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Biotransformation of (*R*)-(+)- and (*S*)-(−)-limonene to α-terpineol by *Penicillium digitatum*— investigation of the culture conditions

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This paper is dedicated to Prof. Dr. Gabor Bernath on the occasion of his 70th birthday

Abstract

The biotransformation of (*R*)-(+)- and (*S*)-(−)-limonene by *Penicillium digitatum* was investigated. One strain of *P. digitatum* was able to convert (*R*)-(+)-limonene to pure (*R*)-(+)-α-terpineol in 8 h with a yield of up to 93%. It was found that (*R*)-(+)-limonene was converted much better into α-terpineol than (*S*)-(−)-limonene, and that no significant chemical conversion of the substrate occurred in control flasks at pH 3.5. The culture conditions involved such as the type and concentration of co-solvent applied and the sequential addition of substrate were investigated, taking into account some findings on the physical behaviour of the system. The highest bioconversion yields were obtained when the substrate was applied as a diluted solution in EtOH.
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1. Introduction

Extensive research has been devoted to the biotechnological production of flavours and fragrances (Berger, 1995). *Via* biotransformation or bioconversion "natural" flavours can be produced, applying to the consumers' demand for natural products (Imhof & Bosset, 1994).

Terpenes and especially their oxygenated derivatives are extensively used in the flavour industry. Via biotransformation, monoterpene precursors are converted into their more valuable oxygenated derivatives (van der Werf, de Bont, & Leak, 1997).

The monoterpene hydrocarbon limonene is a popular starting product for bioconversions because of its widespread and cheap availability (Krasnobajew, 1984). (*R*)-(+)-limonene is the main compound in essential oils of citrus fruits, where it occurs in a concentration of more than 90% and in enantiomerically pure form (Bauer, Garbe, & Surburg, 1990). Annually, approximately 36 million kg of (*R*)-(+)-limonene are recovered as a by-product of the citrus industry (Nonino, 1997).

An interesting end product resulting from the bioconversion of limonene, is the monoterpene alcohol α-terpineol. Bioconversion of limonene to α-terpineol as the main end product has been described, using a wide range of microorganisms as catalyst: a *Cladosporium* strain (Kraidman, Mukherjee, & Hill, 1969), a *Penicillium* sp. isolated from orange peel (Mattison, McDowell, & Baum, 1971), *Penicillium digitatum* (Abraham, Hoffmann, Kieslich, Reng, & Stumpf, 1985; Tan & Day, 1998a; Tan, Day, & Cadwallader, 1998), *Pseudomonas gladioli* (Cadwallader & Braddock, 1992; Cadwallader, Braddock, Parish, & Higgins, 1989) and *Escherichia coli* expressing a thermostable limonene hydratase (Savithiry, Cheong, & Oriel, 1997).

α-Terpineol has a lilac odour and is one of the most commonly used fragrance compounds (Fenaroli, 1975). It is mainly produced chemically, starting from pinene or crude turpentine oil by acid hydration to terpine, followed by partial dehydration (Teisseire, 1994). In this way, α-terpineol is commercially available at relatively low price. Therefore, a microbial process must ensure high yields of α-terpineol in order to be competitive. However, a biotransformation process has the potential to deliver chiral α-terpineol in high enantiomeric excess.

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Both α-terpineol enantiomers have different sensory qualities. (*R*)-(+)-α-Terpineol has a floral, typically lilac odour, while (*S*)-(−)-α-terpineol has a coniferous odour character (Boelens, Boelens, & van Gemert, 1993).

It is in this point of view that optimisation of the different parameters of the bioconversion process was pursued in order to obtain high yields of α-terpineol in an enantiomerically pure form. Several culture conditions can be adapted in order to circumvent certain problems, known to hamper the commercialisation of the biotransformation of monoterpenes, such as their low aqueous solubility, their volatility and toxicity.

Previously we reported the biotransformation of (*R*)-(+)-limonene and (*S*)-(−)-limonene to α-terpineol by shaking cultures of *Penicillium digitatum* (Demyttenaere, Van Belleghem, & De Kimpe, 2001). This paper describes more detailed further studies in this area, reports the bioconversion of (*R*)-(+)-limonene to (*R*)-(+)-α-terpineol by *P. digitatum* and its optimisation and discusses the influence of many culture conditions on the conversion capacity starting from some exploring experiments on the physical behaviour of limonene.

2. Materials and methods

2.1. Microorganisms and cultivation

Five *Penicillium digitatum* strains were used in this study. The fungi were either isolated from a spoiled tangerine (strain marked CLE, i.e. *P. digitatum* ATCC 201167) and a spoiled mandarin (strain CMC), or obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) (strain PDD, *P. digitatum* DSM 62840). The cultures 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).

2.2. Bioconversion by liquid cultures

Biotransformation experiments by submerged liquid cultures of *P. digitatum* were run during 5–8 days. The fungi were cultivated in 250 or 500-ml conical flasks, filled with 50 resp. 100 ml of liquid medium [YMPG: yeast extract 0.5%, malt extract 1%, bacteriological peptone 0.5%, glucose 1%—pH 6.3; MYB (after Tan et al., 1998): malt extract 2%, glucose 1%, bacteriological peptone 1%, yeast extract 0.3%—pH 6.1; MEB: malt extract 2%, glucose 2%, bacteriological peptone 0.1%—pH 5.4] as described earlier (Demyttenaere & Willemen, 1998). Inoculation was performed with spore suspensions as described earlier (Demyttenaere et al., 2001). The test substrates [(*R*)-(+)-limonene and (*S*)-(−)-limonene] 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 analysed by GC/FID. 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.

2.3. Comparison of different fungal strains and culture broths

The bioconversion of (*R*)-(+)-limonene by three different strains of *P. digitatum*, marked CLE, CMC and PDD, was compared, using two culture broths, YMPG and MYB. Conical flasks of 250 ml were used, filled with 50 ml culture broth, inoculated with 1 ml spore suspension of 1.6, 1.3 and 2.6×10^7 CFU/ml for strain CLE, CMC and PDD, resp. After 64 h of growth 250 μl of a solution of 20% (*R*)-(+)-limonene in MeOH (v/v) was added to the cultures. The first series of samples was taken after 8 h and was followed by a new substrate addition (500 μl of the same solution). The second series of samples was taken 15 h after the second substrate addition and followed by a third substrate addition (250 μl of the solution). The third and fourth series of samples were taken 8 and 24 h after the third substrate addition, resp.

2.4. Comparison of the bioconversion of (R)-(+)- and (S)-(−)-limonene

Three *Penicillium digitatum* strains, CLE, CMC and PDD, were cultivated in triplicate in 100 ml of MYB medium. To two cultures of every strain 100 μl of (*S*)-(−)-limonene was added, while in parallel 100 μl of (*R*)-(+)-limonene was added to the third culture. Substrate addition was performed starting 40 h after inoculation in three steps, to overcome substrate inhibition. A first addition (40 h after inoculation) of 250 μl of a 20% (v/v) limonene/EtOH solution was followed by two additions (41.5 and 43 h after inoculation) of 250 μl of a 10% (v/v) limonene/EtOH solution. Samples were taken 9 and 24 h after the first addition.

2.5. Physical behaviour of limonene in shaking cultures

In a first experiment, six conical flasks were filled with 100 ml of distilled water to which 500 μl of a solution of 20% (*R*)-(+)-limonene (v/v) in EtOH was added (100 μl per flask). Samples were taken every 2 h. Three flasks were closed with a glass stopper to prevent evaporation, three other flasks were closed with a cellulose stopper, to enable aeration and evaporation. The flasks were shaken at 150 rpm at 26 °C. At the end of the experi-

ment, for every solution, a total extraction with CH_2Cl_2 was carried out. Every flask was carefully rinsed with CH_2Cl_2 and these solutions were combined with the respective extracts.

In a second experiment, three conical flasks of 250 ml were filled with 50 ml of water, to which 250 μl of a 20% solution (v/v) of (*R*)-(+)-limonene in EtOH was added. The flasks were closed with glass stoppers and shaken at 150 rpm at 26 °C. After 8 h of shaking, 5-ml samples were taken and extracted with Et_2O, after which the content of each flask was poured in a separatory funnel and extracted with CH_2Cl_2. The empty flasks were rinsed with CH_2Cl_2 and these extracts were analysed separately.

In a third experiment, the course of the limonene concentration in liquid medium was monitored. Four 250-ml conical flasks were filled with 50 ml of sterile MYB medium. Two of the flasks were silylated and two were not. The purpose was to investigate a possible adsorption of limonene onto the glass wall. Silylation was carried out by rinsing subsequently the flasks with a solution of 5% (v/v) chlorotrimethylsilane in dry CH_2Cl_2, rinsing with dry CH_2Cl_2 and drying. To the four flasks 250 μl of a 20% (v/v) solution of (*R*)-(+)-limonene in EtOH was administered. From all the flasks 5-ml samples were taken after 2, 4, 6 and 8 h.

2.6. Influence of co-solvent and its concentration on the bioconversion

To test the influence of the co-solvent on the bioconversion, an experiment was carried out with *P. digitatum* strain CMC in 100 ml of MYB medium. Three different co-solvents were used, namely methanol, ethanol and acetone. Three different solvent concentrations were tested, a low, medium and high concentration, so as to obtain a final solvent concentration in the liquid broths of 0.6, 0.9 and 1.4% (v/v), respectively. To all the cultures the same amount of substrate, (*R*)-(+)-limonene, was applied. For the cultures treated with a low solvent concentration, two additions of 250 μl and two of 125 μl of a 20% (v/v) limonene/solvent solution took place (=750 μl solution), whereas the cultures treated with a medium solvent concentration were supplied with two additions of 250 μl of a 20% substrate solution and two additions of 250 μl of a 10% solution (=1000 μl solution). To the cultures with a high solvent concentration, two times 500 μl and two times 250 μl of a 10% substrate solution were administered (=1500 μl solution). The first and second substrate additions took place 40 and 42 h after inoculation, respectively. The first series of samples was taken 7 h after the second substrate addition. The third and fourth substrate addition took place 15 and 17 h after the first sampling period, respectively. A second series of samples was taken 22 h after the fourth substrate addition.

In order to examine the influence of the addition of an ester to solubilize the nonpolar substrate in the medium, an experiment was performed, using three *P. digitatum* strains, CLE, CMC and PDD. Each strain was cultivated in triplicate in 100 ml of MYB medium. Prior to the first substrate addition, 1 ml of ethyl decanoate (1% v/v) was added to two cultures of each strain. All the cultures were supplied with 250 μl of a 20% (*R*)-(+)-limonene/MeOH solution and samples were taken 8 h later. Immediately following the first sampling, a second substrate addition of 250 μl of a 20% (*R*)-(+)-limonene/MeOH solution took place. A second and third series of samples were taken 17 and 24 h after this second addition, respectively.

2.7. Sequential addition of substrate

The influence of a sequential addition of substrate was tested, using three *P. digitatum* strains, CLE, CMC and PDD, cultivated in triplicate in 100 ml of MYB medium. The same amount of substrate was added to each of three equal cultures, but divided in one, two or three additions. At first, a total amount of 100 μl of (*R*)-(+)-limonene was added starting 40 h after inoculation. When a onefold addition was applied, 500 μl of a solution of 20% (*R*)-(+)-limonene (v/v) in MeOH was added once. In case of a twofold addition, 250 μl of the same substrate solution was added twice with a time difference of 2 h. Finally, in case of a threefold addition, twice 200 μl and once 100 μl of the same substrate solution were administered to the cultures with a time interval of 1.5 h. Samples 1 and 2 were taken 8 and 24 h resp. after the first amount of substrate added.

An analogous time schedule was followed for the second addition of substrate, but using a 10% limonene/MeOH solution, i.e. adding in total 50 μl (*R*)-(+)-limonene. Samples 3 and 4 were taken 8 and 24 h after the second substrate addition, respectively.

2.8. Chemical compounds

The substrates used for the biotransformation experiments were (*R*)-(+)-limonene (puriss. ⩾99%, 99% ee, Fluka, Belgium) and (*S*)-(−)-limonene (97%, 80% ee, Fluka, Belgium). As reference compounds (±)-α-terpineol (99% pure, containing 70% (*R*)-(+)- and 30% (*S*)-(−)-α-terpineol, Acros, Belgium), (*R*)-(+)-α-terpineol (95% ee, Fluka, Belgium) and (*S*)-(−)-α-terpineol (75% ee, Fluka, Belgium) were used.

2.9. 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, Agilent), equipped with a CIS-4 PTV (Programmed Temperature Vaporisation)

Injector (Gerstel, Mülheim a/d Ruhr, Germany), and a HP5-MS capillary column (30 m length × 0.25 mm i.d.; coating thickness 0.25 μm) was used. Working conditions were: Injector 250 °C, Transfer Line to MSD 250 °C, oven temperature: start 50 °C, programmed from 50 to 120 °C at 5 °C min^{-1}, from 120 to 200 °C at 20 °C min^{-1}, hold 2 min; carrier gas (He) 1.0 ml min^{-1}; split 1/20; ionisation: 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^{-1}, from 160 to 220 °C at 15 °C min^{-1}, hold 5 min. GC-analyses were performed with a HP 6890 GC Plus (Agilent), equipped with a split/splitless-injector and an FID-detector and an EC-5 column (30 m length × 0.25 mm i.d.; coating thickness 0.25 μm). Working conditions were: Injector 250 °C, Detector 300 °C (make-up gas He 10 ml min^{-1}), oven temperature: start 50 °C, programmed from 50 to 120 °C at 5 °C min^{-1}, from 120 to 180 °C at 20 °C min^{-1}, hold 2 min; carrier gas (He) 0.8 ml min^{-1}; 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). Working conditions were: Injector 250 °C, Detector 300 °C (make-up gas He 10 ml min^{-1}), oven temperature: start 110 °C, programmed from 110 to 150 °C at 2 °C min^{-1}, from 150 to 160 °C at 5 °C min^{-1}, hold 2 min; carrier gas (He), column head pressure 206.8 kPa; split 1/50.

Substances were identified by comparison of their mass spectra and retention indexes (Kováts Indexes) with those of reference substances (where possible) and with literature (Adams, 1995) and by comparison with the NIST Mass Spectral Library (Version 1.6d, 1998).

3. Results and discussion

3.1. Comparison of different fungal strains and culture broths

In former experiments (Demyttenaere et al., 2001), it was found that the most interesting strains for the bioconversion of (*R*)-(+)-limonene to α-terpineol, were *P. digitatum* strains. In a first experiment, the bioconversion of (*R*)-(+)-limonene by three different strains of *P. digitatum*, marked CLE, CMC and PDD, was compared, using two different culture broths, namely YMPG and MYB (see Section 2). The yields of α-terpineol (amount of α-terpineol produced as compared to the amount of limonene added) obtained from samples 1, 2 and 4 taken after the first, second and third substrate addition respectively, are displayed in Table 1. It is clear that strain CMC gives the highest yield of bioconversion product and the lowest recovery of non-converted substrate. The influence of the culture broth on the bioconversion yields is not obvious. Whereas yields were higher when medium MYB was used for the strains CMC and PDD, medium YMPG seemed better for strain CLE. It can be concluded that strain CMC was able to convert the substrate, (*R*)-(+)-limonene, added to an extent of 0.1% (v/v), in 8 h time. When more substrate was added, the yields dropped and inhibition and substrate accumulation were noticed.

In a second experiment, the three strains CLE, CMC and PDD were compared for their bioconversion capacity, using the two media MEB and MYB. From the results (data not shown), it could be concluded that again the highest yields were obtained with strain CMC and that medium MYB was better (yield 63.3±2.3%; substrate recovery 0.5%) than medium MEB (yield 46.7±0.1%; substrate recovery 3.0±0.3%) for the bioconversion of (*R*)-(+)-limonene.

It can be concluded that the best fungal strain for bioconversion of (*R*)-(+)-limonene to α-terpineol was *P. digitatum* strain CMC (isolated from mandarin) and the best culture broth was medium MYB.

3.2. Influence of the medium composition on the fungal growth

To test the influence of the medium composition on the fungal growth and biomass production, six cultures of *P. digitatum* (strain CMC) were grown in duplicate in the three culture media, YMPG, MEB and MYB. Exactly 48 h after inoculation of the broths with 1 ml of a spore suspension (1.8×10^7 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 6.33±0.11, 5.95±0.23 and 6.73±0.13 g/l, respectively. Hence, the best medium leading to the highest fungal growth (biomass production) was medium MYB, which has also proven to be the best medium for bioconversion. On the contrary, medium MEB, which gave low bioconversion yields, gave the lowest cell dry weight. Therefore, it can be concluded that there is a correlation between best fungal growth and best bioconversion yield.

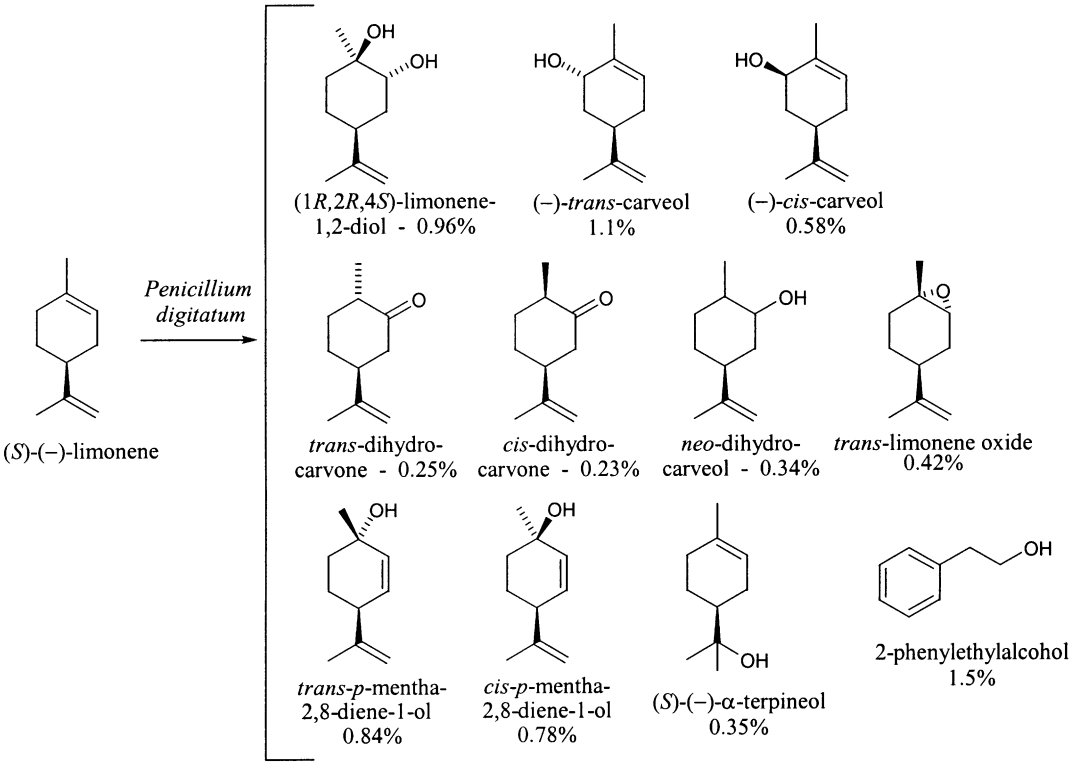
3.3. Comparison of the bioconversion of (R)-(+)- and (S)-(−)-limonene

Using three *P. digitatum* strains, the bioconversion of (*R*)-(+)-limonene and (*S*)-(−)-limonene was compared. Strains CLE, CMC and PDD were cultivated in triplicate: to two cultures of every strain, (*S*)-(−)-limonene was added, while the third culture received (*R*)-(+)-limonene. Results of sampling of the cultures clearly indicated that, in contrast to the bioconversion of (*R*)-(+)-limonene, no significant conversion of (*S*)-(−)-limonene to α-terpineol occurred. These data are in agreement with the findings of Tan et al. (1998) that the

Table 1
Yield of α-terpineol (%) and amount of remaining nonconverted limonene (%) after bioconversion of (*R*)-(+)-limonene by liquid cultures of different *Penicillium digitatum*-strains—samples 1, 2 and 4

Strain	Medium	Sample 1		Sample 2		Sample 4	
		α-Terpineol	Limonene	α-Terpineol	Limonene	α-Terpineol	Limonene
CLE[a]	YMPG[b]	32.51	5.65	10.78	26.23	8.07	4.54
CLE	YMPG	38.64	7.97	12.96	32.05	8.74	3.64
CLE	MYB[c]	30.85	10.97	9.91	31.27	7.08	8.43
CLE	MYB	23.00	13.91	7.36	27.93	5.26	6.98
CMC[d]	YMPG	66.56	1.18	18.40	23.04	14.10	7.84
CMC	YMPG	73.51	1.28	26.97	29.36	16.81	3.57
CMC	MYB	75.91	0.75	30.46	20.02	22.34	3.28
CMC	MYB	79.67	0.88	20.38	21.95	15.82	3.93
PDD[e]	YMPG	13.58	15.30	4.77	32.53	3.41	10.78
PDD	YMPG	18.21	11.67	6.56	28.17	4.47	2.25
PDD	MYB	48.03	7.03	13.75	26.44	9.95	4.92
PDD	MYB	26.18	12.41	4.63	25.08	3.60	7.28

[a] CLE = *Penicillium digitatum* ATCC 201167 isolated from spoiled tangerine.
[b] YMPG = Yeast extract Malt extract Peptone Glucose broth.
[c] MYB = Malt extract Yeast extract Broth.
[d] CMC = *Penicillium digitatum* isolated from spoiled mandarin.
[e] PDD = *Penicillium digitatum* DSM 62840.



Scheme 1. Structures and yields of different metabolites recovered after bioconversion of (*S*)-(−)-limonene by *Penicillium digitatum*.

fungus does not convert (*S*)-(−)-limonene. The result also clarifies the results of former experiments, where in some cases a limited bioconversion of (*S*)-(−)-limonene into (*S*)-(−)-α-terpineol was described (Demyttenaere et al., 2001). Different other metabolites were formed from the bioconversion of (*S*)-(−)-limonene in trace amounts. The structures of these metabolites and their average yields are displayed in Scheme 1.

Bioconversion of limonene to α-terpineol appears to be highly enantioselective: (*R*)-(+)-limonene is converted into pure (*R*)-(+)-α-terpineol (ee > 99%). The chirality of α-terpineol was confirmed by chiral GC analyses. As outlined in the introduction, (*R*)-(+)-α-terpineol is the most interesting enantiomer since it provides the most pleasant odour.

3.4. Physical behaviour of limonene in shaking cultures

The substrate limonene is nonpolar and hence not soluble in water (solubility 13.8 mg/l at 25 °C; Howard & Meylan, 1997). To increase the solubility of limonene in the water phase, a co-solvent is applied. In previous bioconversion experiments however, it was noticed that the concentration of the substrate limonene always showed a very rapid decrease as a function of time (Demyttenaere et al., 2001). Therefore, some experiments were performed in order to shed more light on the physical parameters responsible for this phenomenon.

In a first experiment, the evaporation of limonene from aqueous limonene solutions in flasks closed with a cellulose stopper was compared with the decreasing concentrations of limonene in flasks closed with glass stoppers, by taking liquid samples every two hours (see Section 2).

The samples indicated very low concentrations of limonene in the medium and the concentrations decreased rapidly as a function of time (Fig. 1). This was the case for both the flasks closed with glass stoppers and the flasks with the cellulose stoppers, meaning that evaporation of the substrate was not the (only) reason for the low recovery.

At the end of the experiment, every solution was extracted with CH_2Cl_2 and the flasks were carefully rinsed with CH_2Cl_2 (see Section 2). From the flasks closed with a glass stopper, 78±11% of the initially added limonene was recovered this way, whereas from the flasks closed with cellulose stoppers, the recovery of limonene was 61±3%. The difference in these recoveries indicates the amount of limonene that had really evaporated during shaking. From the flasks closed with glass stoppers, stripping of limonene could only occur when the flasks were opened during sampling.

During the experiment, it was noticed that limonene forms a nonpolar film on the water surface, due to its insolubility. This means that the bioconversion system is of biphasic nature and that the co-solvent does not allow full limonene solubilisation. Considering this, Fig. 1 in fact describes the phase separation process. It shows that phase separation is not an instantaneous phenomenon and equilibrium is reached only after about 2 h. The limonene concentration obtained then is 5.7±0.5 mg/l, which is lower than the maximum solubility of limonene in water, and remains constant until the excess precursor is exhausted. The headspace of the solutions is saturated with limonene, in the case of glass stoppers as well as when cellulose stoppers are used. This gives lead to evaporation losses of limonene during sampling.

Similar experiments were performed in liquid MYB medium. Analogous decreasing concentration profiles were found (Fig. 2). The final limonene concentration found was, however, higher (63±9 mg/l), meaning that the solubility of limonene with ethanol as co-solvent is substantially higher in these media than in pure water. It is expected that the solubility of limonene in full-

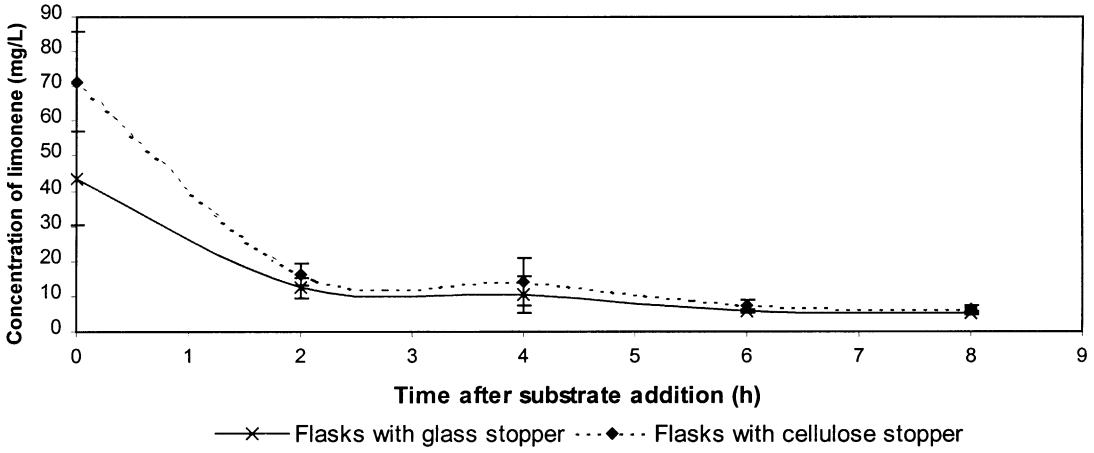


Fig. 1. Decrease as a function of time of the limonene concentration in water solutions in conical flasks, closed with glass or cellulose stoppers—monitoring with 5-ml samples.

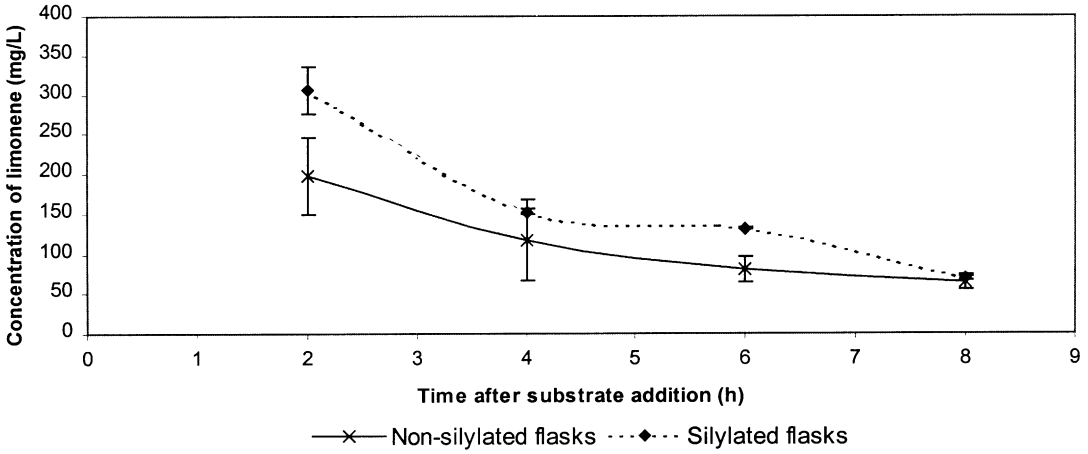


Fig. 2. Decrease as a function of time of the limonene concentration in MYB broth in conical flasks, silylated or nonsilylated—monitoring with 5-ml samples.

grown pellet cultures will be even higher. The standard deviations, however, were also higher. This is probably due to the higher complexity of the medium and interaction with dissolved particles.

α-Terpineol is more soluble in water (1.98 g/l at 25 °C; Howard & Meylan, 1997) than limonene. In liquid medium and with the presence of the co-solvent, this solubility will increase, so no problems should be expected in yield calculations. The yield is calculated as the amount of α-terpineol produced, as compared to the amount of limonene added. This amount of limonene, however, is not immediately totally available for the biocatalyst, but the converted limonene is continuously replaced with limonene from the excess precursor added.

In a second experiment, the results obtained after taking 5-ml samples were compared with the extraction of the solutions with CH_2Cl_2 (see Section 2). The 5-ml samples indicated concentrations in the water close to the solubility of limonene. From the total CH_2Cl_2-extracts carried out in the separatory funnels, however, 40±4% of the initially added limonene was recovered, and rinsing of the flasks yielded another 42±4% of the limonene. In a biphasic system, it is possible that part of the upper layer is transferred to the separatory funnels with the bulk liquid, and part of the upper liquid film stays behind in the flask.

In result of this experiment, a possible adsorption of limonene on the glass wall was considered, since a substantial part of the amount of limonene added could be recovered by rinsing the glass wall with solvent. It was believed that silylation of the glassware would alter the interaction of the glass with the nonpolar limonene. Silylation of the glassware was carried out and the concentration of limonene in MYB medium was monitored taking 5-ml samples. At first, the limonene concentrations in silylated flasks were somewhat higher than in nonsilylated flasks, but after 8 h, there was no significant difference. Probably, the shaking water solutions undo the effect of the silylation of the glassware. The concentrations of limonene thus found still indicate a biphasic system, which shows that adsorption of the glassware is only a minor factor of influence in the low recovery of limonene.

3.5. *Influence of co-solvent and its concentration on the bioconversion*

To improve the availability of the water-insoluble substrate limonene for the biocatalyst, which is present in the water phase, a co-solvent is used. This water miscible solvent is applied to increase the solubility of the substrate in the medium, which will enhance the mass transfer and the bioconversion rate. However, a number of solvents is toxic for microorganisms. A solvent parameter expressing this toxicity of the solvent for microorganisms is the log *P*-value, which is the logarithm of the octanol/water distribution coefficient. Based on many studies with single solvents, it can be concluded that, to a reasonable approximation, the stability of the biocatalyst decreases as the log *P*-value increases, reaching a minimum for log *P*-values between 2 and 4 for microorganisms. After this minimum, increasing log *P* of the solvent results in increased biocatalyst stability (Salter & Kell, 1995). The right choice of solvent implies finding a compromise between solvating power and cytotoxicity.

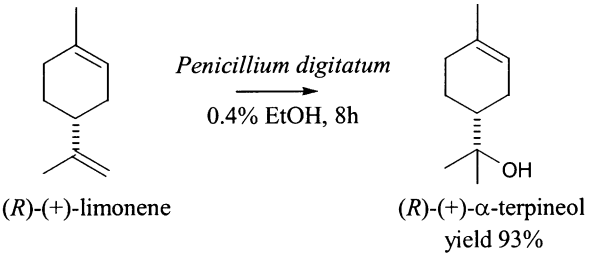
In a first experiment, carried out with *P. digitatum* (strain CMC) in 100 ml of MYB medium, three different co-solvents were used, namely methanol, ethanol and acetone at three different solvent concentrations (see Section 2). The results with the yields of α-terpineol and amounts of nonconverted limonene are displayed in Table 2. A conversion scheme is given in Scheme 2.

From the results displayed in Table 2, it can be concluded that the differences between the yields obtained

Table 2
Yield of α-terpineol (%) and amount of remaining nonconverted limonene (%) after bioconversion of (*R*)-(+)-limonene by liquid cultures of *Penicillium digitatum* with different co-solvents at different concentrations- first and second series of samples

Co-solvent	Co-solvent concentration[a]	Sample 1		Sample 2	
		α-Terpineol	Limonene	α-Terpineol	Limonene
Methanol	low	87.99	0.72	61.32	1.18
Methanol	medium	86.18	0.69	61.05	1.90
Methanol	high	87.95	0.99	61.35	1.25
Ethanol	low	93.24	0.96	65.72	1.48
Ethanol	medium	89.25	0.98	62.28	1.50
Ethanol	high	91.70	1.05	64.85	1.10
Acetone	low	84.83	1.02	56.98	1.18
Acetone	medium	89.15	0.85	61.62	1.52
Acetone	high	82.29	3.63	60.59	1.74

[a] Low = 0.6%, medium = 0.9% and high = 1.4%.



Scheme 2. Bioconversion of limonene to α-terpineol by *Penicillium digitatum* using EtOH as co-solvent.

when different co-solvents were used, were rather small. Generally, the yields with EtOH as co-solvent were slightly higher, and with acetone the yields were lower. No clear correlation could be found between the different co-solvent concentrations applied (0.6, 0.9 and 1.4%) and the bioconversion yields obtained. Although MeOH was expected to be less toxic to the biocatalysts than EtOH and acetone due to its lower log *P*-value (−0.77 for MeOH compared to 0.31 for EtOH and 0.24 for acetone; Howard & Meylan, 1997), none of the three co-solvents applied, seemed to cause any inhibition of the fungal biocatalyst.

Tan and Day (1998b) investigated the effect of organic co-solvents on the bioconversion of (*R*)-(+)-limonene to (*R*)-(+)-α-terpineol by *P. digitatum*. They found that ethanol caused inhibition of the bioconversion at a concentration of 2%. Methanol on the other hand was shown to positively influence the bioconversion, with an optimal concentration of 0.5%, but causing cytotoxic effects at concentrations higher than 2%. The effect of acetone was not checked by these authors. The results obtained in the present research show that MeOH, EtOH as well as acetone have a positive effect on the bioconversion, when applied in concentrations of 0.5–1.5%.

Tan and Day (1998b) also studied the effect of the addition of esters as co-solvents on the bioconversion of limonene by *P. digitatum*. The strongest increase in bioconversion yields was noticed when dioctyl phthalate or ethyl decanoate were added to the culture broths as co-solvent. With an ester concentration of 1.5%, yields were increased more than twofold. In the following experiment, the effect of the addition of small amounts of ethyl decanoate to the cultures on the bioconversion capacity was investigated.

Three strains, CLE, CMC and PDD, were cultivated in triplicate in MYB. To two cultures of every strain, 1 ml of ethyl decanoate was added. Two substrate additions took place and three series of samples were taken (see Section 2), the results of which are depicted in Table 3. It becomes clear that in the presence of 1% ethyl decanoate, nonconverted limonene is present in substantially higher concentrations than found with the other co-solvents. This indicates that limonene is better solubilised in the medium. Still, the α-terpineol yields achieved in the cultures with ester addition are considerably lower than in the untreated cultures, in contrast to the positive results obtained by Tan and Day (1998b). This can be explained by the high log *P*-value of ethyl decanoate (4.79; Howard & Meylan, 1997), leading to a better solubilisation of limonene, but exerting a toxic effect on the fungal cultures.

3.6. Sequential addition of substrate

One of the main problems commonly encountered with biotransformation experiments of monoterpenes, is the toxicity of these compounds for microorganisms. Addition of substrate in high concentrations leads to inhibition. Besides, an important amount of the substrate added, is often lost due to evaporation. In order to provide the fungal cells with appropriate amounts of substrate at each time, the effect of a sequential addition of substrate was tested.

Table 3
Yield of α-terpineol (%) and amount of remaining nonconverted limonene (%) after bioconversion of (*R*)-(+)-limonene by liquid cultures of *Penicillium digitatum* with or without addition of 1% ethyl decanoate- first, second and third series of samples

Strain	Addition of ester	Sample 1		Sample 2		Sample 3	
		α-Terpineol	Limonene	α-Terpineol	Limonene	α-Terpineol	Limonene
CLE[a]	yes	12.26	29.86	16.82	54.87	18.72	50.01
CLE	yes	10.17	25.38	14.41	56.98	17.05	55.20
CLE	no	40.30	13.38	23.45	7.55	24.29	1.75
CMC[b]	yes	12.12	13.06	10.96	11.01	15.94	23.30
CMC	yes	9.94	18.75	13.07	34.93	14.73	36.60
CMC	no	65.30	0.00	51.80	0.00	49.90	0.00
PDD[c]	yes	8.33	13.84	8.23	19.69	9.90	25.20
PDD	yes	0.00	28.80	5.66	31.68	6.26	31.19
PDD	no	45.08	3.90	27.26	2.86	26.52	0.00

[a] CLE = *Penicillium digitatum* ATCC 201167 isolated from spoiled tangerine.
[b] CMC = *Penicillium digitatum* isolated from spoiled mandarin.
[c] PDD = *Penicillium digitatum* DSM 62840.

Again, three *P. digitatum* strains were cultivated in 100 ml of MYB medium. An equal amount of (*R*)-(+)-limonene as a solution in MeOH was administered to the cultures in one of three different ways: divided in one, two or three additions. Samples 1 and 2 were taken after a first substrate addition of 100 μl of limonene, while samples 3 and 4 were taken after a second addition consisting of 50 μl of limonene. The results of samples 1, 2 and 4 are depicted in Table 4.

Especially from the first series of samples, the beneficial effect of a sequential addition of limonene becomes obvious. Yields of the first and second series of samples are considerably higher when a sequential addition of substrate was applied. Considering the final yields however, little difference is noticed between substrate addition in one or in several times. Whether a twofold or a threefold addition is preferable, remains unclear.

3.7. Stability tests of limonene

Since the pH of the culture broths dropped in most cases dramatically after bioconversion, a possible acid catalysed conversion of limonene needed to be considered. In previous experiments (Demyttenaere et al., 2001) it was shown that no acid catalysed conversion products from limonene could be recovered from the headspace extracts. One more control test was run with the substrate dissolved in sterile broths, acidified to pH 3.5 with HOAc. The control flasks were shaken for 5 days and samples were taken and extracted at different

Table 4
Yield of α-terpineol (%) and amount of remaining nonconverted limonene (%) after bioconversion of (*R*)-(+)-limonene by liquid cultures of *Penicillium digitatum* testing different ways of sequential substrate addition- first, second and fourth series of samples

Strain	Substrate addition	Sample 1		Sample 2		Sample 4	
		α-Terpineol	Limonene	α-Terpineol	Limonene	α-Terpineol	Limonene
CLE[a]	Onefold	61.60	7.75	66.35	0.60	45.60	1.27
CLE	Twofold	83.12	0.62	83.70	0.00	51.25	1.26
CLE	Threefold	80.37	1.16	79.74	0.00	52.21	1.00
CMC[b]	Onefold	74.81	3.45	76.62	0.45	61.98	0.73
CMC	Twofold	86.90	0.76	87.03	0.36	60.84	1.44
CMC	Threefold	87.74	0.89	83.57	0.30	59.53	1.71
PDD[c]	Onefold	37.03	17.67	49.37	0.96	41.67	0.38
PDD	Twofold	64.44	2.92	51.10	0.60	47.60	7.87
PDD	Threefold	80.47	1.92	81.88	0.27	52.35	0.90

[a] CLE = *Penicillium digitatum* ATCC 201167 isolated from spoiled tangerine
[b] CMC = *Penicillium digitatum* isolated from spoiled mandarin
[c] PDD = *Penicillium digitatum* DSM 62840

time intervals. Also in the liquid extracts, no acid catalysed conversion of the substrate was noticed and no traces of α-terpineol were recovered. Therefore, it can be concluded that the substrate limonene is very stable in acidic conditions at a pH up to 3.5.

4. Conclusions

It can be concluded that the best fungal strain for bioconversion of (*R*)-(+)-limonene to α-terpineol was *P. digitatum* strain CMC (isolated from mandarin) and the best culture broth was medium MYB. No acid catalysed conversion of the substrate was observed at pH 3.5.

The bioconversion of limonene to α-terpineol was highly enantioselective since (*R*)-(+)-limonene was converted into pure (*R*)-(+)-α-terpineol (ee > 99%), which is the most interesting enantiomer providing the most pleasant odour.

Study of the physical behaviour of limonene in shaking cultures indicated that the bioconversion occurs mainly in a biphasic system. When different co-solvents were used to dissolve the substrate limonene into the medium, the differences between the bioconversion yields were rather small, although the highest yields were obtained with EtOH as co-solvent. Addition of 1% of ethyl decanoate had a negative influence on the bioconversion capacity.

From the economical and practical point of view, when a faster bioconversion is pursued, a sequential substrate addition at low concentration is advised.

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