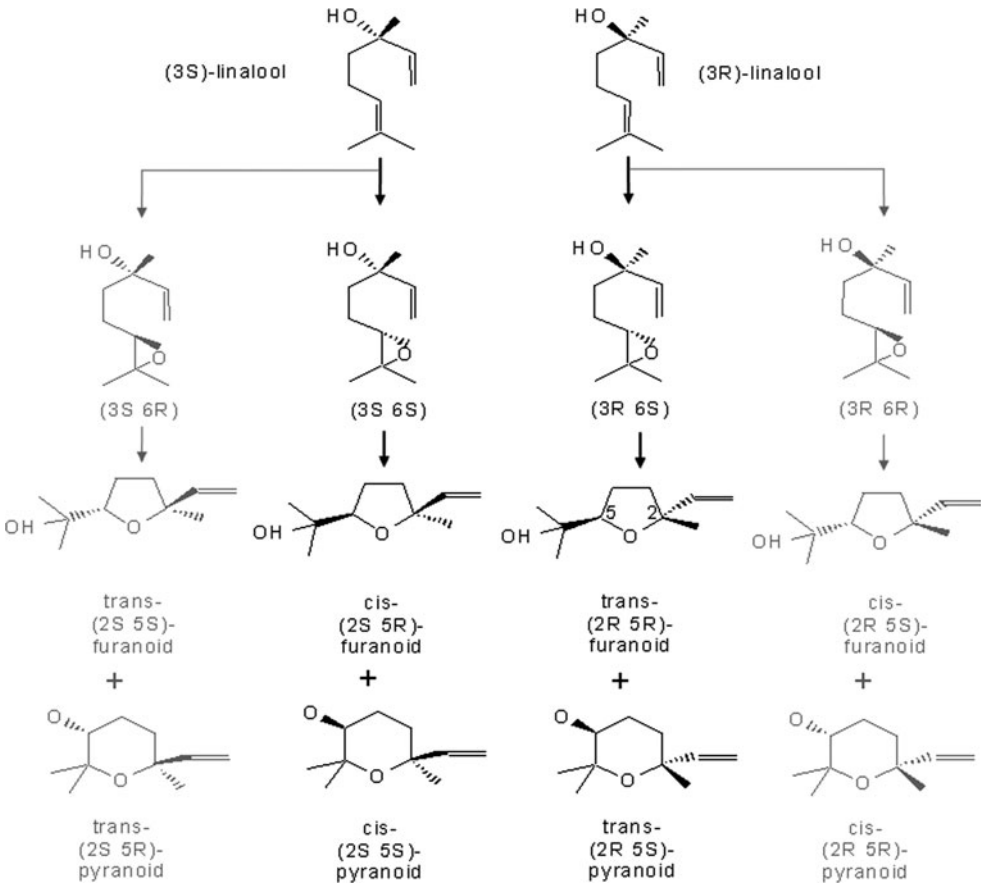

Fig. 1 Postulated microbial biosynthetic pathway for the oxidation of linalool to linalool oxide. Products produced by *Corynespora cassiicola* DSM 62475 are shown in *black*. Adapted from Mirata et al. [21]

accessed 17 October 2011) and might be even cheaper when obtained in bulk. In contrast, natural linalool oxide has a price of about 750 US$ kg^{-1} (Advanced Biotech, Paterson, NJ, personal correspondence). The value of enantiopure natural linalool oxide would be even higher. This illustrates the market potential of this biotransformation.

In order to produce natural linalool oxide according to US and EU legislation, natural linalool has to be oxidised by biological means. Several *Aspergillus niger*, *Botrytis cinerea* and *Streptomyces albus* strains are capable of performing this biotransformation [5, 7, 9]. Unfortunately, all these microorganisms oxidise linalool at a slow pace. Gatfield et al. [11] reported much higher volumetric productivities when linalool was oxidised in the presence of a *Candida antarctica* lipase, but did not describe the enantiomeric composition of the product.

Recently, Mirata et al. [21] identified *Corynespora cassiicola* DSM 62475 to be capable of performing this biotransformation. Compared to several *A. niger* and *B. cinerea* strains, product was formed at a higher rate. Moreover, *C. cassiicola* performed this biotransformation in a highly stereospecific manner, producing preferentially four of eight isomers.

While linalool inhibited growth of all investigated microorganisms at higher concentrations, *C. cassiicola* was one of the most tolerant species. No growth inhibition occurred up to a concentration of 150 mg l^{-1} . For these reasons, *C. cassiicola* DSM 62475 is a promising microorganism for industrial scale oxidation of linalool to linalool oxide.

Both linalool and linalool oxide are hydrophobic substances with a mildly hydrophilic character (log *P* values 3.5 and 2.4, respectively [12]. Substances with log *P* values between 1 and 5 are generally considered as cytotoxic [29]. While the toxicity of linalool has already been shown, linalool oxide is most likely to be cytotoxic as well. To reduce inhibitory effects in microbial biotransformations, several techniques can be applied. Substrate can be added by a feedback controlled pump to maintain a subinhibitory level. As online measurement of terpene concentrations is difficult, feedback control is unfeasible for the time being. To prevent accumulation of toxic product in the immediate aqueous microenvironment of the cells, in situ product removal (ISPR) techniques have been applied successfully [4, 10, 20]. Two-phase liquid–liquid and liquid–solid systems, as well as membrane separation are the most

commonly used ISPR techniques. These methods work most successfully when substrate and product have considerably different physicochemical properties (e.g. charge, volatility, considerable log *P* change). Otherwise there is a risk of removing substrate from the process.

However, in processes where substrate and product have similar physicochemical properties, a combined substrate feeding product removal (SFPR) approach is favourable [14]. To achieve this effect, adsorber is preloaded with substrate and introduced into the process. Substrate will desorb into the aqueous phase until equilibrium is established. In the presence of biocatalyst, substrate will be transformed to product, which is adsorbed. To reestablish equilibrium conditions, more substrate will desorb into the liquid phase, thus constantly supplying substrate according to the metabolic demand of the catalyst and removing toxic product from the aqueous phase [6, 14–17, 25].

While the same can be done with a two-phase liquid–liquid system [13, 24], the use of hydrophobic liquids has disadvantages. Organic solvents are often toxic to microorganisms while hydrophobic adsorbers are usually biocompatible. Moreover, many organic phases tend to build emulsions, especially when organisms produce surfactants, which complicates downstream processing [22, 28]. Adsorbers, in contrast, can be separated easily from the culture broth by filtration, eluted with a suitable solvent and reused.

This work will further elucidate the stereochemistry of the biotransformation by investigating the conversion of linalool to linalool oxides with *C. cassiicola* DSM 62475. The substrates chosen are racemic linalool and the commercially available (R)-(−)-linalool. Application of a combined SFPR approach based on hydrophobic adsorbers, transfer from shake flask to bioreactor conditions and process optimisation will be described.

Materials and methods

Strain and maintenance

Corynespora cassiicola DSM 62475 was obtained from DSMZ (Braunschweig, Germany) and grown on malt extract agar (MEA) at 24 °C [21].

Chemicals

The experiments were executed with the commercially available (R)-(−)-linalool and racemic linalool. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany), Fluka (Ulm, Germany) or Carl Roth (Karlsruhe, Germany). Purity of (±)-linalool and racemic linalool oxide was >97 %, purity of (R)-(−)-linalool was >98.5 %. Oxide standards for stereochemical discrimination per GC-FID were kindly provided and synthesised by the Mosandl group (Johann Wolfgang Goethe University Frankfurt/Main, Germany) according to Weinert et al. [30] and Askari and Mosandl [1].

Fermentation procedure

Preparation of preculture

Spore suspension (1 % v/v) and 200 ml malt yeast broth (MYB) (both described by Mirata et al. [21]) were combined in a 1,000 ml Erlenmeyer flask and incubated at 24 °C and 200 rpm. All shake flask experiments were executed at an amplitude of 25 mm.

Determination of linalool/linalool oxide toxicity

Glass vials for solid phase microextraction (40 ml) were filled with 9.5 ml MYB and inoculated with 500 µl of a 7 day old preculture (homogenised with an Ultra-Turrax, IKA, Staufen, Germany, for 30 s). Linalool and linalool oxide were added from stock solutions in ethanol (3 g l^{-1} for 0.5 and 1 mM, 30 g l^{-1} for 2, 4 and 8 mM). Cell dry weight was determined after inoculation and after 24 h.

Adsorption experiments

Preparation

All adsorbers were conditioned by washing with about two volumes of methanol and deionized H_2O and dried for 24 h at 105 °C. All weights given are dry weights.

Adsorption isotherms of linalool

To determine adsorption isotherms, 20 mg adsorber were added to 20 ml of an aqueous linalool solution (100–1,000 mg l^{-1}) and incubated in a 20 ml screw cap vial at 24 °C. After 24 h, a sample was taken and the remaining aqueous linalool concentration was determined by GC-FID analysis. Investigated adsorbers were Amberlite XAD2, XAD4, XAD7 and XAD16 (Rohm and Haas, Philadelphia, PA), Lewatit VP OC 1163 (Lanxess, Leverkusen, Germany) and Diaion HP-2MG (Mitsubishi Chemical, Tokyo, Japan).

Linalool oxide affinity

Adsorber (0.1 % w/v) was added to 20 ml of an aqueous solution containing both linalool and linalool oxide (500 mg l^{-1} each) and incubated in a 20 ml screw cap vial at 24 °C for 24 h.

Biotransformations

Fed-batch biotransformation on shake flask scale

In a 2,000 ml Erlenmeyer flask, 50 ml of a 7-day-old homogenised preculture was added to 450 ml MYB and cultivated at 24 °C and 200 rpm. Every 24 h, 150 mg l^{-1} linalool (30 g l^{-1} stock solution in ethanol) and 5 g l^{-1} glucose (750 g l^{-1} stock solution) were added before a sample was taken. Both quantitative and stereochemical analysis were done by GC-FID.

SFPR biotransformation on shake flask scale

Lewatit VP OC 1163 (dried for 1 h at 105 °C to prevent contamination) was loaded with 250 mg linalool (load 0.56 g g^{-1}) in 45 ml MYB for 24 h in a 300 ml Erlenmeyer flask. A 7-day-old homogenised preculture (10 % v/v) was added to start the biotransformation (24 °C, 200 rpm). Glucose and terpene concentrations were measured every 24 h and glucose was accordingly adjusted to 10 g l^{-1}.

Biotransformation in a small scale bioreactor

The biotransformation was carried out in a 4 × 1 l parallel bioreactor fedbatch-pro (DASGIP, Jülich, Germany), where 450 ml MYB were inoculated with 50 ml of a 7-day-old homogenised preculture. The reactor was operated at 24 °C, 500 rpm and aerated at 0.3 vvm with air. The air was saturated with linalool prior to injection into the reactor by passing it through a fritted washing flask filled to one-third with linalool. Glucose was added at a rate of 15 g l^{-1} day^{-1}. For SFPR-cultures, 8.9 g adsorber were loaded with 5 g linalool in 100 ml MYB (load 0.56 g g^{-1}) for 24 h, filtered, added to the bioreactor and stirred for 1 h at 500 rpm prior to inoculation to ensure equilibrium conditions. For fed-batch-cultures, 150 mg l^{-1} linalool were added every 24 h.

Elution of adsorber

The adsorber was separated from the culture broth by filtration (TE 38 membrane filter, 5 µm, Whatman Schleicher & Schuell, Maidstone, UK). It was eluted with 10 and 50 ml ethanol (shake flask and bioreactor scale, respectively) for 1 h. This procedure was repeated five times. The eluates were combined and analysed by GC-FID. Terpene concentrations given are always based on culture volume.

Analytics

Sample preparation for GC analysis

Aqueous samples were extracted 1:2 with MTBE. The extract was dried over sodium sulfate and analysed by GC-FID. Prior to extraction, 2-octanol (5 % v/v of a 2 g l^{-1} stock solution in ethanol) was added as an internal standard. Ethanol based samples were analysed without further preparation after addition of internal standard.

Sample Analysis by GC-FID

Linalool and linalool oxides were analysed by gas chromatography (GC 17A equipped with FID, Shimadzu, Tokyo, Japan). For quantification, a DB-WAX_{etr} column (30 m × 0.25 mm × 0.25 µm, Agilent, Santa Clara, CA) was used. Parameters were as follows: carrier gas, helium; split, 1:41, column flow, 0.8 ml min^{-1}; temperature: 120 °C (7 min), to 250 °C at 30 °C min^{-1},250 °C (3 min). For stereochemical analysis, a Chiraldex B-DM (30 m × 0.25 mm × 0.12 µm, Sigma–Aldrich, Schnelldorf, Germany) was used with the following parameters: carrier gas, helium; split, 1:20, column flow, 1.1 ml min^{-1}; temperature: isothermal 95 °C (30 min). Elution order on achiral DB-WAXetr was: trans-furanoid linalool oxide, cis-furanoid linalool oxide, linalool, trans-pyranoid linalool oxide, cis-pyranoid linalool oxide as determined by comparison with reference compounds. On the chiral Chiraldex B-DM column, the elution order was: trans-(2R,5R)-furanoid linalool oxide, trans-(2S,5S)-furanoid linalool oxide, cis-(2R,5S)-furanoid linalool oxide, cis-(2S,5R)-furanoid linalool oxide, (R)-(−)-linalool, (S)-(+)-linalool, trans-(2S,5R)-pyranoid linalool oxide, trans-(2R,5S)-pyranoid linalool oxide + cis-(2S,5S)-pyranoid linalool oxide (coelution), cis-(2R,5R)-pyranoid linalool oxide as determined by comparison with reference compounds. To quantify terpene concentrations, a response factor in relation to 2-octanol was determined.

Concentration of coeluted trans-(2R,5S)-pyranoid linalool oxide and cis-(2S,5S)- pyranoid linalool oxide was determined by substraction of trans-(2S,5R)-pyranoid linalool oxide or cis-(2R,5R)-pyranoid linalool oxide concentration from the respective total pyranoid diastereomer concentration, determined by GC analysis on achiral DB-WAX_{etr}.

Cell dry weight determination

Culture broth samples (10 ml) were filtered through a preweighed 0.45 µm cellulose nitrate filter (Whatman, Maidstone, UK). The filter was dried overnight at 105 °C. Biomass was determined gravimetrically.

Glucose analysis

Glucose concentrations were determined enzymatically (2,700 Select, YSI, Yellow Springs, OH).

Results and discussion

Stereochemistry of biotransformation products

Corynespora cassiicola DSM 62475 was cultivated in the presence of (±)-linalool and (R)-(−)-linalool. Culture broth samples were analysed by GC-FID on an enantioselective column (Fig. 2). The biotransformation of (±)-linalool resulted in a mixture of (2R)- and (2S)-configured furanoid and pyranoid linalool oxides and was in accordance with the findings of Mirata et al. [21]. The main products were trans-(2R,5R) and cis-(2S,5R) furanoid linalool oxide as well as trans-(2R,5S) and cis-(2S,5S) pyranoid linalool oxide. When (R)-(−)-linalool was used, >93 % of the biotransformation products were (2R)-configured. The main products were trans-(2R,5R) furanoid linalool oxide and trans-(2R,5S) pyranoid linalool oxide. The two minor products with a (2S) configuration may be attributed to (S)-(+)-linalool contaminations in the substrate (R)-(−)-linalool, which was available only at >98.5 % purity. The stereochemical composition of products did not change over the course of the biotransformation. These results confirm the microbial biosynthetic pathway postulated by Mirata et al. [21]. Comparing the results from the biotransformation of (±)-linalool with the results of the (R)-(−)-linalool biotransformation, it can be concluded that almost pure (2S)-configured linalool oxides can be produced from (S)-(+)-linalool. Since linalool is naturally available in both enantioforms with high enantiomeric excess, a bioprocess with *C. cassiicola* DSM 62475 could produce two different sets of natural linalool oxides, each with a specific olfactorial character dependent on the substrate used.

Kinetics of fed-batch biotransformation

A fed-batch cultivation in shake flasks was carried out in order to determine the productivity of the biotransformation. For this, 150 mg l^{-1} day^{-1} (±)-linalool and (R)-(−)-linalool was added to the culture broth (Fig. 3). This was the critical concentration determined by Mirata et al. [21] for *C. cassiicola*. Over the course of 14 days, 1,180 and 1,110 mg l^{-1} linalool oxides were produced from (±)-linalool and (R-)(−)-linalool, respectively. This corresponds to a productivity of about 80 mg l^{-1} day^{-1} and a molar yield of approximately 55 %. The low yield may result from substrate losses due to the high volatility of linalool. This is in accordance with Demyttenaere et al. [8], who reported losses of up to 60 % in liquid culture over the course of 1 week due to evaporation. Substrate consumption and product formation kinetics were similar for both substrates. Thus, the stereochemistry of the substrate seems to have little—if any—influence on the biotransformation. Over the course of 11 days, substrate in all cultures was fully depleted within 24 h after addition. This suggests that substrate limitation is the factor constricting this process.

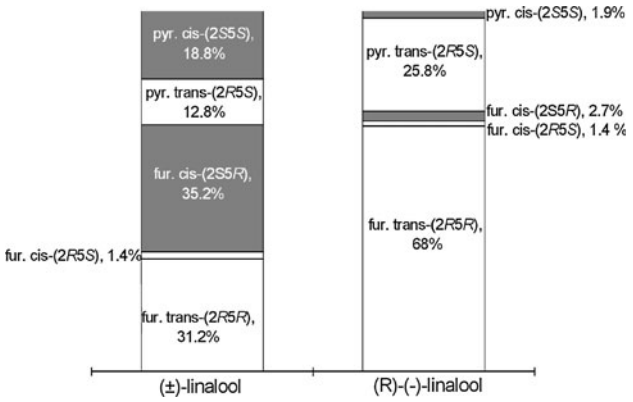

Fig. 2 Stereochemical composition of biotransformation products furanoid linalool oxide (fur.) and pyranoid linalool oxide (pyr.) from (±)-linalool and (R)-(−)-linalool after 14 days of cultivation. *White columns* (2R)-configured products, *grey columns* (2S)-configured products

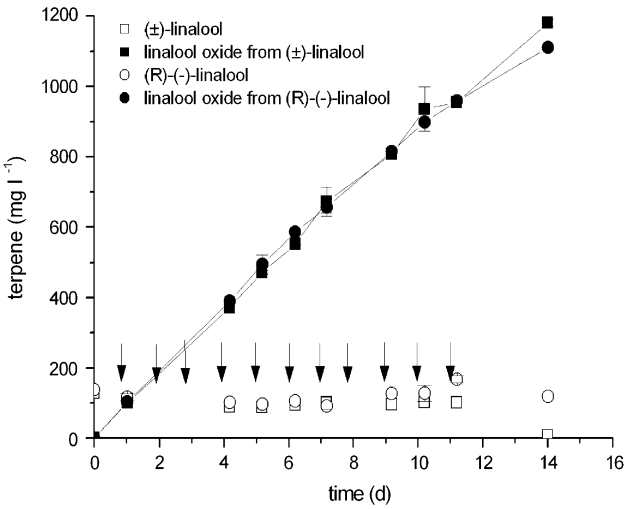

Fig. 3 Product formation and substrate consumption kinetics of a linalool biotransformation on 2-l shake flask scale with racemic and (R)-(−)-configured substrate. *Arrows* indicate addition of 150 mg l^{-1} linalool. Mean values of double experiments are given, *error bars* show individual results

Substrate and product toxicity

Since linalool and its oxides are structurally and therefore physicochemically similar, not only substrate inhibition but

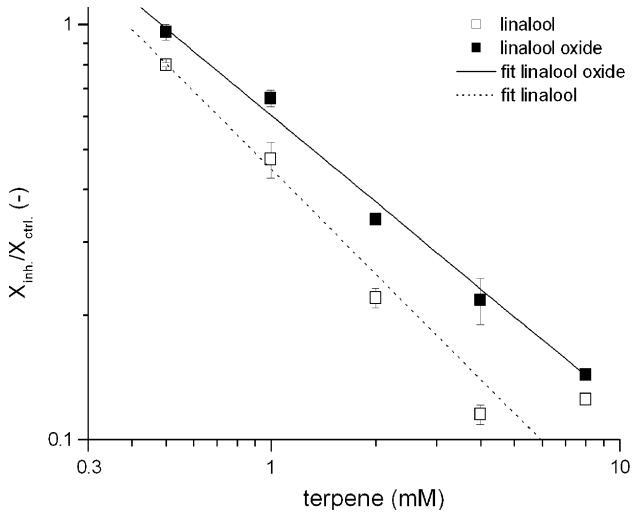

Fig. 4 Growth inhibition of *C. cassiicola* in the presence of linalool and linalool oxide. Biomass reduction is expressed as fraction of biomass produced under inhibited conditions ($X_{inh.}$) per biomass of an uninhibited control ($X_{ctrl.}$). Values were determined after 24 h of cultivation in 40 ml flasks. Both inhibitions could be described by a power function $y = c^{-a} \cdot b$ in which y is the fraction of biomass produced at an inhibitor concentration c; a and b are constants depicting the toxicity of the inhibitors. Curve fitting produced for linalool: $a = 0.84$, $b = 0.45$ ($R^2 = 0.98$); for linalool oxide: $a = 0.69$, $b = 0.61$ ($R^2 = 0.99$). Mean values of double experiments are given, *error bars* show individual results

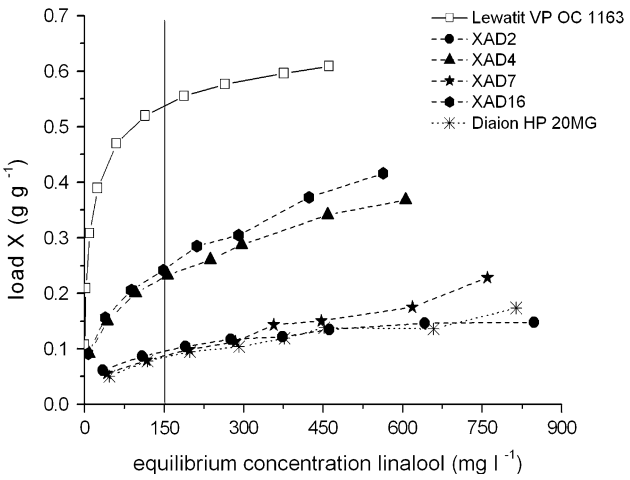

Fig. 5 Adsorption isotherms of linalool on hydrophobic adsorbers. *Vertical line* Critical aqueous linalool concentration and the equilibrium load of the adsorber

also product inhibition may interfere with the biotransformation. Hence, the influence of elevated substrate and product concentrations on the growth was investigated.

Incubation of *C. cassiicola* under the influence of linalool and linalool oxide showed the severe toxicity of both substances even at low concentrations (Fig. 4). It became obvious that the decrease in biomass followed a power law, when the data was plotted on double logarithmic axes. After regression analysis, IC_{50} (50 % inhibitory concentration) values were determined. These values were 0.88 mM (136 mg l^{-1}) for linalool and 1.33 mM (230 mg l^{-1}) for linalool oxide. The less toxic effect of linalool oxide is not surprising since oxidation of hydrophobic molecules can be seen as part of a cell's detoxification mechanism [21, 23]. Mirata et al. [21] determined a threshold concentration of 150 mg l^{-1} for linalool, below which they did not notice any significant inhibition. The main differences in their experimental setup were the use of spore suspension and an incubation time of 4 days. In contrast, cultures were inoculated with homogenised preculture and cultivated for only 24 h in the present work. During the longer period of incubation, linalool may have evaporated or been oxidised to the less toxic linalool oxide. This might explain the slightly lower toxic effect described by Mirata et al. [21].

Nonetheless, 150 mg l^{-1} linalool was chosen as a suitable concentration for further experiments since it was in the range of the IC_{50} value. Lower concentrations might have been difficult to control due to the high volatility of the substrate.

It became apparent that linalool had to be dosed carefully to achieve constant substrate supply while maintaining low concentrations. Furthermore, accumulating product had to be removed constantly from the process to prevent growth inhibition. For this reason, a combined substrate delivery and product removal system based on hydrophobic adsorbers had to be established.

Screening of adsorbers

Solid phases for a substrate feeding product removal approach need to show affinity for both substrate and product. It should be possible to load the adsorber with high amounts of substrate while maintaining a low aqueous concentration. Moreover, the resin should release most of the adsorbed substrate into the liquid phase. To investigate substrate affinity, adsorption isotherms of linalool on different hydrophobic adsorbers were determined (Fig. 5). Methacrylate-based adsorbers Diaion HP20MG and Amberlite XAD7 showed the lowest loading capacities together with polystyrene-divinylbenzene based adsorber Amberlite XAD2. Amberlite XAD4 and XAD16 showed very similar adsorption isotherms. At a concentration of 150 mg l^{-1} linalool, Lewatit VP OC 1163 had an equilibrium loading capacity of about 0.56 g g^{-1} which was more than twice as much as Amberlite XAD4 and XAD16, and more than five times the loading capacities of Amberlite XAD2, XAD7 and Diaion HP20MG at this concentration. It was found that the loading capacity increased proportionally with the specific surface area, which corresponds to the findings of Krings et al. [19].

Moreover, the affinity of linalool oxide in comparison to linalool towards the adsorbers was explored by incubating the adsorbers in a solution with similar amounts of both linalool and linalool oxide (data not shown). Amberlite XAD2, XAD7 and Diaion HP20MG adsorbed only linalool. Amberlite XAD4, XAD16 and Lewatit VP OC1163 adsorbed both terpenes, but the amount of adsorbed linalool was always more than three times the amount of linalool oxide. Interestingly, the mildly hydrophilic methacrylate adsorbers (Diaion HP20MG, Amberlite XAD7) did not perform better at adsorbing the more hydrophilic linalool oxide compared to the unpolar adsorbers with a styrene/divinylbenzene matrix.

Since Lewatit VP OC 1163 showed superior adsorption characteristics for linalool and similar adsorption characteristics for linalool oxide compared to Amberlite XAD4 and XAD16, it was chosen for use in a SFPR-bioprocess.

Application of SFPR in the bioprocess

At first, the application of simultaneous substrate feeding and product removal was tested at the shake flask scale (Fig. 6a). At the beginning of the biotransformation the aqueous linalool concentration was higher than 150 mg l^{-1}. The equilibrium state, predicted by the previously described adsorption isotherm, was not achieved, and since loading of the adsorber was done in situ, excess substrate remained in the culture broth. On the one hand, this may be attributed to the different linalool-medium and adsorber-medium ratios compared to the experiments done for adsorption isotherms. On the other hand, the presence of glucose and other components in the culture broth may have impaired the adsorption process.

Nonetheless, product formation could be observed. After 1 day, the aqueous linalool concentration had decreased from 250 to 40 mg l^{-1} and an aqueous linalool oxide concentration of 480 mg l^{-1} was determined. The aqueous molar product concentration after 24 h exceeded the initial aqueous substrate concentration. This clearly indicated that substrate from the adsorber had desorbed into the liquid phase. After 3.7 days no more substrate was detected in the aqueous phase. Elution of the adsorber showed that 1,200 mg l^{-1} linalool oxide had been adsorbed during the biotransformation, which was twice the amount of product in the aqueous phase. Thus, it was possible to produce 1,800 mg l^{-1} linalool oxide in 3.7 days, which corresponds to a productivity of 486 mg l^{-1} day^{-1}. This accounts for a molar yield of 33; 28 % of the substrate was recovered from the resin and 39 % was lost, most likely due to evaporation. Compared to the fed-batch approach, volumetric productivity was six times higher.

Because of differences in mass transfer, transition from shake flask to bioreactor can have a significant impact on a bioprocess. Preliminary experiments suggested that the volatility of linalool may limit substrate supply in an aerated bioreactor. Stripping experiments with an aeration rate of 0.3 vvm showed that more than 500 mg l^{-1} linalool could be blown out of the culture broth within 1 day. In contrast, less than 2 % of 300 mg l^{-1} linalool oxide were lost over the course of 3 days. To compensate for the loss of substrate via the air stream, the air supply was saturated by passing it through a linalool filled fritted washing flask. The bioprocess was carried out with SFPR and conventional fed-batch substrate supply in parallel (Fig. 6b, c). For both setups, a similar increase in aqueous linalool oxide concentration was observed. For the SFPR setup, the linalool concentration decreased from an initial 200 to 14 mg l^{-1} over the course of 5 days. For the fed-batch-setup, substrate concentrations (measured 24 h after every substrate addition) varied between 20 and 60 mg l^{-1}. The constant presence of substrate in this setup is most likely due to substrate entry through aeration. After 5 days, the biotransformation was stopped and the SFPR resin was eluted. In addition to the 1,000 mg l^{-1} linalool oxide dissolved in the aqueous phase, 3,600 mg l^{-1} product were recovered from the adsorber. Unfortunately, linalool did not desorb completely into the aqueous phase. About 35 % linalool was eluted at the end of the process. The incomplete desorption of substrate and adsorption of product is understandable since the hydrophobic adsorber has a higher affinity for the more hydrophobic compound. Once again,

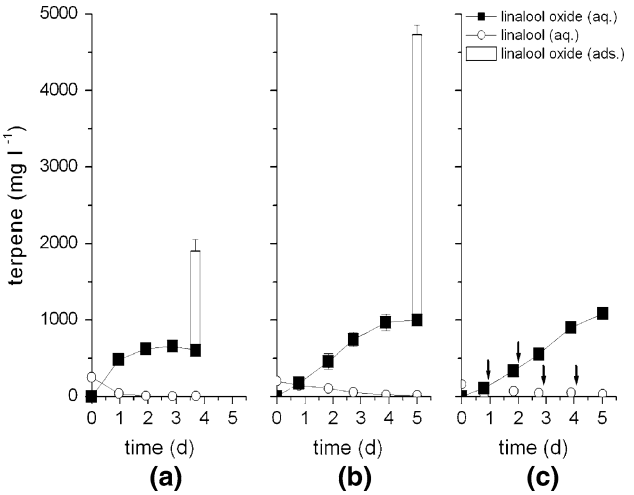

Fig. 6 Shake flask scale substrate feeding product removal (SFPR)-biotransformation (**a**) and bioreactor scale SFPR- (**b**) and fed-batch-biotransformation (**c**). Product was eluted from the adsorber at the end of the biotransformation (*white column*). Adsorber was loaded with **a** 5 g l^{-1}, **b** 10 g l^{-1} linalool (load 0.56 g g^{-1} each). **c** 150 mg l^{-1} linalool was fed manually every 24 h as indicated by *arrows*. For **b** and **c**, additional substrate may have been introduced through a linalool-saturated airstream. **a** and **b** show mean values of double experiments, *error bars* indicate deviation of single experiments (for deviations >60 mg l^{-1}). Experiments **b** and **c** were done in parallel

it becomes obvious that the development of high specific area, mildly hydrophilic adsorbers is important to improve ISPR and SFPR application in microbial biotransformations, as outlined by Straathof [26]. Nonetheless, 42 % of the substrate was oxidised over the course of 5 days, which corresponds to a productivity of 921 mg l^{-1} day^{-1}. In comparison to the fed-batch process, this is an increase in productivity by factor 4.6.

Conclusion

Microbial oxidation products of (R)-(−)-linalool by *C. cassiicola* DSM 62475 are predominantly trans-(2R, 5R) furanoid and trans-(2R, 5S) pyranoid linalool oxides. Comparison with the results of the biotransformation of (±)-linalool shows that the corresponding (2S)-configured linalool oxides can be expected if pure (S)-(+)-linalool is fed. This biotransformation therefore allows stereospecific access to the desired pure products with uniform olfactorial properties. Application of a liquid–solid two-phase SFPR technique greatly reduced the toxic effects of substrate and product. At the same time, continuous substrate supply and facilitated downstream processing has been achieved, resulting in the highest product concentrations and productivities reported so far for this microbial biotransformation.

References

1. Askari C, Mosandl A (1991) Stereoisomeric flavour compounds. LII: separation and structure elucidation of the furanoid linalool oxide stereoisomers using chirospecific capillary gas chromatography and nuclear magnetic resonance spectroscopy. Phytochem Anal 2(5):211–214
2. Bedoukian PZ (1986) Perfumery and flavoring synthetics. Allured, Carol Stream
3. Berger R (2009) Biotechnology of flavours—the next generation. Biotechnol Lett 31(11):1651–1659
4. Bluemke W, Schrader J (2001) Integrated bioprocess for enhanced production of natural flavors and fragrances by *Ceratocystis moniliformis*. Biomol Eng 17(4–5):137–142
5. Bock G, Benda I, Schreier P (1986) Biotransformation of linalool by *Botrytis cinerea*. J Food Sci 51(3):659–662
6. Conceiço GJA, Moran PJS, Rodrigues JA (2003) Highly efficient extractive biocatalysis in the asymmetric reduction of an acyclic enone by the yeast *Pichia stipitis*. Tetrahedron Asymmetry 14(1): 43–45
7. David L, Veschambre H (1984) Preparation d'oxydes de linalol par bioconversion. Tetrahedron Lett 25(5):543–546
8. Demyttenaere JC, Adams A, Vanoverschelde J, De Kimpe N (2001) Biotransformation of (S)-(+)-linalool by *Aspergillus niger*: an investigation of the culture conditions. J Agric Food Chem 49:5895–5901
9. Demyttenaere JCR, Willemen HM (1998) Biotransformation of linalool to furanoid and pyranoid linalool oxides by *Aspergillus niger*. Phytochemistry 47(6):1029–1036
10. Etschmann MM, Sell D, Schrader J (2005) Production of 2-phenylethanol and 2-phenylethylacetate from L-phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. Biotechnol Bioeng 92(5):624–634
11. Gatfield IL, Hilmer JM, Betram HJ (2006) A facile enzymatic conversion of linalool to linalool oxide catalysed by the lipase B from *Candida antarctica*. Anal Bioanal Chem 28(3):139–141
12. Griffin S, Wyllie SG, Markham J (1999) Determination of octanol-water partition coefficient for terpenoids using reversed-phase high-performance liquid chromatography. J Chromatogr A 864(2):221–228
13. Held M, Panke S, Kohler HPE, Feiten HJ, Schmid A, Schmid A, Wubbolts MG, Witholt B (1999) Solid phase extraction for biocatalytic production of toxic compounds. BioWorld 5:2–9
14. Hilker I, Alphand VÃ, Wohlgemuth R, Furstoss R (2004) Microbial Transformations, 56. Preparative scale asymmetric Baeyer-Villiger oxidation using a highly productive "two-in-one" resin-based in situ SFPR concept. Adv Synth Catal 346(2-3):203–214
15. Hilker I, Baldwin C, Alphand V, Furstoss R, Woodley J, Wohlgemuth R (2006) On the influence of oxygen and cell concentration in an SFPR whole cell biocatalytic Baeyer-Villiger oxidation process. Biotechnol Bioeng 93(6):1138–1144
16. Hilker I, Gutierrez MC, Alphand V, Wohlgemuth R, Furstoss R (2004) Microbiological transformations 57. Facile and efficient resin-based in situ SFPR preparative-scale synthesis of an enantiopure "unexpected" lactone regioisomer via a Baeyer-Villiger oxidation process. Org Lett 6(12):1955–1958
17. Hilker I, Wohlgemuth R, Alphand V, Furstoss R (2005) Microbial transformations 59: first kilogram scale asymmetric microbial Baeyer–Villiger oxidation with optimized productivity using a resin-based in situ SFPR strategy. Biotechnol Bioeng 92(6): 702–710
18. Klein E, Farnow H, Rojahn W (1964) Die Chemie der Linalooloxide. Liebigs Ann Chem 675(1):73–82
19. Krings U, Kelch M, Berger RG (1993) Adsorbents for the recovery of aroma compounds in fermentation processes. J Chem Tech Biotechnol 58:293–299
20. Mirata MA, Heerd D, Schrader J (2009) Integrated bioprocess for the oxidation of limonene to perillic acid with *Pseudomonas putida* DSM 12264. Process Biochem 44(7):764–771
21. Mirata MA, Wuest M, Mosandl A, Schrader J (2008) Fungal biotransformation of (±)-linalool. J Agric Food Chem 56(9): 3287–3296
22. Morrish JL, Brennan ET, Dry HC, Daugulis AJ (2008) Enhanced bioproduction of carvone in a two-liquid-phase partitioning bioreactor with a highly hydrophobic biocatalyst. Biotechnol Bioeng 101:768–775
23. Onken J, Berger RG (1999) Biotransformation of citronellol by the basidiomycete *Cystoderma carcharias* in an aerated-membrane bioreactor. Appl Microbiol Biotechnol 51(2):158–163
24. Schewe H, Holtmann D, Schrader J (2009) P450(BM-3)-catalyzed whole-cell biotransformation of alpha-pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system. Appl Microbiol Biotechnol 51:158–163
25. Simpson HD, Alphand VÃ, Furstoss R (2001) Microbiological transformations: 49. Asymmetric biocatalysed Baeyer-Villiger oxidation: improvement using a recombinant *Escherichia coli* whole cell biocatalyst in the presence of an adsorbent resin. J Mol Catal B Enzym 16(2):101–108
26. Straathof AJ (2003) Auxiliary phase guidelines for microbial biotransformations of toxic substrate into toxic product. Biotechnol Prog 19(3):755–762
27. Surburg H, Panten J (2006) Common fragrance and flavor materials: preparation, properties and uses, 5th edn. Wiley-VCH, Weinheim

28. Valadez-Blanco R, Ferreira FC, Jorge RF, Livingston AG (2008) A membrane bioreactor for biotransformations of hydrophobic molecules using organic solvent nanofiltration (OSN) membranes. J Memb Sci 317(1–2):50–64
29. van der Werf MJ, De Bont JAM, Leak DJ (1997) Opportunities in microbial biotransformation of monoterpenes. In: Scheper T (ed), 55th edn, Springer, Berlin, p 283
30. Weinert B, Wüst M, Mosandl A, Hanssum H (1998) 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 9(1):10–13
31. Wüst M, Mosandl A (1999) Important chiral monoterpenoid ethers in flavours and essential oils—enantioselective analysis and biogenesis. Eur Food Res Technol 209:3–11