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Biotransformation of Limonene-1,2-epoxide to Limonene-1,2-diol by *Rhodococcus erythropolis* Cells

An Introductory Approach to Selective Hydrolysis and Product Separation

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Summary

Cells of *Rhodococcus erythropolis* DCL14, when grown on terpenes, show a limonene epoxide hydrolase (LEH) activity which enables them to convert *cis*-limonene-1,2-epoxide to limonene-1,2-diol. The *trans* isomer is only converted when the *cis* isomer is not available. The transformation of limonene-1,2-epoxide ('limox') by whole cells of the above mentioned strain was used as a reaction model for the biotransformation of terpenes. The inclusion in the reaction system of an organic phase in which limox was dissolved led to high diol production rates. A diol production of *m* (diol)/*m* (protein)= 72.40 was achieved in a 500 mL mechanically stirred reactor with an external loop for product separation. A downstream process, based on the affinity of limox for the organic phase and that of the diol for the aqueous phase, was developed.

Key words: limonene epoxide hydrolase, *cis* and *trans* limonene-1,2-epoxide

Introduction

The market of flavoured and fragranced products is growing and requires new production strategies, specially those that use inexpensive, readily available and renewable natural precursors (*1*). Terpenes, which are natural unsaturated hydrocarbons derived from isoprene units, are potential substrates (e.g. limonene, α-pinene). Their oxygenated derivatives, called terpenoids, are widely used as flavours and fragrances. Since ancient times, perfume producers have developed fragrances based on terpenes and terpenoids from nature. For instance, several oils, which are basically composed of terpenoids are obtained by distillation of plant and flower tissues (*2*). The classification of biotechnological generated compounds as »natural« has increased the attention to biotransformation systems.

The main obstacles in the biotransformation of terpenes are (*i*) instability and low solubility of substrates and/or products in water and (*ii*) substrate and/or product inhibition. However, biotransformation is a valuable tool, because biocatalytic processes have the advantage of being regio-, stereo- or enantioselective and specific properties of terpenoids often depend on their absolute configuration (*1,3*).

This paper presents the development of a microbial reaction system for the biotransformation of terpenes (generally applicable to other strains and/or terpene transformations) that overcomes the toxicity, low solubility and instability of substrates and/or products. As a model reaction we chose the transformation of limonene-1,2-epoxide ('limox') to limonene-1,2-diol by whole

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cells of *Rhodococcus erythropolis* DCL14, because limox is unstable and has a low solubility in aqueous solutions. This strain, when grown on limonene, contains a limonene epoxide hydrolase (LEH) that transforms *cis*-limox to the diol. *Trans*-limox is only converted when no *cis*--limox is present (*4*,*5*). Therefore, this biotransformation besides producing the diol, provides the simultaneous resolution of *cis*/*trans*-limox mixtures.

Materials and Methods

Microorganism: Rhodococcus erythropolis DCL14 was obtained from the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands, within the frame of the European Project BIO4-CT95-0049.

Growth: Cells were grown at 30 °C and 400 rpm in a 2.0 L fermenter containing 1.5 L of mineral medium (*6*). Limonene, the growth substrate, was supplied through the gas phase (*4*) by bubbling the inlet air at 100 mL/min through the sintered glass sparger of a glass bottle containing limonene. Growth was followed by measuring the absorbance at 600 nm. When the absorbance was higher than 1.5, cells were harvested and the fermenter was refilled with fresh mineral medium.

Partition studies: Limox was dissolved in the organic solvent (concentration range = 0–50 mM) and analysed prior to the addition of the 50 mM phosphate buffer (K_2HPO_4/KH_2PO_4) in a volume ratio = 1:5. After 2 h at 30 °C and 200 rpm, the organic phase was analysed. Diol partition assays were carried out similarly, except that the diol was dissolved in the aqueous phase (0–20 mM).

Reactions: Reactions were carried out at room temperature in a 500 mL magnetically stirred reactor containing 300 mL of phosphate buffer, 60 mL of cyclohexane, 50 mM of limox (referred to the aqueous phase) and 50 mg (dry weight) of cells. The time-course of the reactions was followed by monitoring the diol accumulation in the aqueous phase.

Chemicals: The terpenes used were (*R*)-(+)-limonene (97 %) and (+)-limonene oxide (97 %) from Aldrich Chemicals (Milwaukee, U.S.A.). The organic solvents tested in biphasic systems were *n*-hexadecane (99 %) purchased from Sigma (St. Louis, U.S.A.) and *iso*-octane (> 99.5 %) from Riedel-de Haën (Seelze, Germany). In the purification procedures ethyl acetate (99.5 %) was from Riedel-de Haën (Seelze, Germany), acetone (99 %) from J. M. G. dos Santos (Odivelas, Portugal), and LiChroprep® RP-18 (40–63 μm) from Merck (Darmstadt, Germany).

Analysis: The aqueous phase was sampled every 0.5 h. The diol in the samples was extracted with ethyl acetate (volume fraction = 1:1), which was subsequently analysed by gas chromatography (GC) on a Hewlett Packard 5890 gas chromatograph (U.S.A.) connected to a HP3394 integrator. The limox consumption was followed by analysis of the organic reaction phase. The oven temperature of the gas chromatograph was 150 °C and that of the injector and detector was 250 °C. The enantiomeric composition of the epoxide was determined with an oven temperature of 90 °C.

Microbial adhesion to hydrocarbon (*MATH*) *test*: Cell hydrophobicity was assessed using the MATH test as follows: various volumes of *n*-hexadecane were added to 1.2 mL of washed cells suspended in phosphate buffer (pH=7) in test tubes. Following 10 min preincubation at 30 °C, the mixtures were agitated at full speed for 120 s on a vortex (Heidolph REAX 2000, Germany). After 15 min, the organic phase was removed and the absorbance was measured at 600 nm (*7*).

Cell migration velocity: 0.2 mL of *n*-hexadecane were added to 1.2 mL of washed cells suspended in phosphate buffer (pH=7) in test tubes. These were agitated at full speed on a vortex (Heidolph REAX 2000, Germany) for 45 s and allowed to stand still for 15 s. Every minute the absorbance was measured and the cycle repeated.

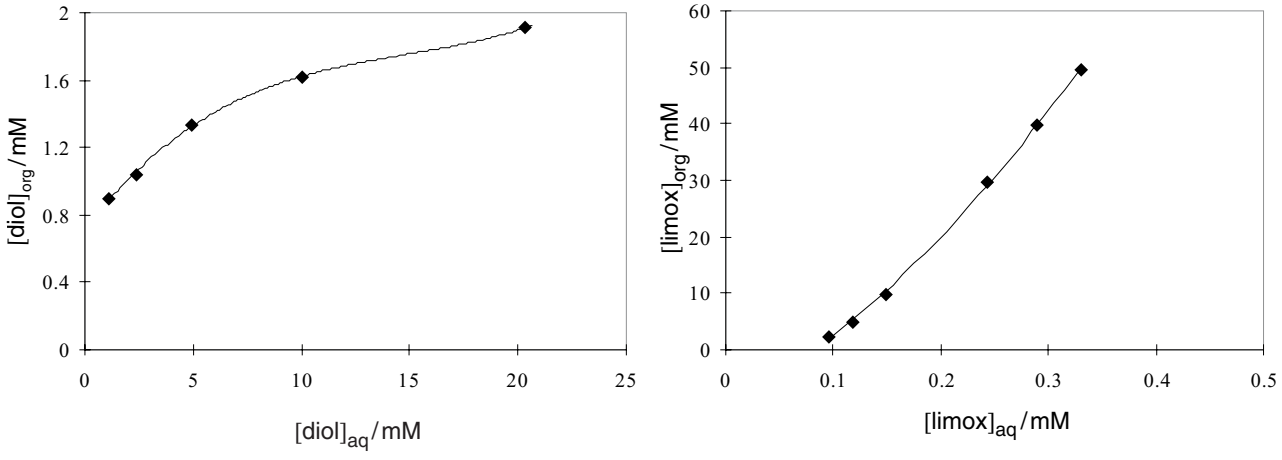

Fig. 1. Diol and limox equilibrium concentrations in iso-octane/aqueous systems (30 °C; *V*(org) : *V*(aq) = 1:5)

Table 1. Coefficients of Eqs. /1/ and /2/ for different organic/buffer systems (volume ratio = 1 : 5)

Solvent	A	B	C	D	E	F	G
Cyclohexane	0.0003	–0.0152	0.2879	0.1314	383.17	45.635	0.032
Iso-octane	0.0002	–0.0097	0.1658	0.7167	237.50	104.33	–10.363
n-Hexadecane	0.0003	–0.0169	0.3173	–0.2357	330.72	29.814	2.601

Results and Discussion

Partition studies

In organic/buffer systems the diol partitioned preferentially to the aqueous phase and the limox to the organic phase (Fig. 1). The equilibrium concentration of diol and limox in the organic phase $[x]_{org}$ fits Eqs. /1/ and /2/, respectively:

$$[x]_{org}=A\,[x]_{aq}^3 + B\,[x]_{aq}^2 + C\,[x]_{aq} + D \qquad /1/$$

$$[x]_{org}=E\,[x]_{aq}^2 + F\,[x]_{aq} + G \qquad /2/$$

where $[x]_{aq}$ is the equilibrium concentration in the aqueous phase. The coefficients of Eqs. /1/ and /2/ are given in Table 1 for cyclohexane, *iso*-octane and *n*-hexadecane. Their values indicate that the tested solvents and the buffer solution serve as appropriate repositories and as extraction phase for the substrate and the product, respectively. Other solvents, e.g. ethyl butyrate, trichloromethane, *n*-hexane, 1-dodecanol and *n*-dodecane, behaved similarly.

Data in Table 1 show that in the case of cyclohexane, even though a higher diol concentration is attained in the organic phase, it is possible to achieve a higher limox partition than with *iso*-octane and *n*-hexadecane. Further advantages of cyclohexane are its high vapour pressure, which facilitates product recovery, and its low price.

MATH test and Cell migration velocity

Rhodococcus erythropolis DCL14 cells were found to be rather hydrophobic (Fig. 2), *i.e.* in biphasic systems, cells migrated towards the organic/aqueous interface. The aqueous phase became practically biomass free after *c.* 15 min (84 % reduction in absorbance). The initial velocity of cell migration expressed in dry weight per time was 0.46 mg/h (Fig. 3).

Reaction studies

The solubility of limox in water is only 4.6 mM at 25 °C (*8*), limiting the amount of limox available in batch reaction systems. This limitation was overcome by the use of an organic phase as epoxide repository. In organic-aqueous systems (with *iso*-octane or hexadecane as solvents), a 3-fold increase of the initial hydrolysis rate was observed, as compared to their aqueous counterpart. This was possibly due to the increased contact area between the biocatalyst and the organic substrate repository, resulting from cell migration to the interface. In addition, the partition of the epoxide to the organic phase reduced the rate of chemical hydrolysis 30- to 60-fold (*5*).

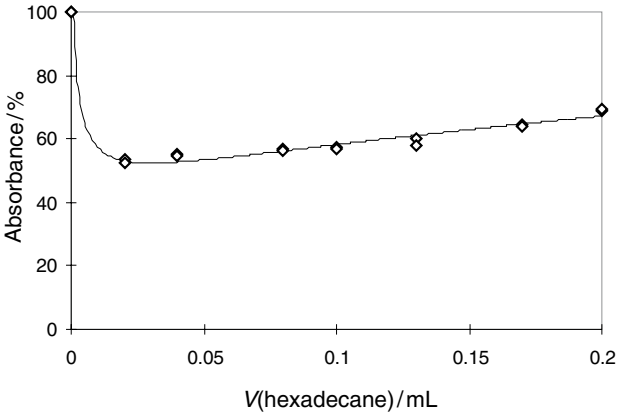

Fig. 2. Results of the MATH test at various hexadecane : aqueous volume ratios

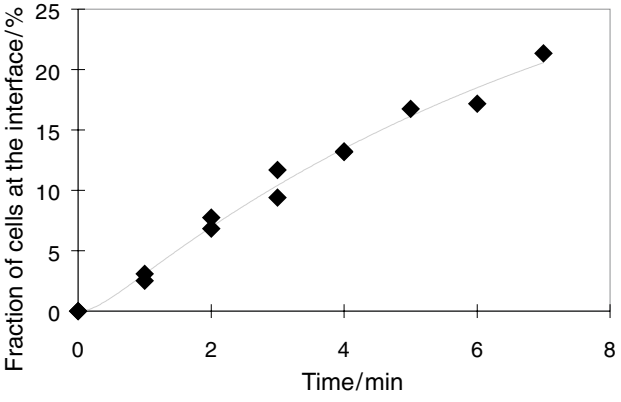

Fig. 3. Effect of time on the fraction of cells gathered at the interface

The advantage of the observed bacterial hydrophobicity was exploited in a 500 mL magnetically stirred tank reactor *V* (org.) : *V* (aq.) = 1 : 5. Cell migration in this reactor resulted in a bulk aqueous phase free of biomass. This enabled recirculation of the aqueous phase through a column (filled with 70 g of Lichroprep® RP-18) to achieve *in situ* diol recovery. The aqueous phase contained low amounts of *trans*-limox which were also adsorbed on the RP-18 material. With this set-up, a diol production *m* (diol)/*m* (protein) = 72.4 was achieved in 4.2 days. Furthermore, 98.5 % of unreacted *trans*-limox was recovered from the organic phase by evaporating the solvent (cyclohexane) in a rotavapor. The downstream methods for each product are shown in Fig. 4. Both products were obtained with a purity higher than 99 %, according to gas chromatography data (Fig. 5).

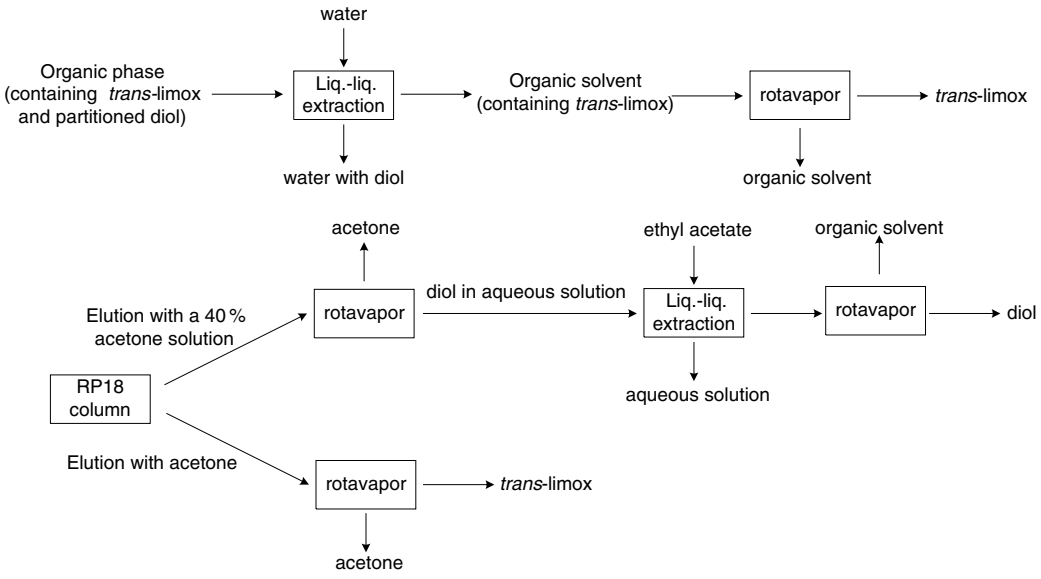

Fig. 4. Methods used for the recovery and purification of the diol and the epoxide

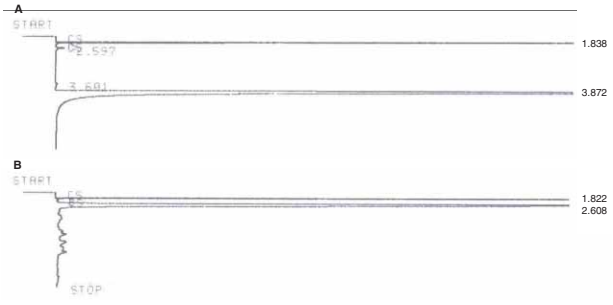

Fig. 5. Gas chromatographic analysis of the purified diol (retention time=3.872 min), A, and of the purified *trans*-limonene epoxide (retention time=2.608 min), B, dissolved in ethyl acetate (retention time=1.838 min)

Conclusions

Partition studies in organic/buffer systems using cyclohexane, *iso*-octane and *n*-hexadecane as solvent, indicated the preference of diol and limox for the aqueous and the organic phase, respectively. The cells, by gathering at the organic/aqueous interface (as shown by the MATH test), were allowed to contact easily with the organic substrate repository.

These results were used to set up a 500 mL magnetically stirred reactor with aqueous phase recirculation, through a column, enabling the *in situ* recovery of the diol. In this system, a conversion of 97.5 % of the *cis*-epoxide was achieved, resulting in a specific productivity expressed as m (diol)/(m (protein)·t) = 17.2 g·g^{-1}·day^{-1}.

With the downstream methods which were developed, both limonene-1,2-diol and the unreacted *trans*-limox were obtained with high purity.

Acknowledgements

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Biotransformacija limonen-1,2-epoksida u limonen-1,2-diol s pomoću stanica *Rhodococcus erythropolis*

Pokušaj selektivne hidrolize i izdvajanje proizvoda

Sažetak

Stanice *Rhodococcus erythropolis* DCL14 kada rastu na terpenima imaju limonen-epoksid hidrolaznu (LEH) aktivnost koja im omogućava da prevode *cis*-limonen-1,2-epoksid u limonen-1,2-diol. *Trans*-izomer prevodi se u *cis*-izomer samo ako nema *cis*-izomera. Transformacija limonen-1,2-epoksida (limox) s pomoću cijelih stanica navedenoga soja korištena je kao model za biotransformaciju terpena. Primjenom organske faze u reakcijskom sustavu u kojem se otapa »limoks« postiže se veliko iskorištenje na proizvodu diolu. Masa prozvedenog diola po masi proteina od 72,40 g postignuta je u reaktoru od 500 mL s miješalicom koji ima vanjski odvojak za izdvajanje proizvoda. Razrađen je postupak što se zasniva na razdvajanju limonen-1,2-epoksida u organsku, a diola u vodenu fazu.