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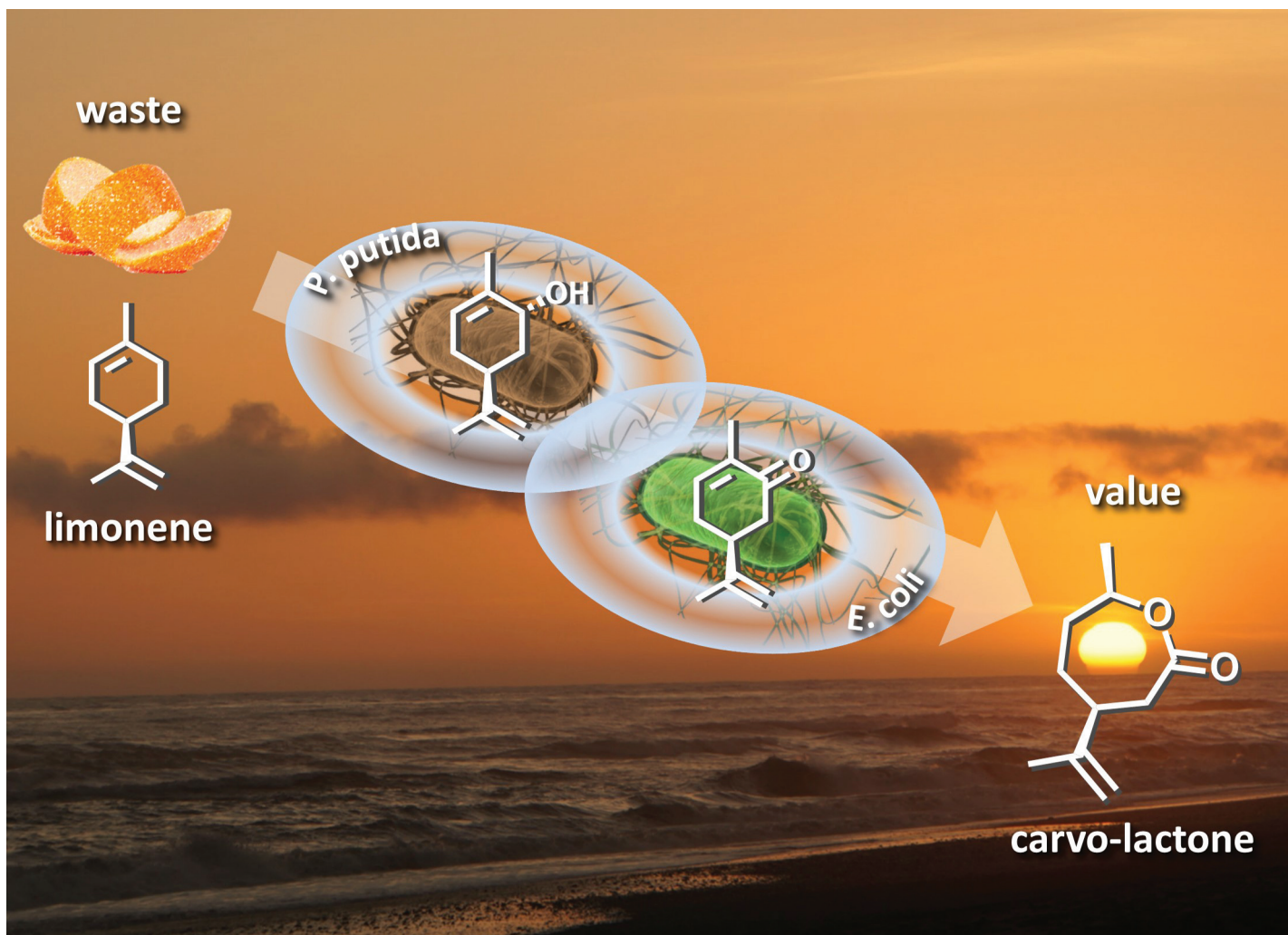
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An article presented by Oberleitner *et al.* of the TU Wien.

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

Aiming for a valuable chiral lactone from a sustainable waste product, a one-pot valorization of orange peel towards chiral carvolactone was achieved by applying a 4 step biocatalytic cascade reaction. Four different concepts were applied to augment limonene availability either based on water extraction solely, addition of extraction enhancers or biomass dissolution. The presented enzyme cascade yielded an enantiopure carvolactone, which is a valuable monomer for chiral thermoplastic material.

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From waste to value – direct utilization of limonene from orange peel in a biocatalytic cascade reaction towards chiral carvolactone†

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

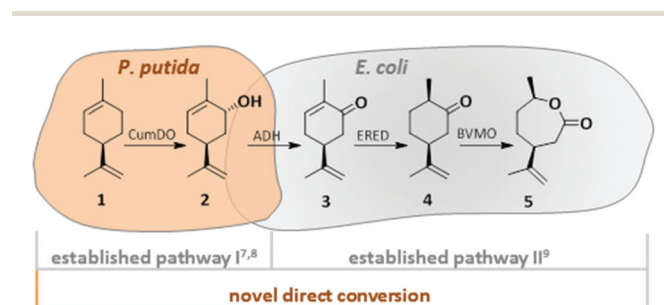
In this proof of concept study we demonstrate direct utilization of limonene containing waste product orange peel as starting material for a biocatalytic cascade reaction. The product of this cascade is chiral carvolactone, a promising building block for thermoplastic polymers. Four different concepts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

Depletion of fossil resources and increasing demand for platform chemicals have given rise to utilization of renewable biomass as a 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 a by-product of the citrus fruit industry annually. *R*-(+)-Limonene (limonene, **1**), the main component of most citrus oils, is industrially isolated from orange peel by energy intensive steam distillation or cold expression.³ Recent research has opened 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, carveone) 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 the starting material. By this new combination limonene (**1**) could be directly transformed to chiral carvolactone (**5**) via carveol (**2**), carveone (**3**) and dihydrocarvone (**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 system¹² 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 systems¹³ (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



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.

^aInstitute of Applied Synthetic Chemistry, TU Wien, Getreidemarkt 9/163, 1060 Wien, Austria. E-mail: florian.rudroff@tuwien.ac.at

^bMolecular Enzymology Group, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

^cInstitute of Biochemistry, Dept. of Biotechnology & Enzyme Catalysis, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany

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‡ These authors contributed equally to this work.

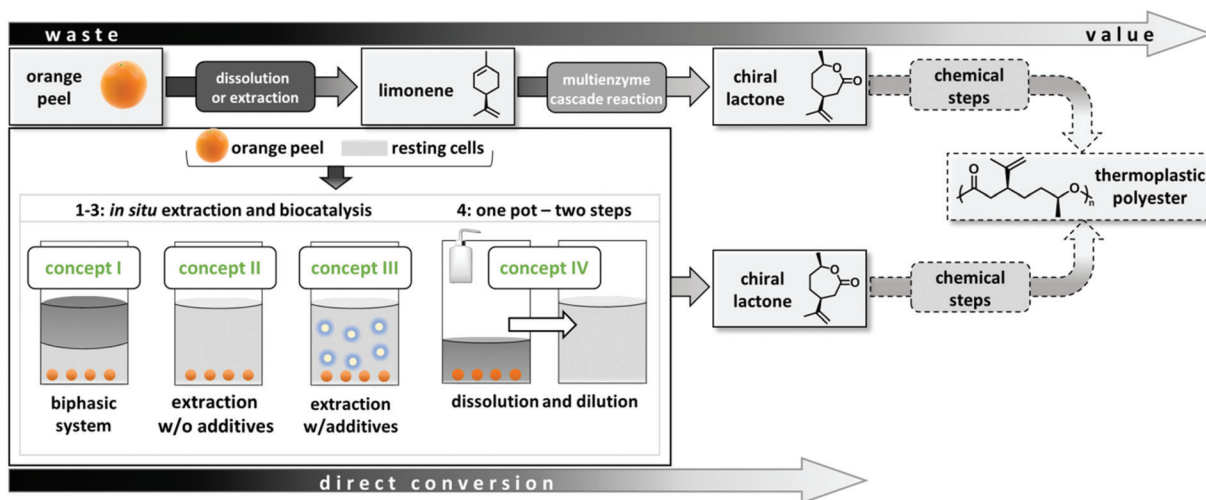


Fig. 1 Different strategies for the direct conversion of limonene (1) present in orange peel to chiral carvulactone (5).

limonene, due to its high $\log P$ 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.

Another possibility is the application of the SFPR (substrate feed product removal) approach,¹⁶ taking advantage of the orange peel itself as a 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 pretreat lignocellulosic biomass even in mixtures with water¹⁷ make ILs promising additives that have already been applied in several whole-cell biotransformations.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.

The 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 taking a closer look at the total amount of limonene per gram biomass, only 2–6% of limonene (see ESI, Fig. S1;† ref. 4) is available. A suitable method to obtain limonene concentrations in this 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 level²⁰ for both microbial hosts and in a suitable concentration range for our biocatalytic cascade.

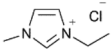
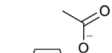
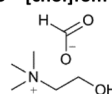
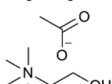
Besides water (concept II), two hydrophilic 1-ethyl-3-methyl-imidazolium-based ILs and two biocompatible choline ([chol]) ILs^{21,22} were chosen as additives for possible limonene extraction enhancement (concept III). 1-Ethyl-3-methyl-imidazolium acetate [C₂mim]OAc was investigated as it is known for its excellent ability to extract limonene from orange peel.¹⁹ 1-Ethyl-3-methyl-imidazolium chloride [C₂mim]Cl and [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 the viability of both bacterial strains based on growth rates, shown in Table 1.

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. A pronounced influence of the concentration of ILs can be seen in the case of *E. coli* BL21(DE3) where 50 mM [C₂mim]OAc were well tolerated but 100 mM [C₂mim]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 [C₂mim]OAc and [chol]-

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

IL [mM]	50		100	
	growth [%]			
Entry - IL	<i>E. coli</i> BL21(DE3)		<i>P. putida</i> S12	
1 - [C₂mim]Cl	63 ± 5	35 ± 7	87 ± 2	77 ± 2
				
2 - [C₂mim]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
				

OAc, but not with [C₂mim]Cl and [chol]fom. [Chol]fom had the least effect on the viability of both bacterial strains, at either 50 or 100 mM concentration, and was consequently selected as the best candidate for subsequent whole-cell biocatalysis.

First biotransformation tests with 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, [C₂mim]OAc, which was not compatible with the growing cells of *P. putida* S12, showed nearly no interference with the biotransformation in resting cells as can be seen in Fig. 2a.

Also [C₂mim]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 (50 mM, concept **III**)

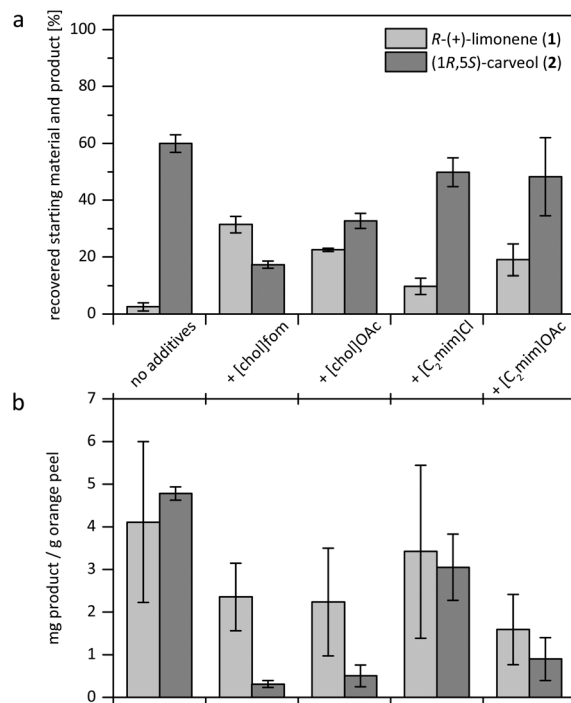


Fig. 2 Transformation of (a) 0.5 mM *R*-(+)-limonene (**1**) and (b) approx. 3% (w/v) orange peel (limonene [c] = 13.8 mg ± 4.0 mg g⁻¹ biomass) to (1*R*,5*S*)-carveol (**2**) by CumDO in *P. putida* S12 resting cells in the presence of ILs (50 mM) within 12 h reaction time. Results are GC yields and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

(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 [C₂mim]Cl gave acceptable results as it showed just minor inhibition of the reaction. Based on these results a clear preference for concept **II**, the simple use of orange peel in water, was gained.

Finally we dissolved the biomass in pure ILs, as proposed in concept **IV**, and fed the extract to resting cells expressing 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 the 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).

However, a sequential approach was devised, where hydroxylation of 1 mM limonene – to reach the same concentration of the 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 these 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⁻¹ 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⁻¹ biomass) could be produced. To ascertain 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⁻¹ 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 the 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⁻¹ biomass) as can be seen in Fig. 4. This promising result, 29% carvolactone from limonene over 4 biocatalytic steps (73% per step), thus only relies on orange peel as the substrate reservoir in aqueous buffer without additives, consequently avoiding any additional parameters increasing the 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 [C₂mim]Cl showed promising results as it hardly interfered with the biotransformation, product formation was not improved by the addition of ILs.

The most facile and economic approach (concept II), making use of orange peel as the substrate reservoir in a SFPR manner in aqueous buffer, emerged with 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 not only avoids tedious limonene extraction and purification, but also limits volatility problems with the starting material. Acting as a substrate reservoir, orange peel constantly releases limonene to the aqueous phase where it can be directly converted *via* the multi-step biotransformation within a principal proof-of-concept.

Studies on the improvement of parameters for the set-up of a 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 concentrations, could be handled by adaptation of *P. putida* S12²⁶ or introduction of the hydroxylation reaction into 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 for any additives and it underlines the power of cascade biocatalysis.

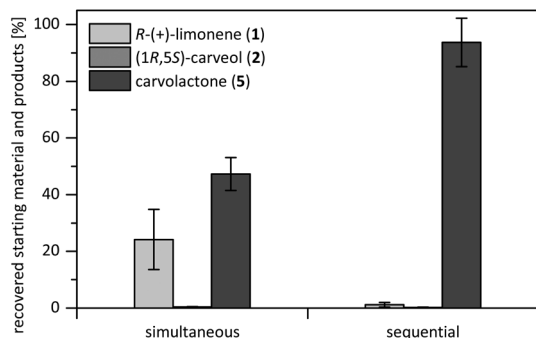


Fig. 3 Production of carvolactone (5) from 0.5 mM limonene (1) with simultaneous and 1 mM 1 with sequential addition of *P. putida* S12 and *E. coli* BL21(DE3) resting cells after 20 h reaction time.

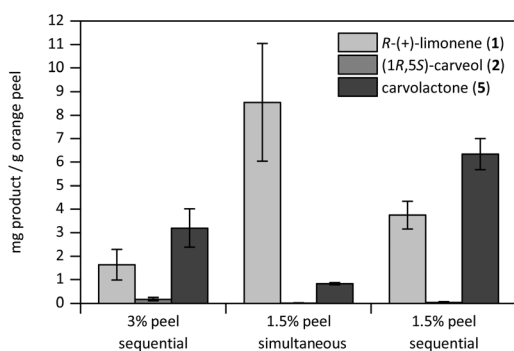


Fig. 4 Production of carvolactone (5) from orange peel (limonene [c] = 17.9 mg ± 3.7 mg g⁻¹ biomass) in different approaches and with altered biomass loadings.

Acknowledgements

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Notes and references

- 1 J. Alonso-Gutierrez, R. Chan, T. S. Batth, P. D. Adams, J. D. Keasling, C. J. Petzold and T. S. Lee, *Metab. Eng.*, 2013, **19**, 33–41.
- 2 N. J. Mueller, C. Stueckler, B. Hauer, N. Baudendistel, H. Housden, N. C. Bruce and K. Faber, *Adv. Synth. Catal.*, 2010, **352**, 387–394.
- 3 R. Ciriminna, M. Lomeli-Rodriguez, P. Demma Cara, J. A. Lopez-Sanchez and M. Pagliaro, *Chem. Commun.*, 2014, **50**, 15288–15296.
- 4 M. Pourbafrani, G. Forgács, I. S. Horváth, C. Niklasson and M. J. Taherzadeh, *Bioresour. Technol.*, 2010, **101**, 4246–4250.
- 5 A. M. Balu, V. Budarin, P. S. Shuttleworth, L. A. Pfaltzgraff, K. Waldron, R. Luque and J. H. Clark, *ChemSusChem*, 2012, **5**, 1694–1697.
- 6 W. A. Duetz, H. Bouwmeester, J. B. van Beilen and B. Witholt, *Appl. Microbiol. Biotechnol.*, 2003, **61**, 269–277.
- 7 W. A. Duetz, A. H. M. Fjällman, S. Ren, C. Jourdat and B. Witholt, *Appl. Environ. Microbiol.*, 2001, **67**, 2829–2832.
- 8 M. Groeneveld, H. L. van Beek, W. A. Duetz and M. W. Fraaije, *Tetrahedron*, 2016, DOI: 10.1016/j.tet.2015.12.061.
- 9 N. Oberleitner, C. Peters, J. Muschiol, M. Kadow, S. Saß, T. Bayer, P. Schaaf, N. Iqbal, F. Rudroff, M. D. Mihovilovic and U. T. Bornscheuer, *ChemCatChem*, 2013, **5**, 3524–3528.
- 10 S. C. Knight, C. P. Schaller, W. B. Tolman and M. A. Hillmyer, *RSC Adv.*, 2013, **3**, 20399–20404.
- 11 J. R. Lowe, M. T. Martello, W. B. Tolman and M. A. Hillmyer, *Polym. Chem.*, 2011, **2**, 702–708.
- 12 S. Schmidt, H. C. Büchenschütz, C. Scherkus, A. Liese, H. Gröger and U. T. Bornscheuer, *ChemCatChem*, 2015, **7**, 3951–3955.
- 13 E. Deziel, Y. Comeau and R. Villemur, *Biodegradation*, 1999, **10**, 219–233.
- 14 S. Cornelissen, M. K. Julsing, J. Volmer, O. Riechert, A. Schmid and B. Bühler, *Biotechnol. Bioeng.*, 2013, **110**, 1282–1292.
- 15 J. Sikkema, J. A. de Bont and B. Poolman, *J. Biol. Chem.*, 1994, **269**, 8022–8028.
- 16 F. Rudroff, V. Alphand, R. Furstoss and M. D. Mihovilovic, *Org. Process Res. Dev.*, 2006, **10**, 599–604.
- 17 A. Brandt, M. J. Ray, T. Q. To, D. J. Leak, R. J. Murphy and T. Welton, *Green Chem.*, 2011, **13**, 2489–2499.
- 18 L. L. Fan, H. J. Li and Q. H. Chen, *Int. J. Mol. Sci.*, 2014, **15**, 12196–12216.
- 19 K. Bica, P. Gaertner and R. D. Rogers, *Green Chem.*, 2011, **13**, 1997–1999.
- 20 V. Chubukov, F. Mingardon, W. Schackwitz, E. E. Baidoo, J. Alonso-Gutierrez, Q. Hu, T. S. Lee, J. D. Keasling and A. Mukhopadhyay, *Appl. Environ. Microbiol.*, 2015, **81**, 4690–4696.
- 21 R. S. Boethling, E. Sommer and D. DiFiore, *Chem. Rev.*, 2007, **107**, 2207–2227.
- 22 P. Nockemann, B. Thijs, K. Driesen, C. R. Janssen, K. Van Hecke, L. Van Meervelt, S. Kossmann, B. Kirchner and K. Binnemans, *J. Phys. Chem. B*, 2007, **111**, 5254–5263.
- 23 N. Wood, J. L. Ferguson, H. Q. N. Gunaratne, K. R. Seddon, R. Goodacre and G. M. Stephens, *Green Chem.*, 2011, **13**, 1843–1851.
- 24 E. Gonzalez García, A. K. Ressmann, P. Gaertner, R. Zirbs, R. L. Mach, R. Krska, K. Bica and K. Brunner, *Anal. Bioanal. Chem.*, 2014, **406**, 7773–7784.
- 25 A. K. Ressmann, E. Gonzalez Garcia, D. Khlan, P. Gaertner, R. L. Mach, R. Krska, K. Brunner and K. Bica, *New J. Chem.*, 2015, **39**, 4994–5002.
- 26 F. J. Weber, L. P. Ooijkaas, R. M. Schemen, S. Hartmans and J. A. de Bont, *Appl. Environ. Microbiol.*, 1993, **59**, 3502–3504.
- 27 M. Frederix, K. Hütter, J. Leu, T. S. Batth, W. J. Turner, T. L. Rüegg, H. W. Blanch, B. A. Simmons, P. D. Adams, J. D. Keasling, M. P. Thelen, M. J. Dunlop, C. J. Petzold and A. Mukhopadhyay, *PLoS One*, 2014, **9**, e101115.
- 28 J. Volmer, C. Neumann, B. Bühler and A. Schmid, *Appl. Environ. Microbiol.*, 2014, **80**, 6539–6548.