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

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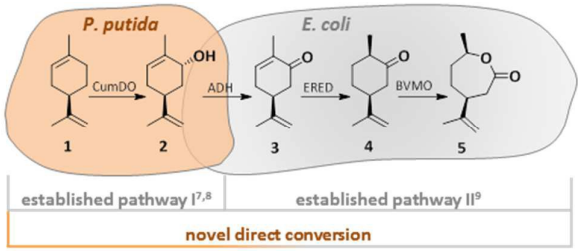
N. Oberleitner,[†a] A. K. Ressmann,[†a] K. Bica,[a] P. Gärtner,[a] M. W. Fraaije,[b] U. T. Bornscheuer,[c] F. Rudroff*[a] and M. D. Mihovilovic[a]

In this proof of concept study we demonstrate direct utilization of limonene containing waste product orange peel as starting material for a biocatalytic cascade reaction. Product of this cascade is chiral carvolactone, a promising building block for thermoplastic polymers. Four different concepts were applied to augment limonene availability either based on water extraction solely, addition of extraction enhancers or biomass dissolution.

Depletion of fossil resources and increasing demand for platform chemicals has given rise to utilization of renewable biomass as sustainable feedstock. To overcome the food vs. feed problem, valorisation of food supply chain waste (FSCW) can offer a sustainable route to cheap starting materials for syntheses of valuable compounds.[1, 2] More than 15 million tons of orange peel waste accumulates as by-product of citrus fruit industry annually. *R*-(+)-Limonene (limonene, **1**), main component of most citrus oils, is industrially isolated from orange peel by energy intensive steam distillation or cold expression.[3] Recent research opens the possibility for concerted production of biofuels, pectin and limonene from citrus peel waste.[4, 5] Limonene and its oxygenated derivatives (menthol, perillyl alcohol, carveol, carvone) have great market potential as solvents, fine chemicals, flavours, fragrances or even fuels.[1] However achieving regio- and stereospecific hydroxylation by chemical means is difficult, therefore biocatalytic transformation of limonene has been studied extensively since the 1960s.[6] Duetz et al. showed regio- and stereospecific hydroxylation of limonene (**1**) by using toluene-grown *Rhodococcus opacus* PWD4 cells and obtained 97% (+)-*trans*-carveol (**2**).[7] The gene cluster coding for the enzyme, potentially responsible for this reaction, cumene dioxygenase (CumDO) was recently cloned into *Pseudomonas putida* S12 allowing toluene-free enzyme production.[8]

In a one-pot resting cell mixed culture approach (Scheme 1) we connected this selective hydroxylation by CumDO expressed in *P. putida S12* with our previously established synthetic mini-pathway in *Escherichia coli* BL21(DE3)[9], where carveol can serve as starting material. By this new combination limonene (**1**) could be directly transformed to chiral carvolactone (**5**) *via* carveol (**2**), carvone (**3**) and dihydro-carvone (**4**). Carvolactones, interesting building blocks for syntheses of bioactive or natural products, can also serve as monomers for polymer production as they can be subjected to ring-opening polymerisation and their olefinic side chains can be easily functionalized and crosslinked.[10, 11] Only recently enzymatic oligomerisation of chiral lactones was achieved, notably in an aqueous system[12] and this may be applicable also for carvolactones.

We considered different concepts to utilize FSCW orange peel as starting material for our biocatalytic cascade towards carvolactone (Fig. 1). Most commonly liquid biphasic systems[13] (concept **I**) are applied with hydrophobic substrates such as limonene[14]. Unfortunately, this concept is not feasible for *in situ* conversion of limonene from orange peel as limonene, due to its high *logP* value[15], would accumulate in the hydrophobic solvent. With a reasonable biomass loading (ratio of orange peel to liquid volume) limonene concentrations in the aqueous phase required for biotransformations cannot be attained.



Scheme 1 Cascade from limonene (**1**) to carvolactone (**5**), consisting of cumene dioxygenase (CumDO), an alcohol dehydrogenase (RR-ADH), an enoate reductase (XenB) and a Baeyer-Villiger monooxygenase ($CHMO_{Acineto}$) in a mixed-culture set-up.

[a] *Institute of Applied Synthetic Chemistry, TU Wien, Getreidemarkt 9/163, 1060 Wien, Austria, E-mail: florian.rudroff@tuwien.ac.at*
[b] *Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*
[c] *Institute of Biochemistry, Dept. of Biotechnology & Enzyme Catalysis, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany*
[†] These authors contributed equally to this work.
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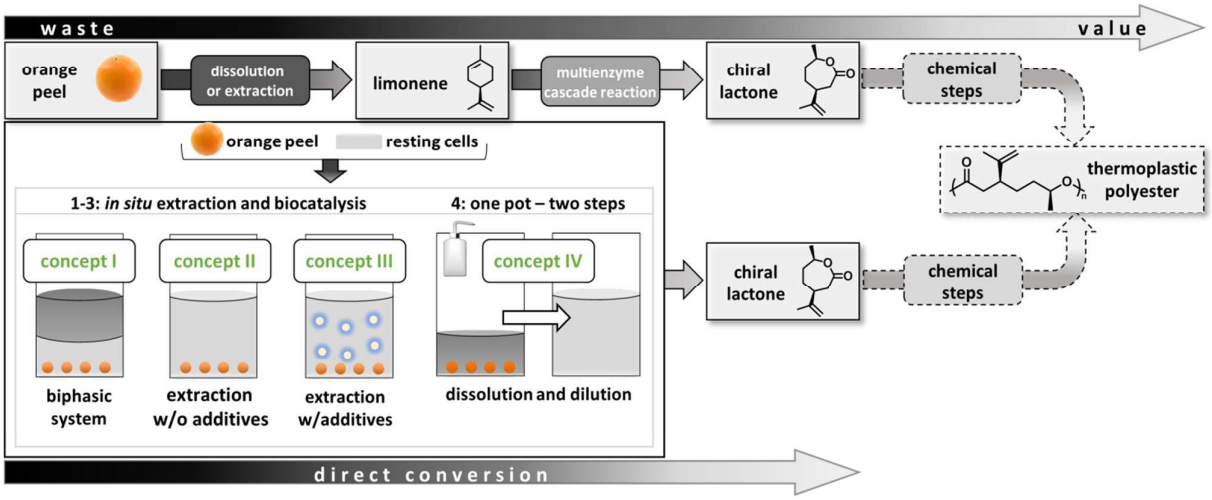


Fig. 1 Different strategies for the direct conversion of limonene (**1**) present in orange peel to chiral carvolactone (**5**).

Another possibility is the application of the SFPR (substrate feed product removal) approach[16], taking advantage of the orange peel itself as substrate reservoir, constantly feeding the reaction with low amounts of water insoluble limonene. Therefore, mixing orange peel with the resting cells in aqueous buffer would be the most facile approach (concept **II**). Here, *in situ* conversion could be enhanced by variation of the reaction solvent or rather the addition of water miscible solvents. Due to intolerance of microbial expression hosts to organic solvents we opted for the use of hydrophilic ionic liquids (ILs) as additives in concept **III**, as the limited solubility of many organic compounds in water could be enhanced in well-defined aqueous IL solutions. Moreover, their ability to pre-treat lignocellulosic biomass even in mixtures with water[17] make ILs promising additives that were already applied in several whole-cell bio-transformations.[18] Partial or complete dissolution of biomass in pure ILs should enable enhanced extraction efficiency of limonene from orange peel, as it was previously shown by Bica *et al.*[19] (concept **IV**). In contrast to *in situ* concepts **I-III**, the latter requires additional dilution of the dissolved biomass with resting cells after the initial extraction.

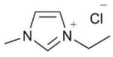
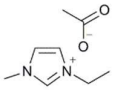
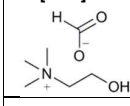
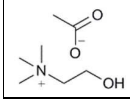
For the set-up of a multi-component system potential bottlenecks should be ruled out upfront. We investigated influencing parameters such as (i) performance of limonene hydroxylation, (ii) compatibility of extraction additives with both whole-cell biocatalysts, and (iii) compatibility of the two microbial hosts among themselves.

Concentration of starting material **1** is a relevant parameter for the biocatalytic cascade, especially for the hydroxylation step. We investigated different concentrations of limonene in the first hydroxylation reaction and could improve the yield from 40% at 4 mM limonene (**1**) to almost 80% at 0.5 mM **1** (ESI, Fig. S2). The latter concentration seemed to be very low and unfeasible for further biotechnological applications, but having a closer look at the total amount of limonene per gram biomass, only 2-6% of limonene (see ESI, Fig. S1; reference[4]) are available. A suitable method to obtain limonene concentrations in that range would be concepts **II** and **III** where orange peel itself serves as substrate reservoir. Thus, the overall substrate concentration would be below any toxicity level[20] for both microbial hosts and in a suitable concentration range for our biocatalytic cascade.

Besides water (concept **II**), two hydrophilic 1-ethyl-3-methyl-imidazolium-based ILs and two biocompatible choline ([chol]) ILs[21, 22] were chosen additives for possible limonene extraction enhancement (concept **III**). 1-Ethyl-3-methyl-imidazolium acetate [C_2mim]OAc was investigated as it is known for its excellent extraction ability of limonene from orange peel.[19] 1-Ethyl-3-methyl-imidazolium chloride [C_2mim]Cl as well as [chol]OAc were reported to have no growth inhibitory effect on *E. coli*[23] and were therefore included in our study. Choline formate [chol]fom was tested as it previously showed superior biomass extraction performance.[24, 25]

First we evaluated the influence of ILs on viability of both bacterial strains based on growth rates, shown in Table 1.

Table 1 Bacterial growth in presence of ILs. Values given in percentage related to growth without addition of ILs.

IL [mM]	*50*	*100*	*50*	*100*
Entry – IL	***E. coli* BL21(DE3) growth [%]**		***P. putida S12* growth [%]**	
1 – [C_2mim]Cl 	63±5	35±7	87±2	77±2
2 – [C_2mim]OAc 	83±5	9±3	2±1	1±1
3 – [chol]fom 	81±4	60±6	95±3	86±3
4 – [chol]OAc 	99±6	96±6	0	0

Due to economic reasons, IL concentrations of 50-100 mM were tested. Growing *E. coli* BL21(DE3) and *P. putida S12* responded differently towards addition of ILs as can be retrieved from the data in Table 1. Pronounced influence of the concentration of IL can be seen in case of *E. coli* BL21(DE3) where 50 mM [C_2mim]OAc were well tolerated but 100 mM [C_2mim]OAc strongly impaired growth (Table 1, entry 2).

P. putida S12 is known to be sensitive to higher acetate concentrations if not adapted to it.[26] This was also observed here as growth was inhibited by addition of [C_2mim]OAc and [chol]OAc, but not with [C_2mim]Cl and [chol]fom. [Chol]fom had the least effect on viability of both bacterial strains, at either 50 or 100 mM concentration, and was consequently elected as the best candidate for subsequent whole-cell biocatalysis.

First test biotransformations in the presence of growing cells and pure limonene (**1**) led to a massive loss of material due to the immiscibility and high volatility of **1** (data not shown).Therefore, we changed from growing to resting cells and explored the influence of aqueous buffer (concept **II**) and aqueous buffer + ILs (concept **III**) on the biotransformation performance. Hence, we investigated the hydroxylation of limonene by CumDO expressing resting cells of *P. putida S12* in the presence of 50 mM and 100 mM IL. In this pre-experiment the 50 mM showed no interference whereas 100 mM IL strongly impaired the reaction performance (ESI, Fig. S3).

Consequently 0.5 mM limonene were subjected to hydroxylation in CumDO expressing resting cells of *P. putida S12* with and without the addition of 50 mM IL. Interestingly [C_2mim]OAc, which was not compatible with growing cells of *P. putida S12*, showed nearly no interference with the biotransformation in resting cells as can be seen in Fig. 2a.

Also [C_2mim]Cl had hardly any impact on limonene hydroxylation whereas both choline ILs reduced the conversion to carveol significantly. Nevertheless the best results could be obtained with resting cells in aqueous buffer without additives (Fig. 2a, concept **II**).

In order to investigate direct utilization of the waste product by *in situ* conversion of limonene, we used orange peel instead of pure limonene in the presence of aqueous buffer (concept **II**) and aqueous buffer + ILs (concept **III**) (50 mM, concept **III**) (Fig. 2a). Orange peel, from a batch with 13.8 mg ± 4.0 mg limonene per g biomass (based on classical EtOAc extraction of triplicates) was added to CumDO expressing resting cells of *P. putida S12* with a biomass loading of 3% (w/v), which should result in an acceptable concentration of limonene in the aqueous phase. As limonene contents in orange peel may vary, we settled on representation of our results in mg product per g orange peel. As can be seen in Fig. 2b the conversion of limonene (**1**) from orange peel to carveol (**2**) performed best in the aqueous system without additives with 4.8 mg carveol per g orange peel to be detected (GC yield). The addition of ILs led to lower yields of carveol, where only [C_2mim]Cl gave acceptable results as it showed just minor inhibition of the reaction. Based on those results a clear preference for concept **II**, the simple use of orange peel in water, was gained.

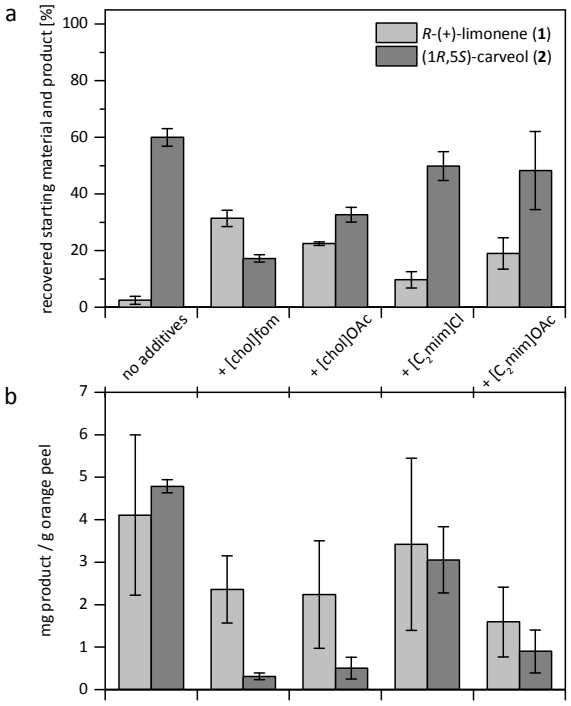


Fig. 2 Transformation of **a)** 0.5 mM *R*-(+)-limonene (**1**) **b)** approx. 3 % (w/v) orange peel (limonene [c] = 13.8 mg ± 4.0 mg / g biomass) to (1*R*,5*S*)-carveol (**2**) by CumDO in *P. putida* S12 resting cells in presence of ILs (50 mM) within 12 h reaction time. Results are GC yields and deviations and material loss are due to limonene volatility.

Finally we dissolved the biomass in pure ILs, as proposed in concept **IV**, and fed the extract to resting cells expressed CumDO to 50 mM final concentration of ILs. This required not only an additional handling step, but also reproducibility was lowered and did not result in sufficient amounts of product (data not shown).

To extend concept **II**, we combined *P. putida S12* cells expressing CumDO with *E. coli* BL21(DE3) cells expressing RR-ADH, XenB and $CHMO_{Acineto}$ in a mixed culture approach (Scheme 1) in the presence of 0.5 mM limonene. Simultaneous combination of the bacterial strains in one pot, despite moderate material loss, yielded about 47% of carvolactone (**5**) after 20 h (Fig. 3, stagnation of product formation after 10 h).

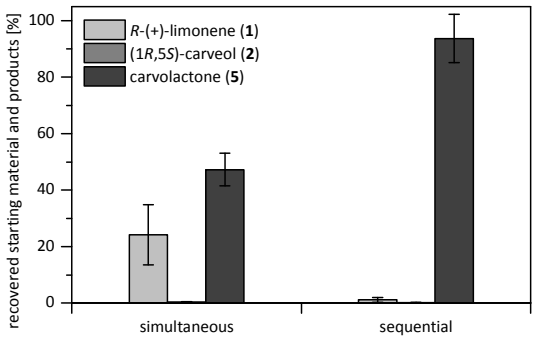


Fig. 3 Production of carvolactone (**5**) from 0.5 mM limonene (**1**) with simultaneous and 1 mM **1** with sequential addition of *P. putida* S12 and *E. coli* BL21(DE3) resting cells after 20 h reaction time.

However, a sequential approach was devised, where hydroxylation of 1 mM limonene – to reach the same concentration of final product after dilution – by CumDO was performed first and *E. coli* BL21(DE3) resting cells were only added to the reaction vessel after 10 h. This enabled nearly full conversion to carvolactone (**5**) in 20 h (Fig. 3). Inspired by those results, we finally explored the direct valorisation of waste product orange peel to chiral carvolactone in the mixed-culture system applying concept **II**. From a biomass loading of about 3% (w/v), which yielded in 4.8 mg carveol per g orange peel (limonene [c] = 13.8 mg ± 4.0 mg / g biomass) through hydroxylation with CumDO in *P. putida S12* (Fig. 2b), 3.2 mg carvolactone per g orange peel (limonene [c] = 17.9 mg ± 3.7 mg / g biomass) could be produced. To acertain no orange peel overloading or to avoid a toxic effect limiting the reaction, a lower biomass loading of 1.5% (w/v) orange peel (limonene [c] = 17.9 mg ± 3.7 mg / g biomass) was tested with concept **II**. In a simultaneous addition approach only low amounts of carvolactone could be detected. However, combination of the mixed-culture sequential combination set-up, which proved feasible with limonene as starting material, and the lower orange peel loading, yielded 6.3 mg carvolactone per g orange peel (limonene [c] = 17.9 mg ± 3.7 mg / g biomass) as can be seen in Fig. 4. This promising result, 29% carvolactone from limonene over 4 biocatalytic steps (73% per step), is thus only relying on orange peel as substrate reservoir in aqueous buffer without additives, consequently avoiding any additional parameters increasing complexity of the overall process.

Conclusions

We successfully combined two established biotransformation pathways[8, 9] gaining access to a novel direct conversion of natural product limonene (**1**) to chiral carvolactone (**5**). This was realized in a one-pot sequential biocatalyst addition approach where almost full conversion of limonene concentrations in the mM range could be achieved.

In advanced investigations we explored different concepts for the valorisation of FSCW orange peel. Several ILs were considered as additives to enhance *in situ* conversion of limonene from orange peel. We monitored the impact of the ILs on the growth of our bacterial expression hosts as well as on biotransformation activity. Although [C_2mim]Cl showed promising results as it hardly interfered with the bio-transformation, product formation was not improved by the addition of ILs.

The most facile and economic approach (concept **II**), making use of orange peel as substrate reservoir in a SFPR manner in aqueous buffer, emerged in promising results. With a biomass loading of 1.5% (w/v) we detected the production of 6.3 mg carvolactone per g orange peel (29% yield over 4 steps) in a one-pot sequential biocatalyst addition approach. This direct utilization of waste product orange peel is not only avoiding tedious limonene extraction and purification, but also limits volatility problems with the starting material. Acting as substrate reservoir, orange peel constantly releases limonene to the aqueous phase where it can be directly converted in the multi-step biotransformation within a principal proof-of-concept.

Studies on improvement of parameters for the set-up of the multi-component system will be part of future research. Bacterial strains could be engineered for tolerance to increased IL concentrations as already shown for *E. coli.*[27] Higher orange peel loadings, resulting in higher limonene concentration, could be handled by adaptation of *P. putida* S12[26] or introduction of the hydroxylation reaction in a constitutive solvent tolerant bacterial host[28].

Through assembly of a biocatalytic cascade *in vivo* we demonstrated the valorisation of waste product orange peel to chiral carvolactone, a promising chiral polymer building block. This direct multi-step conversion was performed in a one-pot whole cell biotransformation cascade in aqueous buffer without the need of any additives and underlines the power of cascade biocatalysis.

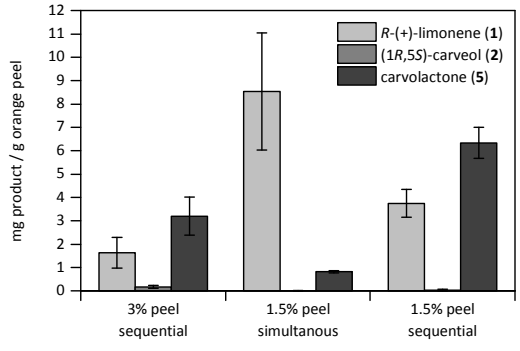


Fig. 4 Production of carvolactone (**5**) from orange peel (limonene [c] = 17.9 mg ± 3.7 mg / g biomass) in different approaches and with altered biomass loadings.

Acknowledgements

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TOC

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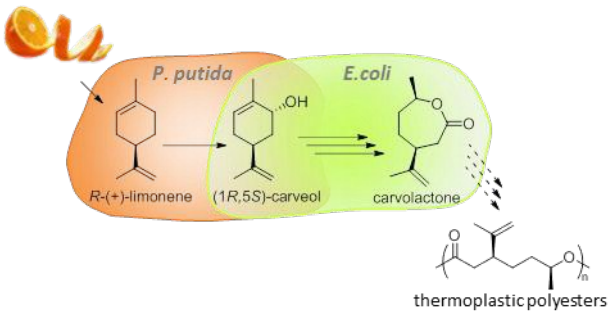
Nikolin Oberleitner,[†a] Anna K. Ressmann,[†a] Katharina Bica,[a] Peter Gärtner,[a] Marco W. Fraaije,[b] Uwe T. Bornscheuer[b], Florian Rudroff*[a] and Marko D. Mihovilovic[a]



From waste to value: We investigated the valorisation of limonene containing waste product orange peel, and performed a biocatalytic cascade for the production of chiral carvolactone, which can serve as building block for thermoplastic polymers. Overall, we were able to produce carvolactone starting from small pieces of orange peel in a 4-step biocatalytic cascade in 30% yield, based on continued extraction solely with water.

[a] *Institute of Applied Synthetic Chemistry, TU Wien, Getreidemarkt 9/163, 1060 Wien, Austria, E-mail: florian.rudroff@tuwien.ac.at*
[b] *Molecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*
[c] *Institute of Biochemistry, Dept. of Biotechnology & Enzyme Catalysis, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany*
[†] These authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: Experimental work and additional results are described in the ESI. See DOI: 10.1039/x0xx00000x