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Supplementary information

From waste to value - Direct utilization of limonene from orange peel in biocatalytic cascade reaction towards chiral carvolactone

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1 Experimental procedures

1.1 Synthesis of ionic liquids

1-Ethyl-3-methylimidazolium acetate ([C_2mim]OAc,> 95%) and was purchased from Iolitec (Heilbronn, Germany) and dried before use *in vacuo* (0.01 mbar) with stirring for 24-48 hrs. 1-Ethyl-3-methylimidazolium chloride ([C_2mim]Cl, >98%) was purchased from Iolitec (Heilbronn, Germany) and used as received.

1.1.1 General procedure for choline-ionic liquids *via* neutralization

The exact concentration of choline hydrogen carbonate was determined *via* titration prior to use. ILs were prepared by dropwise addition of the base to the corresponding acid (1:1) in an appropriate solvent, e.g. water. The solution was stirred at ambient temperature and pressure for 30 min to 4 h. The solvent was removed and the IL was dried *in vacuo* (0.01 mbar) overnight.

1.1.2 Choline formate [chol]fom

Synthesis was accomplished according to the general procedure using formic acid (3.03 g, 65.85 mmol), choline hydrogen carbonate (77% solution in water) (14.13 g, 65.85 mmol) and 5 mL water. A colourless solid was obtained in quantitative yield (9.82 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H =3.12 (s, 9H, $N(CH_3)_3$), 3,40-3.49 (m, 2H, N-CH_2), 3.80-3.87 (m, 2H, O-CH_2), 8.52 (s, 1H, HCOO)

Analytical data was in accordance with literature. [1]

1.1.3 Choline acetate [chol]OAc

Synthesis was accomplished according to the general procedure using HOAc (1.00 g, 16.66 mmol), choline bicarbonate (77% solution in water) (3.57 g, 16.66 mmol) and 3 mL water. A colourless solid was obtained in quantitative yield (2.72 g).

^{1}H-NMR (200 MHz, d_6-DMSO): δ_H = 1.58 (s, 3H, CH_3COO), 3.12 (s, 9H, $N(CH_3)_3$), 3.39-3.44 (m, 2H, N-CH_2), 3.78-3.86 (m, 2H, O-CH_2)

Analytical data was in accordance with literature.[1]

1.2 Limonene extraction

HPLC analysis for *R*-(+)-limonene (limonene) was performed on a Jasco HPLC unit equipped with a PDA detector. For the determination of limonene a Maisch ReproSil 100 C18 250 x 4.6, 5 µm was used with MeOH:H_2O(0.1% TFA) = 87:13 as solvent and a flow of 1 mL min^{-1}; detection was done at 210 nm, at 30 °C column oven temperature, 25 °C tray temperature. Retention times were 7.0 min for the internal standard and 9.5 min for limonene.

200 mg of small pieces of orange peel (approx. 7 pieces) were stirred with 800 mg of [C_2mim]OAc for 1.5 h at 80 °C. The solution was filled up to 10 ml methanol (incl. 1-methyl-1-cyclohexene), filtered and analyzed *via* HPLC.

1.3 Bacterial cultivation

Bacterial strains were routinely cultured in LB medium (adapted from Bertani[2]: 10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) in orbital shakers (InforsHT Multitron 2Standard) supplemented with the appropriate antibiotics at specific conditions as shown in Table S1.

Table S1: Enzymes, bacterial strains and plasmids and antibiotic concentrations

Enzyme (origin)	Expression strain (plasmids)	Antibiotics, temperature and shaking speed	Ref.
CumDO (*Pseudomonas putida* PWD32)	*Pseudomonas putida* S12 (pBTBX-2_CumDO)	50 µg mL^{-1} kanamycin (kan), 30 °C at 200 rpm	[3]
RR-ADH (*Rhodococcus ruber*), XenB (*Pseudomonas putida*) and $CHMO_{Acineto}$ (*Acinetobacter calcoaceticus*)	*E. coli* BL21(DE3) (pRRADH + pGASTON_XenB + pET28a_CHMO)	34 µg mL^{-1} chloramphenicol (clam), 100 µg mL^{-1} ampicillin (amp), 100 µg mL^{-1} kan, 37 °C at 200 rpm	[4]

1.3.1 Growth in presence of ILs

LB medium supplemented with the appropriate antibiotics (Table S1) was inoculated with overnight culture to an OD_{590} of 0.05, mixed with the ILs to obtain a concentration of either 50 mM or 100 mM and aliquoted in triplicates of 1 mL each to 96-square-deep-well plates. Growth was started at 30 °C for *P. putida,* at 37 °C for *E. coli* at 200 rpm and monitored via optical density measurements. A sample of 50 µL was taken every hour and analysed using a plate reader (Anthos Zenyth 3100) at a wavelength $\lambda = 595$ nm.

1.3.2 Resting cells

After production of the enzymes as described in Table S2, the cell cultures were centrifuged at 3600 x g at 4 °C for 15 min. The supernatant was discarded and the cell pellet gently washed in 50 mM TRIS-HCl pH=7.5 and centrifuged again. After discarding the supernatant the cells were concentrated to a specific calculated OD_{590} in 50 mM TRIS-HCl pH=7.5 supplemented with 1% (w/v) D-glucose and stored at 4 °C until further use.

Table S2: Enzyme production protocols

Expression strain (plasmids)	Expression protocol	Concentrated to OD_{590}	Ref.
Pseudomonas putida S12 (pBTBX-2_CumDO)	TB_{kan}, 30 °C, 200 rpm; at OD_{590} 0.6 + 2% (w/v) L-arabinose; 30 °C 200 rpm, 6 h	20	adapted from[3]
E. coli BL21(DE3) (pRRADH + pGASTON_XenB + pET28a_CHMO)	$TB_{clam+amp+kan}$ 37 °C, 120 rpm; at OD_{590} 0.3 + 1 mM $ZnCl_2$; 30 min 25 °C; + 0.1 mM IPTG, 20 h, 25 °C	100	[4]

1.4 Biotransformations

1.4.1 General procedures

All reactions were performed as triplicates in 20 mL screw cap glass vials and shaken in an orbital shaker at 25 °C and 200 rpm. Extraction was performed with EtOAc supplemented with 1 mM methyl benzoate as GC standard. The phases were separated, if needed by transfer of the emulsion to an Eppendorf vial and a short centrifugation step, the organic layer dried over Na_2SO_4 and subjected to GC analysis (see 1.5).

1.4.2 Parameter evaluation

1.4.2.1 Limonene concentration

P. putida resting cells (as prepared in 1.3.2) were filled in the glass vials and supplemented with 1 M ethanolic stock of limonene to concentrations of 0.5 mM, 1 mM and 4 mM in a total volume of 2 mL. Reaction was performed as described in 1.4.1 for 12 h.

1.4.2.2 IL concentration

[C_2mim]OAc was weighed into the vials to concentrations of 50 mM and 100 mM. *P. putida* resting cells were added, supplemented with 4 mM limonene to a volume of 2 ml and reaction performed for 12 h.

1.4.3 *P. putida* with CumDO + limonene

The vials were loaded with IL if applicable, 1.999 mL of *P. putida* resting cells and 1 µL of a 1 M ethanolic stock of limonene to obtain a concentration of 0.5 mM. The reaction was shaken for 12 h.

1.4.4 *P. putida* with CumDO + orange peel

1.4.4.1 Concept II

Orange peel (50-65 mg) cut into approx. 0.2 cm² pieces was weighed into the vials and mixed with of *P. putida* resting cells (2 mL), to reach a biomass loading of approx. 3% (w/v). Work-up was performed after 12 h.

1.4.4.2 Concept III

The vials were first loaded with IL to a concentration of 50 mM, then orange peel and resting cells were added and reaction performed as described in 1.4.4.1.

1.4.4.3 Concept IV

Limonene extraction was performed using orange peel (196 mg) and [C_2mim]OAc (500 mg, 50 mM). The solution was diluted with *P. putida* resting cells (66 mL) in a 250 mL Schott flask. The reaction was run for 12 h and work-up performed as mentioned above (1.4.1).

1.4.5 Mixed-culture + limonene

1.4.5.1 Simultaneous addition

Resting cells of *P. putida* and *E. coli* as prepared in 1.3.2 were mixed in a glass vial in a 1:1 ratio and supplemented with limonene (0.5 mM) to a total volume of 2.4 mL. A sample (400 µL) was taken after 10 h and the rest worked up after 20 h reaction time.

1.4.5.2 Sequential addition

P. putida resting cells (999 µL) were mixed with ethanolic limonene stock (1 µL, 1 M) and shaken for 10 h. After sampling (400 µL) 600 µL of *E. coli* resting cells were added and the reaction continued for 10 h until final work-up.

1.4.6 Mixed-culture + orange peel

1.4.6.1 Simultaneous addition

Approx. 100 mg orange peel (3% (w/v) biomass loading) were mixed in a glass vial with resting cells of *P. putida* and *E. coli* in a 1:1 ratio to a total volume of 2 mL. A sample (400 µL) was taken after 10 h and the rest worked up after 20 h reaction time.

1.4.6.2 Sequential addition

P. putida resting cells (2 mL) were mixed with approx. 100 mg respectively 50 mg orange peel (3% / 1.5% biomass loading) and shaken for 10 h. After sampling (400 µL) 1600 µL of *E. coli* resting cells were added and the reaction continued for 10 h until final work-up.

1.5 GC analysis

Conversion and product purity were determined by gas chromatography (GC) using a Thermo Finnigan Focus GC equipped with a standard capillary column (BGB5, 30 m x 0.25 mm ID, 0.50 µm film). Method and retention times of intermediates and GC standard are listed below.

Method: 60 °C, 0.6 min hold → 50 °C min^{-1} → 280 °C, 1 min hold; duration 6 min

Retention times: 3.56 min limonene (**1**), 3.78 min methyl benzoate (standard), 4.11 min cis-dihydrocarvones (**4**), 4.14 min trans-dihydrocarvones (**4**), 4.16 min trans-carveols (**2**), 4.19 min cis-carveols, 4.25 min carvone (**3**), 4.74 min normal carvolactone (**5**)

2 Results

2.1 Limonene extraction

Peel from different oranges (a-e) was extracted with [C_2mim]OAc as described in 1.2. As depicted in Fig. S1 limonene content varies from 25-60 mg per g orange peel in different biomass batches.

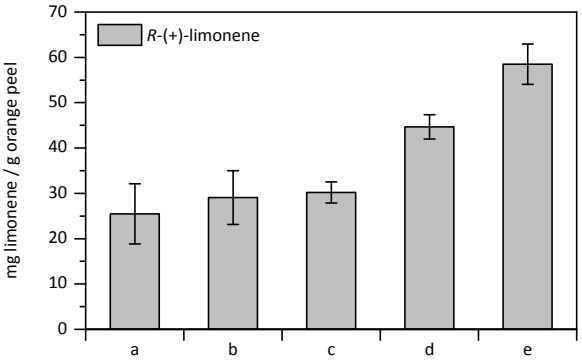

Fig. S1: Different batches of orange peel extracted with [C_2mim]OAc

2.2 Parameters of limonene hydroxylation by CumDO

2.2.1 Influence of limonene concentration

Resting cells of *P. putida* with expressed CumDO were supplemented with different concentrations of limonene. As depicted in Fig. S2, with decreasing concentrations of limonene better conversions to (1*R*,5*S*)-carveol could be achieved.

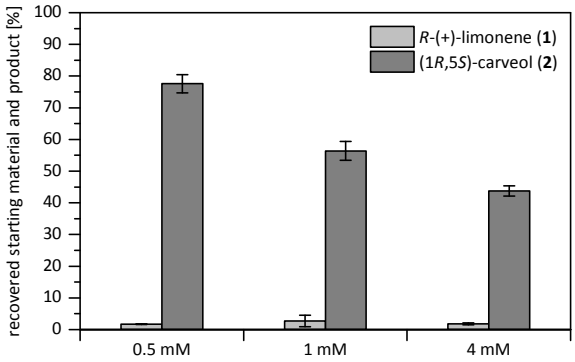

Fig. S2: Influence of limonene concentration on hydroxylation reaction. Resting cells of *P. putida* with CumDO (OD_{590}=20 in 50 mM TRIS-HCl pH 7.5) were incubated in closed glass vials with different concentrations of limonene for 12 h at 200 rpm and 25 °C.

2.2.2 Influence of IL concentration

Hydroxylation of limonene was tested in resting cells of *P. putida* with expressed CumDO in presence of different concentrations of [C_2mim]OAc. 50 mM IL were well tolerated whereas 100 mM interfered with the biotransformation as shown in Fig. S3.

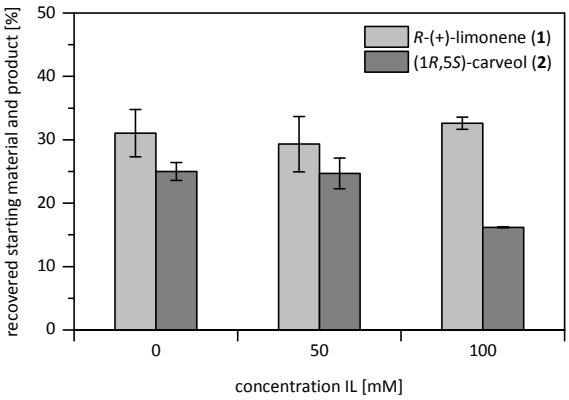

Fig. S3: Hydroxylation of 4 mM limonene with and without the addition of different concentrations of [C_2mim]OAc.

3 References

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