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Towards the bio-production of *trans*-carveol and carvone from limonene: induction after cell growth on limonene and toluene

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Abstract—*Rhodococcus opacus* PW4 cells were found to produce *trans*- and *cis*-carveol and/or carvone as result of limonene metabolism, depending on the type and concentration of the carbon source used for cell growth. In aqueous systems, cells grown on ethanol and toluene only produced *trans*-carveol, whilst cells grown on limonene and on toluene with a larger head-space available produced both *trans*-carveol and carvone. In biphasic systems, limonene was converted to *trans*- and *cis*-carveol as well as to carvone, regardless of the carbon source used, although carveol and carvone production rates were higher in toluene and limonene grown cells, respectively. A good and stable emulsion was obtained in a magnetically stirred two-phase reactor but both *trans*-carveol and carvone were produced at low rates: 0.08 and 0.02 nmol/min mg prot, respectively. No *cis*-carveol was formed. When (−)-carveol was added, carvone production increased 4.7 fold to 0.12 nmol/min mg prot. Using an aerated two-phase reactor, carvone production was enhanced even with cells grown on toluene. The highest *trans*- and *cis*-carveol and carvone production rates were attained with cells grown on limonene by continuously supplying limonene to the reactor through the air stream, carvone production reaching 0.58 nmol/min mg prot. The best *trans*-/*cis*-carveol ratio (2.26) was observed with cells grown on toluene when limonene was supplied in the gas phase. When 50 mM limonene was added initially, carvone was produced 27.9 and 141.4 times faster than *trans*-carveol with cells grown on toluene and limonene, respectively.
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1. Introduction

Terpenes are spread throughout nature and some of them, such as limonene, are readily available at low cost, which motivates their use as reaction substrates. A high number of their oxygenated derivatives, known as terpenoids, are used as flavours and fragrances in the food and perfume industries.

The oxidation of limonene at the 6-position has been reported: production of *cis*- and *trans*-carveol, carvone, *cis* and *trans*-*p*-menth-2,8-dien-1-ol, *p*-menth-1,8-dien-4-ol, perillyl alcohol and *p*-menth-8-ene-1,2-diol by *Penicillium digitatum* and *P. italicum*;[1] production of α-terpineol and 6-hydroxycarveol by the fungus *Armillareira mellae*;[2] formation of isopiperitenone, limonene-1,2-diol, *cis*-carveol, perillyl alcohol, isopiperitenol and α-terpineol by the fungus *Aspergillus cellulosae*;[3] production of carveol, α-terpineol, perillyl alcohol, and perillyl aldehyde by *Bacillus stearothermophilus* BR388;[4] production of *trans* and *cis*-carveol and carvone by enzymes from the plants *Solanum aviculare* and *Dioscorea deltoidea*;[5] formation of *trans* and *cis*-carveol and carvone by the basidiomycete *Pleurotus sapidus*;[6] production of carvone from limonene (produced from geranyl diphosphate) in the fruit of caraway, *Carum carvi*;[7] conversion of (−)- and (+)-limonene into, respectively, (−)- and (+)-carvone in the plant *Mentha cardiaca*;[8] production of perillyl alcohol and α-terpineol, after cloning the genes encoding a thermostable limonene hydratase, in *Escherichia coli*.[9]

Recently, Duetz et al.[10] presented a toluene-degrading strain, *Rhodococcus opacus* PWD4, which is able to produce enantiomerically pure *trans*-carveol with traces of carvone by hydroxylation of D-limonene exclusively at the 6-position. These authors used toluene as the carbon and energy source for cell growth and achieved a maximum specific activity of 14.7 U ($g_{dry\ weight}$ of cells). *R. globerulus* PWD8, also able to degrade toluene, oxidised the *trans*-carveol formed after limonene oxidation in carvone, although at low rates.

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Previous work carried out in our laboratory, concerning the biotransformation of limonene-1,2-epoxide and (−)-carveol carried out with whole cells of *R. erythropolis* DCL14, showed that biotransformation rates could be enhanced by growing the cells in limonene as carbon source and by performing the reaction in an organic:aqueous system.[11–14] The conversion rates depended widely on the carbon source used for cell growth and on the solvent chosen as organic phase. The present work is aimed at finding favourable growth and bioconversion conditions to enhance limonene hydroxylation by *R. opacus* PWD4 cells.

2. Results and discussion

2.1. Carbon source

Duetz et al.[10] found that *R. opacus* PWD4 cells grown on glucose were unable to carry out the formation of *trans*-carveol or any other product resulting from the oxidation of limonene, whilst cells grown on toluene as the sole carbon source produced enantiomerically pure (+)-*trans*-carveol with traces of (+)-carvone.

Herein, it has been found that the production of both *trans*- and *cis*-carveol and carvone depends on the type and concentration of carbon source used for cell growth (Fig. 1). In single aqueous phase systems, cells grown on ethanol and toluene produced only *trans*-carveol from limonene, although cells grown on 0.25% (v/v) toluene produced also a small amount of *cis*-carveol. Cells grown on toluene in flasks with a larger head-space and thus more air (B-Tol) and on limonene produced both *trans*-carveol and carvone. When a concentration of limonene and toluene of 0.125% was used, carvone production rate was around 80% that of *trans*-carveol, whilst for an initial concentration of 0.25%, carvone production rate decreased to 60 and 40% the value of *trans*-carveol production rate for cells that metabolised limonene and toluene, respectively. In biphasic systems, *R. opacus* PWD4 cells converted limonene into *trans* and *cis*-carveol and carvone, independent of the carbon source used for cell growth. Cells grown on ethanol, toluene and 0.125% B-Tol, produced three products at similar rates. The best *trans*-carveol and carvone production rates were attained with cells grown on 0.125% limonene. Carveol selectivity was also improved as the production of *cis*-carveol was very low. When metabolising 0.25% of limonene, the carvone production rate was also higher than that obtained with the other carbon sources, but cells produced more *cis*-carveol as well. Cells grown on 0.25% B-Tol produced *cis*- and *trans*-carveol at similar rates and almost no carvone.

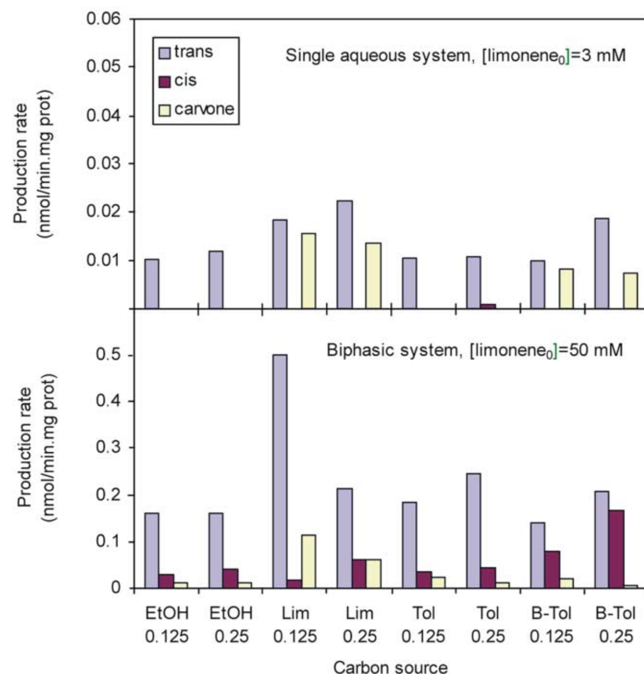


Figure 1. *Trans*- and *cis*-Carveol and carvone production rates in single aqueous and *iso*-octane:aqueous systems with cells grown on 0.125 or 0.25% (v/v) of ethanol (EtOH), limonene (Lim), toluene (Tol) and toluene in flasks with more air available (B-Tol); 30°C and 200 rpm with an initial biomass correspondent to 0.39 g/L of protein; limonene initial concentration referred to the aqueous phase.

The results indicate that if the cells grow on toluene with a higher amount of air available, the conversion of limonene to carvone will be induced in single aqueous systems, probably due to an activation of oxidative steps in the metabolic pathway. On the other hand, in organic:aqueous systems, these cells produced a low amount of carvone whilst *cis*-carveol was produced at a higher rate than when the cells grew with less oxygen available. The ratio between the production rates of carvone and *trans*-carveol obtained with cells grown on limonene was also lower in biphasic systems than in single aqueous systems (data not shown). It could be considered that, since carveol partitions preferentially to the organic phase, in biphasic systems cells could have a more difficult access to this compound, and thus, a lower carvone production. However, since the reaction is carried out inside the cell, carveol should be readily available for carvone production. The fact that *cis*-carveol production is induced in biphasic systems could be the result of a higher initial limonene concentration allowed in these systems, in which the organic phase acts as substrate reservoir.

Degradation of several monoterpenes, namely (+)-(*R*)-limonene, (−)-carveol (mixture of enantiomers), (−)-(*R*)-carvone, (+)-(*S*)-carvone and limonene-1,2-epoxide, in dodecane:aqueous phase systems, indicated the steps used by *R. opacus* cells in the metabolism of these compounds (data not shown). Degradation of limonene with limonene grown cells resulted in the production of carvone and almost no production of either *trans*- or *cis*-carveol, although the production rate of the three compounds increased with increasing initial limonene concentrations. However, the degradation of limonene was strongly inhibited by its initial concentration: almost nothing was produced for an initial limonene concentration of 30 mM, referred to the aqueous phase.

Toluene grown cells produced mainly *cis*- and *trans*-carveol from limonene and almost no carvone.

Bioconversion of (–)-carveol by limonene grown cells resulted in the production of (–)-carvone by degradation of mainly *trans*-carveol. For a carveol concentration range of 3–50 mM, referred to the aqueous phase, little variation in the carvone production rate was observed, the average being 0.15 nmol/min mg prot. Cells grown on toluene produced (–)-carvone, almost independently of the initial carveol concentration, by degradation of *trans*-carveol at a low rate, the average being 0.08 nmol/min mg prot.

When (–)-carvone was added to biphasic systems with limonene grown cells, for concentrations referred to the aqueous phase lower than 16 mM, *trans*-carveol was produced, while for concentrations higher than 30 mM, *R. opacus* cells produced *cis*-carveol. Cells grown on toluene produced both *trans*- and *cis*-carveol (the *cis* isomer at higher rates) for initial carvone concentrations higher than 16 mM. In all cases, the production rates were very low (around 0.007 nmol/min mg prot).

The (+)-(*S*)-carvone tested had a purity of 96%, the 4% being (+)-*trans* and (+)-carveol. When this carvone was used as substrate, it was not metabolised by *R. opacus* cells, but the (+)-carveol present was rapidly consumed, yielding (+)-carvone. Cells grown on limonene showed a 0.87 nmol/min mg prot production rate, whilst toluene grown cells attained 4.35 nmol/min mg prot. The result indicates that *R. opacus* cells are much better degraders of (+)-carveol than of (–)-carveol. Limonene-1,2-diol was formed by conversion of 50 mM (referred to the aqueous phase) limonene-1,2-epoxide at 3.83 and 8.5 nmol/min mg prot by cells grown on limonene and toluene, respectively. In both cases, the diol production rate increased with increasing epoxide concentrations.

Apparently, and considering the results obtained, *R. opacus* PWD4 cells contain at least a limonene hydroxylase (conversion of limonene to carveol), a carveol dehydrogenase (carveol to carvone) and a limonene epoxide hydrolase (limonene epoxide to limonene diol). The conversion of carvone to carveol may be achieved by the same carveol dehydrogenase or by a different carvone hydrogenase. Further studies concerning these enzymes need to be made to elucidate the whole limonene metabolic pathway in this strain.

Assays carried out with cells grown on agar-based mineral medium plates with different amounts of limonene showed that the production of *trans*-carveol and carvone depended on the concentration of limonene used as carbon source (Fig. 2). Carvone production rate decreased with increasing concentrations of limonene in the petri dish, up to a concentration of 0.16% (v/v) limonene, whilst *trans*-carveol production increased. The minimum carvone and maximum carveol production rates were attained when cells had grown in the presence of 40 μL of limonene (0.16%). The converse was observed for higher limonene concentrations. When the cells grew in the presence of more

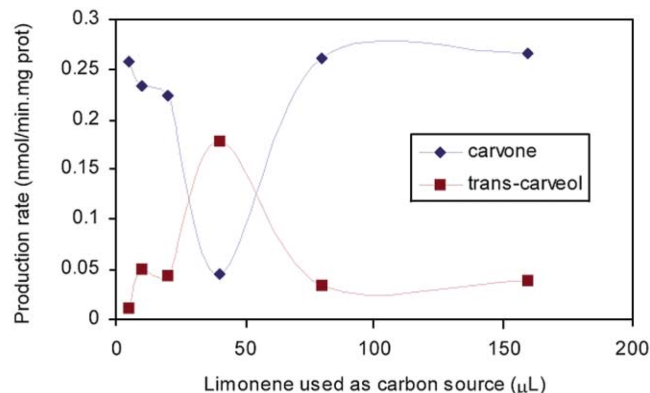


Figure 2. *trans*-Carveol and carvone production rates obtained with cells grown on agar-based mineral medium plates with different initial amounts of limonene.

than 80 μL of limonene (concentrations higher than 0.32%, v/v), *trans*-carveol and carvone production rates were almost constant in the tested concentrations, carvone being produced 7 fold faster than carveol.

Extraction 1:1 (v/v) of the aqueous phase with ethyl acetate showed that limonene-1,2-diol was also produced during the experiments (data not shown). The diol was only detected 24 h after the beginning of the experiment, which could be the reason why Duetz et al. (2001) never detected it. However, the production rate of the diol was nearly 7.9 nmol/min mg prot, by cells grown on the two highest limonene concentrations tested. This shows that *R. opacus* cells also contain a limonene epoxidase which enables them to convert limonene in limonene-1,2-epoxide. Furthermore, when both *R. opacus* and *R. erythropolis* DCL14 (which shows a high carveol dehydrogenase activity and thus should improve carvone production[12]) were used to carry out the conversion of limonene, no carvone or carveol were produced (data not shown). Only accumulation of limonene-1,2-diol was observed, independently of the carbon source used for growing *R. opacus* cells.

In all assays described, no differences were observed when they were carried out with 100 mM glucose as compared to the those assays performed in the absence of glucose. Nevertheless, *trans*-carveol production rates as high as those presented by Duetz et al.[10] were never attained.

2.2. Magnetically stirred two-phase reactor

In order to increase *trans*-carveol and carvone production rates and to evaluate the production of limonene-1,2-diol during the experiments, a magnetically stirred two phase reactor was tested, at 30°C. *Iso*-octane was used as organic phase to follow diol accumulation, as some interference with the diol peak would occur during GC analysis if *n*-dodecane were used.

This reactor allowed the formation of a good and stable emulsion, which permitted a high area of contact between the cells and the organic phase containing

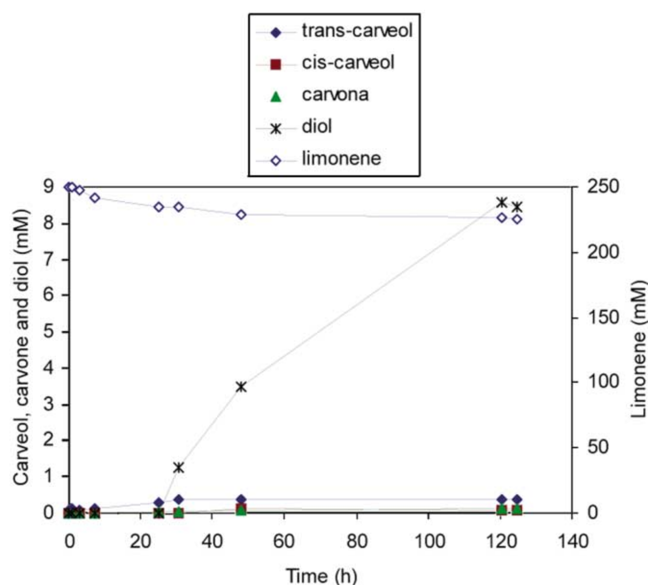
limonene. Nevertheless, *trans*-carveol and carvone were produced at low rates: 0.08 and 0.02 nmol/min mg prot, respectively (Fig. 3). No *cis*-carveol formation was detected. Diol production was only significant after 25 h of bioconversion, but a production rate of 0.53 nmol/min mg prot was attained onwards.

When commercial carveol was added to the system at 126.7 h, carvone production was enhanced 4.7 fold to 0.12 nmol/min mg prot (data not shown), suggesting that the step from limonene to carveol is the limiting step in carvone production.

2.3. Aerated two-phase reactor

The aim of developing an aerated two-phase reactor was both to increase the amount of air available to the cells, so as to increase carvone production, and to feed continuously limonene to the system, while preventing high concentrations that might be toxic to the cells. The highest production rates were attained with cells grown on limonene and when limonene was fed through the air stream (Fig. 4). In this case, cells produced *trans*-carveol nearly 40 times faster than cells grown on toluene while carvone production rate was, on average, 7 fold higher. This also means that carvone production was enhanced even in toluene grown cells, as compared to *trans*-carveol production.

Cells grown on toluene produced *trans*-carveol at similar rates when limonene was added through the air stream and when 50 mM were added initially (maintaining the aeration). However, in this latter case, both *cis*-carveol and carvone were produced at higher rates than when limonene was supplied in the gas phase. Cells grown on limonene, produced the three compounds at much higher rates when limonene was supplied through the air stream.



Figrue 3. Time course of the biotransformation in a magnetically stirred two-phase reactor. Concentrations referred to the organic phase.

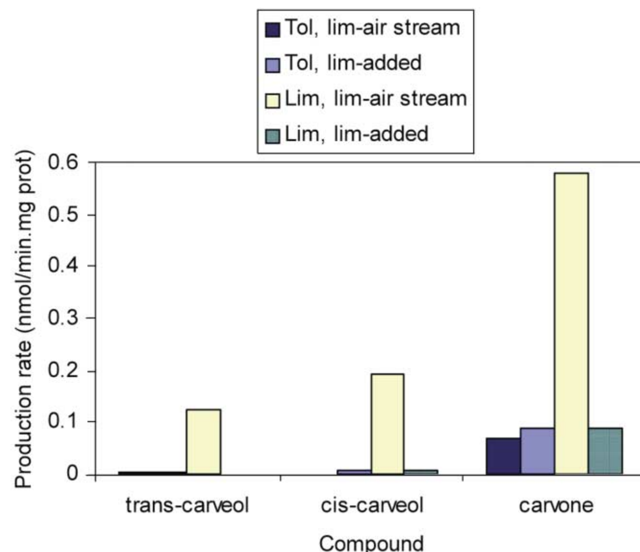


Figure 4. *trans*- and *cis*-Carveol and carvone production rates during the biotransformation of limonene on an aerated two-phase reactor with cells grown on toluene (Tol) or limonene (Lim). Limonene was supplied either through the air stream (lim-air stream) or was added initially (lim-added).

The best *trans*/*cis* selectivity was obtained with cells grown on toluene when limonene was supplied through the air stream, *trans*-carveol being produced at a rate 2.26 times faster than the *cis*-isomer (Fig. 5). Cells grown on toluene and carrying out the conversion in the situation in which limonene was added at the beginning of the assay presented a similar *trans*/*cis* ratio to those grown on limonene but performing the bioconversion of limonene supplied by the gas phase. In these cases, the *cis*-isomer was produced faster than *trans*-carveol. Almost no *trans*-carveol was observed by degradation of limonene, with limonene grown cells, when 50 mM limonene was added at the beginning of the experiment.

In all cases, carvone was always the main product of limonene conversion (Fig. 5). Even when limonene was added through the air stream (which also enhanced *trans*-carveol production), carvone production rate was 18.7 and 4.7 fold higher than that of *trans*-carveol for cells grown on toluene and limonene, respectively. When 50 mM limonene was added initially, carvone was produced 27.9 times faster than *trans*-carveol with cells grown on toluene, the ratio achieving 141.4 for cells grown on limonene.

The blank assay revealed that the time of residence of limonene inside the reactor was very short when limonene was supplied through the air stream, specially when cells were not present (data not shown). This suggested that a larger amount of solvent should be present to maintain higher quantities of limonene inside the reactor. When the organic:aqueous phase ratio was increased to 2:5 the amount of *trans*- and *cis*-carveol and carvone was much lower than in systems with a

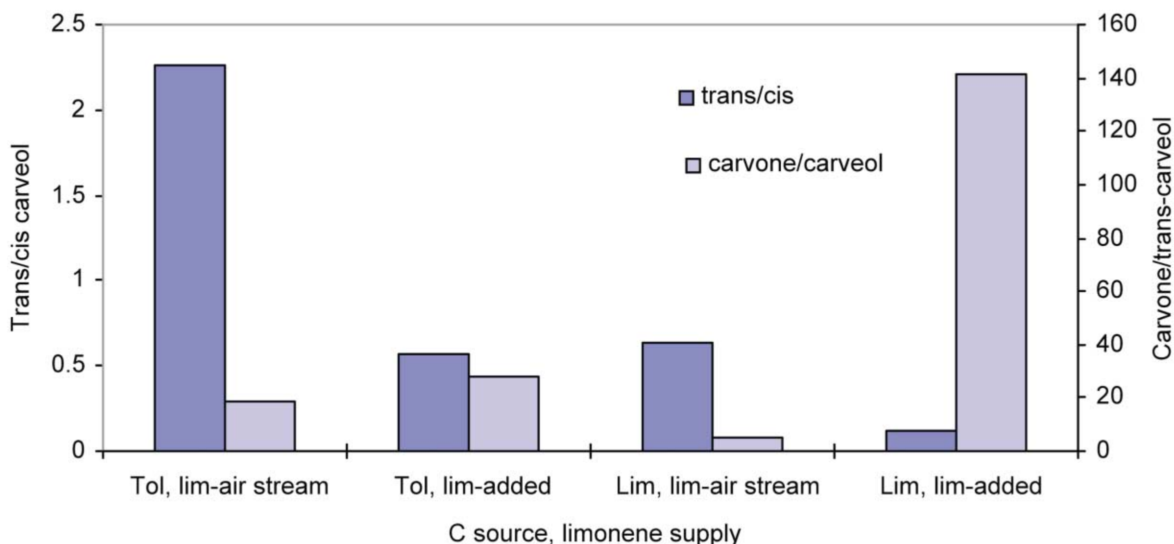


Figure 5. *trans-/cis*-Carveol and carvone/*trans*-carveol production rate ratios observed during limonene conversion in an aerated two-phase reactor. Abbreviations as in Figure 4.

ratio of 1:5 (data not shown). The decrease was almost 10 fold when limonene was supplied through the gas phase, independent of the carbon source used for cell growth. When 50 mM limonene was added initially, the decrease was even more significant reaching almost 30 fold. The results thus show that this phase ratio did not improve limonene oxidation. Although higher amounts of limonene were present, at this phase ratio, only about a quarter of the organic phase was emulsified since the extent of coalescence was also high, and thus the interfacial area was much smaller than in 1:5 systems.

3. Conclusions

Production of *trans*- and *cis*-carveol and/or carvone as result of limonene metabolism by *R. opacus* PW4 cells was found to depend on both the type and concentration of the carbon source used for cell growth. In aqueous systems, cells grown on ethanol and toluene only produced *trans*-carveol. However, cells grown on limonene and on toluene with a larger head-space produced both *trans*-carveol and carvone. In biphasic systems, limonene was converted to *trans*- and *cis*-carveol as well as to carvone, regardless of the carbon source used, although carveol and carvone production rates were higher in toluene and limonene grown cells, respectively.

Degradation of (+)-(*R*)-limonene, (−)-carveol (mixture of enantiomers), (−)-(*R*)-carvone, (+)-(*S*)-carvone and limonene-1,2-epoxide, indicated that *R. opacus* PWD4 cells contain a limonene hydroxylase (conversion of limonene to carveol), a carveol dehydrogenase (carveol to carvone), a limonene epoxidase (conversion of limonene to limonene-1,2-epoxide) and a limonene epoxide hydrolase (limonene epoxide to limonene diol). The conversion of carvone to carveol may be achieved by the same mentioned carveol dehydrogenase or by a different carvone hydrogenase. The results also suggest that *R. opacus* cells degrade (+)-carveol at higher rates than (−)-carveol.

The production rates of *trans*-carveol and carvone depended also on the concentration of limonene used as carbon source. Carvone production rate decreased with increasing initial concentrations of limonene, up to a concentration of 0.16% (v/v), whilst *trans*-carveol production increased. For higher limonene concentrations the converse was observed. At limonene concentrations higher than 0.32% (v/v), *trans*-carveol and carvone production rates were almost constant in the tested concentrations, carvone being produced 7 fold faster than carveol.

A good and stable emulsion was obtained in a magnetically stirred two-phase reactor but both *trans*-carveol and carvone were produced at low rates: 0.08 and 0.02 nmol/min mg prot, respectively. No *cis*-carveol was formed but limonene-1,2-diol was produced from 25 h onwards at 0.53 nmol/min mg prot. When (−)-carveol was added, carvone production increased 4.7 fold to 0.12 nmol/min mg prot.

Using an aerated two-phase reactor, carvone production was enhanced even with cells grown on toluene. The highest production rates were attained with cells grown on limonene and when limonene was fed to the reactor through the air stream. The best *trans-/cis*-carveol ratio (2.26) was observed with cells grown on toluene when limonene was supplied in the gas phase. When 50 mM limonene was added initially, carvone was produced 27.9 and 141.4 times faster than *trans*-carveol with cells grown on toluene and limonene, respectively.

Although our results show that biphasic systems may largely increase *trans*-carveol and carvone productions as compared to single aqueous systems, specially if air

is supplied, the cells used were never able to produce *trans*-carveol at the rate indicated by Duetz et al., which was 14.7 U/g $_{\text{dry weight}}$ of cells, in a single aqueous system.[10]

4. Materials and methods

4.1. Strain

R. opacus PWD4 (DSMZ 44313) was supplied by Duetz and co-workers.

4.2. Growth on solid media

4.2.1. On toluene. Cells were grown on agar-based mineral medium plates, without any added carbon source, and incubated in a desiccator with a solution of 10% (v/v) toluene in *n*-hexadecane. After 4 days at room temperature, the cells were scrapped from the agar surface and suspended in mineral medium with the same composition as described by Wiegant and de Bont.[15]

4.2.2. On limonene. Cells were grown at 30°C on agar-based mineral medium plates to which 5, 10, 20, 40, 80 or 160 μL of limonene were added before solidification of the agar (25 mL per Petri dish). The petri dishes were wrapped in celophane paper to prevent limonene evaporation. Four days after inoculation colonies were already visible on the plates. The cells were harvest and suspended in mineral medium as described previously.

4.3. Growth on liquid media

Cells were grown on 1 L flasks, closed with rubber stoppers, containing 150 mL of mineral medium. 4 mL of cell suspension, previously grown on 1 g/L ethanol, were used as inocculum. The carbon sources used were 0.125% (v/v) ethanol, limonene and toluene. Growth was followed by measuring the optical density (O.D.) at 600 nm. When the value was higher than 1.5, cells were harvested by centrifugation (7000 rpm, 10 min) and used forthwith in the bioconversion studies.

4.4. Bioconversion assays

All assays were carried out at least in duplicate.

4.4.1. Single aqueous systems. To 2 mL of fresh phosphate buffer (50 mM, pH 7.0) containing the cells, 1 or 5 μL of limonene were added on a 10 mL test tube, closed with tin foil and tape to prevent limonene losses. The reactions were carried out at 30°C and 200 rpm. In some of the assays, 100 mM of glucose were added initially.[10] The reaction was followed by gas chromatography (GC) after extraction of substrate and products from the aqueous phase with 0.5 mL of ethyl acetate.

4.4.2. Biphasic systems. To 2 mL of fresh phosphate buffer (50 mM, pH 7.0) containing the cells, 0.4 mL of either *iso*-octane or *n*-dodecane were added. In some assays, 100 mM glucose were also added initially. The reaction started by adding 1, 5 or 17 μL of limonene. The bioconversions were carried out in test tubes closed with tin foil and tape, at 30°C and 200 rpm. The limonene conversion was followed by monitoring limonene consumption and product accumulation in the organic phase by GC analysis.

4.4.3. Biphasic systems magnetically stirred. 4 mL of *iso*-octane containing 50 mM limonene were added to 20 mL of phosphate buffer containing *R. opacus* cells (corresponding to 9.44 mg of protein) and 100 mM glucose in a 100 mL flask closed with rubber bungs wrapped in tin foil. The bioconversion was performed at 30°C and 200 rpm and monitored by GC analysis of the organic phase.

4.4.4. Biphasic systems with aeration. The reaction system consisted of 20 mL of phosphate buffer containing the cells (corresponding to 9.69 mg of protein) and 4 mL of *n*-dodecane. Limonene was either continuously fed by the air stream (by bubbling the inlet air at 158.8 mL/min, through the sintered glass sparger of a glass bottle containing limonene; the air stream entered each reaction flask at 79.4 mL/min) or added initially (170 μL; air was supply directly to the reaction flask at 79.4 mL/min).

4.5. Chemicals

The terpenes used were (+)-(*R*)-limonene (97%), (−)-carveol (97%), (−)-(*R*)-carvone (98%) and (+)-(*S*)-carvone (96%) from Aldrich Chemicals. The ethyl acetate (99.5%) used for extracting the terpenes from the aqueous phase was from Riedel-de Haën. The organic solvents used in biphasic systems were *n*-dodecane (>99%) purchased from Aldrich and *iso*-octane (>99.5%) from Riedel-de Haën.

4.6. Analysis

Limonene consumption and carveol and carvone accumulation was followed by gas chromatography on a Hewlett Packard 5890 gas chromatograph with a FID detector, connected to a HP3394 integrator. The capillary column was a SGE HT5, 25 m in length and with internal and external diameters of 0.22 and 0.33 mm, respectively. The oven temperature was 120°C and that of the injector 200°C. The detector was at 250°C.

4.7. Error analysis

The error associated with the GC quantification of samples, injected without previous treatment, was ±6% and that of aqueous samples, extracted with ethyl acetate prior to injections, was ±8%. The errors were calculated based on the standard deviation and sample mean of seven repeated injections and are quoted for a confidence interval of 95%. Biomass concentration measurements (O.D.) had an associated error of ±8% based on the standard deviation and sample mean of eight repeated samples, quoted for a confidence interval of 95%.

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References

1. Bowen, E. R. *Proc. Fla. State Horti. Soc.* **1975**, 304–308.
2. Draczynska, L. B. *J. Basic Microbiol.* **1987**, *27*, 191–196.
3. Noma, Y.; Yamasaki, S.; Asakawa, Y. *Phytochemistry* **1992**, *31*, 2725–2727.
4. Chang, H. C.; Oriel, P. J. *J. Food Sci.* **1994**, *59*, 660–662.
5. Vanek, T.; Valterova, I.; Vankova, R.; Vaisar, T. *Biotechnol. Lett.* **1999**, *21*, 625–628.
6. Onken, J.; Berger, R. G. *J. Bio-technol.* **1999**, *69*, 163–168.
7. Bouwmeester, H. J.; Gershenzon, J.; Konings, M. C. J. M.; Croteau, R. *Plant Physiol.* **1998**, *117*, 901–912.
8. Rani, K.; Akhila, A. *Fitoterapia* **1998**, *69*, 337–348.
9. Savithiry, N.; Cheong, T. K.; Oriel, P. *Appl. Biochem. Biotech.* **1997**, *63–65*, 213–220.
10. Duetz, W. A.; Fjällman, A. H. M.; Ren, S.; Jourdat, C.; Witholt, B. *Appl. Env. Microbiol.* **2001**, *67*, 2829–2832.
11. de Carvalho, C. C. C. R.; van Keulen, F.; da Fonseca, M. M. R. *Biocatal. Biotrans.* **2000**, *18*, 223–235.
12. de Carvalho, C. C. C. R.; da Fonseca, M. M. R. *J. Mol. Catal. B-Enzym.* **2002**, *19–20*, 389–398.
13. de Carvalho, C. C. C. R.; da Fonseca, M. M. R. *J. Mol. Catal. B-Enzym.* **2002**, *19–20*, 377–387.
14. de Carvalho, C. C. C. R.; van Keulen, F.; da Fonseca, M. M. R. *Tetrahedron: Asymmetry* **2002**, *13*, 1637–1643.
15. Wiegant, W. M.; de Bont, J. A. M. *J. Gen. Microbiol.* **1980**, *120*, 325–331.