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Accepted Article

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To be cited as: *ChemSusChem* 10.1002/cssc.201701146

Link to VoR: http://dx.doi.org/10.1002/cssc.201701146

A Journal of

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Chemo-enzymatic synthesis of a novel borneol-based polyester

S. Roth,[a] I. Funk,[a] M. Hofer,[b] V. Sieber[a,b]

Abstract: Terpenes are a class of natural compounds that have recently moved into the focus as bio-based resource for chemicals productions due to their abundance, their mostly cyclic structure and the presence of olefin or single hydroxyl groups. In order to apply this raw material in new industrial fields, a second hydroxyl group is inserted into borneol by cytochrome P450cam (CYP101) enzymes in a whole cell catalytic biotransformation with *Pseudomonas putida* KT2440. Next, a semi-continuous batch system was developed to produce 5-exo-hydroxyborneol with a final concentration of 0.54 g L^{-1}. Afterwards, bifunctional terpene was used for the synthesis of an all bio-based polyesters by a solvent free polycondensation reaction. The resulting polymer showed a glass transition temperature around 70 °C and a molecular weight in the range of 2,000 – 4,000 g mol^{-1} (M_w). These results show that whole cell catalytic biotransformation of terpenes could lead to bio-based, higher-functionalized monomers, which might be basic raw materials for different fields of application, such as biopolymers.

Introduction

Terpenes are a wide class of natural hydrocarbons derived from isoprene units, which are abundant in nature.[1] Terpenes have a broad range of application possibilities. Some monoterpenes have antibacterial[2] and antifungal[3] properties whereas higher terpenes such as carotenoids are essential for plants, where they act as energy transfer molecules to the chlorophylls,[4] and are used as coloring agents in the food industry.[5] Some terpenes are also used in the pharma industry for example as an ingredient in transdermal drug delivery systems, where terpenes enhance the skin permeation of drugs.[6]

Recently terpenes have moved into the focus for the production of bio-based polymers. There are several reports on α- and β-pinene[7-10] and limonene[11] being used for polymerization resulting in homopolymers or copolymers.[12] These examples make use of the olefinic bonds available in many terpenes, which could be used for cationic or radical polymerizations[13] or further converted to epoxides and lactones to open them for ring opening polymerizations. However, monomers to be used in polycondensations require two functional groups (i.e. carboxyl or hydroxyl), yet typical monoterpenes do not fulfill this criterion. Dihydromyrcenol, for example, has been used for the production of an amphiphilic biopolymer with emulsifying properties by first introducing a thiol group at the double bond.[14]

In contrast, the modification of non-activated C-H bonds of terpenes is more challenging, but could be achieved by enzymes such as P450 enzymes.[15-19] It has also been shown that recombinant *Pseudomonas putida* is able to transform different terpenes likes limonene or α-pinene to products like perillyl alcohol or verbenol respectively, which have a higher commercial value for the industry than the starting material.[20-22] *P. putida* is a ubiquitous, aerobic, Gram-negative bacterium with a great metabolic versatility. In the last decades, it has become a bacterial workhorse for several applications in the field of biotechnology, especially for biotransformation processes.[23-24] Today recombinant *P. putida* is used as a whole cell catalyst to convert various substrates such as styrene,[25] octane[26] or toluene.[27]

Herein we report that parts of the P450cam operon, P450cam (camC, monooxygenase), PdX (camB, putidaredoxin), and PdR (camA, putidaredoxin reductase) can be used to modify borneol in a biotransformation with *Pseudomonas putida*. The terpenoid borneol consists of a bornane scaffold containing one hydroxyl group at C2 and exists in two stereo configurations. As constituent of essential oils of many plants and waste residues, e.g. in spruce needles, borneol has already been known for many years for its wide occurrence.[28] Even more important, (-)-borneol is also available in high amounts at a cost range of ca. 5 USD/kg from turpentine oil of the cellulose industry via established chemical transformation, especially as an intermediate in the synthesis of camphor (production route via bornyl chloride) or derived from synthetic camphor (production route via isobornyl acetate).[29-31] Hence, it is an interesting raw material for bio-based polymers. To achieve this, a second functional group has to be introduced. We show the incorporation of a hydroxyl group at C5 of (-)-borneol by whole cell catalysis of recombinant *P. putida* harboring P450cam enzymes to create a novel monomer that can be used in a polymerization reaction for a new bio-based polyester (Scheme 1).

Results and Discussion

Engineering of a *P. putida* whole cell catalyst for biotransformation of different borneols

The cam operon[32] is responsible for camphor degradation in *P. putida* ATC17453, where in the first step camphor is hydroxylated at C5 by the enzyme P450cam and then further metabolized.[33] For this hydroxylation two more proteins, PdR and PdX, are necessary as they act as an electron transporter system towards the P450cam.[33] In order to obtain a *P. putida* strain capable of borneol hydroxylation without further

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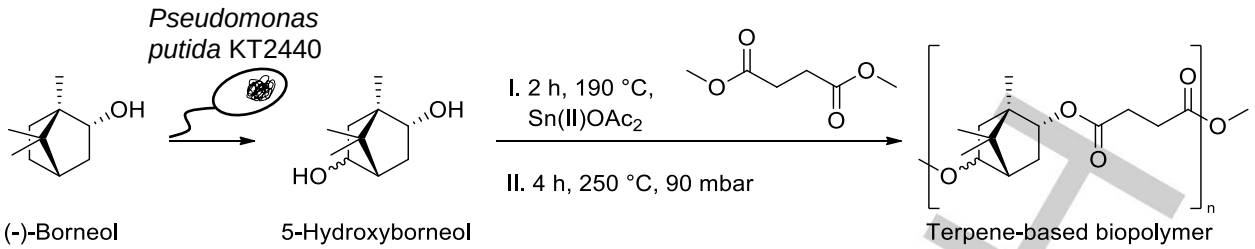

Scheme 1. Biotransformation of (-)-borneol to 5-exo-hydroxyborneol by engineered *Pseudomonas putida* KT2440. The bio-based monomer was subsequently used for polymerization to produce a terpene-based biopolymer.

degradation, parts of the cam operon were cloned into the broad host vector pBBR122. It is known that the camD gene codes for a dehydrogenase transforming the 5-hydroxyl group into a ketone[34-35] and the camR repressor negatively regulates the operon,[34, 36] which would both result in a reduced yield of the hydroxylated borneols. Hence, a synthetic operon was designed, which does not contain these two regions. After successful cloning and sequencing, the plasmid was transferred to *P. putida* KT2440 to yield a whole cell catalytic system. Afterwards, this strain was used to convert (-)-borneol to the corresponding hydroxyborneol in a biotransformation. To determine the stereochemistry of the product and to clarify if isomers are produced, NMR and GC-MS analysis of the purified product and substrates were performed (see Supplement). As there is no 5-endo-hydroxyborneol available, (-)-borneol and its isomer (-)-isoborneol were analyzed by GC-MS and could be differentiated as two separated signals were obtained (Supplement Figure S8). NMR analysis indicated that (-)-borneol is hydroxylated in only one orientation as well (Supplement Figure S1, S7). Furthermore it is reported that hydroxylation of camphor by P450cam enzymes is highly site-specific[37] and solely leads to 5-exo-hydroxycamphor,[37-38]. Therefore the resulting product of the biotransformation should be enantiomeric pure 5-exo-hydroxyborneol.

Optimization of the biotransformation of (-)-borneol

For an optimal production of hydroxylated (-)-borneol the substrate conversion was analyzed in dependence on different temperatures as well as different buffers and buffer systems, as the activity of P450cam enzymes is influence by both. Michizoe et al. tested different buffers at different pH values for the hydroxylation of camphor by the P450cam monooxygenase and obtained an optimum at pH 7.4.[39] For the conversion with the purified enzymes it could be shown that the buffer substance itself had an influence on the stability of *PdX*, which was more stable in Tris-HCl buffer than phosphate buffered systems.[39] Hence, the buffered systems itself, using Tris-HCl, MOPS and sodium-potassium-phosphate (S/P) buffers, as well as the pH values were varied (pH 6.0 – 8.0) to identify the optimal parameters for the conversion of (-)-borneol. Conversion rates of (-)-borneol and yields of 5-exo-hydroxyborneol decreased in all buffers at alkaline pH values (Table 1). Contrary to the pH optimum at pH 7.4 as mentioned above, the optimum for the conversion of (-)-borneol was observed at pH 6. This differences may occur because whole cells are used as catalysts. Nevertheless Spolitak et al. reported similar activities even with purified P450cam at pH 6.2, 7.4 and 8.0 for the reaction with peracids,[40] so pH optimum for each conversion is also dependent on the substrate. Interestingly, in S/P and Tris-HCl buffers the selectivity increased at alkaline pH values, whereas it remains nearly constant in MOPS. The selectivity is also slightly influenced by the buffer substance as high activity was obtaiend in Tris-HCl and phosphate buffer by contrast to the MOPS buffered system. As mentioned above, the purified enzymes showed a lower activity in phosphate buffers.[39] Using whole cells for the conversion protect the enzymes, as the yields are similar in Tris-HCl and phosphate buffered systems at pH 7 and 8 (Table 1). Based on these results the preferred buffer system for the conversion of (-)-borneol is S/P buffer at pH 6.

Table 1. Substrate consumption and product formation in different buffer systems after 6 h at 30 °C. Selectivity is calculated based on conversion and yield.

	Conversion (-)-borneol [%]	Yield 5-exo-hydroxyborneol [%]	Selectivity [%]
S/P buffer pH 6	100 ± 0.0	78.6 ± 11.1	78.6
S/P buffer pH 7	76.3 ± 2.0	69.8 ± 3.8	92.6
S/P buffer pH 8	63.0 ± 1.5	63.6 ± 1.6	100
Tris-HCl buffer pH 7	100 ± 0.0	70.2 ± 2.8	70.2
Tris-HCl buffer pH 8	74.9 ± 5.4	70.7 ± 3.7	94.4
MOPS buffer pH 6	100 ± 0.0	64.8 ± 1.7	64.8
MOPS buffer pH 7	100 ± 0.0	67.1 ± 0.9	67.1
MOPS buffer pH 8	76.6 ± 1.9	51.9 ± 4.0	67.8

Substrate conversion by P450cam enzymes is also influenced by the temperature as shown for whole cell biotransformation of camphor with recombinant *E. coli*.[41] Furthermore, *P. putida* tolerates a wide range of temperatures[42] and so influence of different temperatures on the biotransformation was investigated (Figure 1). Conversion rates were nearly similar in a range of 30 – 40 °C and decreased at lower temperatures. In contrast, product yields decreased at higher temperatures reaching their maximum at 30 °C. Mouri et al. figured out that lower temperatures for the hydroxylation of camphor by P450cam enzymes in recombinant *E. coli* resulted in a faster conversion.[41] The authors used inducible cells for the protein expression, which were harvested, concentrated and then used

for the hydroxylation of 2 mM camphor.[41] Following this procedure an optimal temperature of 20 °C was obtained.[41] In contrast, herein hydroxylation of (-)-borneol was performed using a constitutive expression of the enzymes with growing cells. Therefore 30 °C seemed to be the optimal temperature for the conversion, as it also is the optimal growth temperature of *P. putida*.[42]

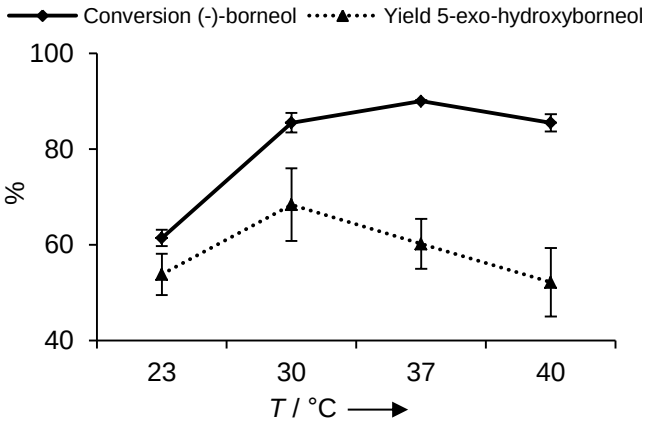

Figure 1. Conversion of (-)-borneol and obtained yield of 5-exo-hydroxyborneol at different temperatures after 5 h reaction time using the P450cam enzymes in recombinant *P. putida* KT2440 as a whole cell catalytic system.

Both experiments showed the formation of 5-oxocamphor as a side-product at lower pH values and higher temperatures respectively. Side product formation is attributed to different effects: (i) products are further converted by P450cam enzymes due to unspecific oxidation as shown for camphor, which is oxidized to 5-oxo-camphor,[43] (ii) a high oxygen level in the media is also responsible for further oxidation of products by P450cam enzymes.[44] To prevent further product oxidation the biotransformation was performed in a two phase system, where the product should be protected by dissolving in the organic phase. Moreover, high volatility as well as insolubility of (-)-borneol in aqueous media as two major challenges could be circumvented by the two phase system as well. As *Pseudomonas* species are known to have a high tolerance to different organic solvents,[45-46] they are excellent candidates for biotransformations in presence of organic solvents. *n*-Hexane and *n*-dodecane, both of which are reported to minimize toxic effects of substrate or product,[47] containing (-)-borneol were used and space-time yields (*STY*) were calculated accordingly. *STYs* for both solvents (*n*-hexane and *n*-dodecane) were similar (*STY* ~ 32 mg L^{-1} h^{-1}) and also comparable to the conversion without an organic solvent ($STY_{P\text{-}media}$ ~ 34 mg L^{-1} h^{-1}). Similar yields were reported for α- and β-pinene (*STY* = 22 and 41 mg L^{-1} h^{-1}), which were converted by resting cells of *Pseudomonas* strain PIN.[48] The major product of the metabolism of this strain was *p*-cymene, which was further metabolized to a broad spectrum of terpenes (limonene, α-terpinolene, α-terpineol).[48]

Schewe et al. investigated the biotransformation of α-pinene using recombinant *E. coli* as a whole cell catalytic system.[49] They make use of the $P450_{BM\text{-}3}$ monooxygenase from *Bacillus megaterium* for the conversion of α-pinene to α-pinene oxide, *trans*-verbenol and myrtenol respectively.[49] As a main product 20 mg g_{cdw}^{-1} α-pinene oxide were obtained in 1.5 h reaction time.[49] They obtained constant conversion rates for the first 30 min, which then decreased. The authors suggested that the main problem was the toxicity of the substrate α-pinene.[49] To determine any toxicity of (-)-borneol or 5-exo-hydroxyborneol to *P. putida*, which would affect conversion rates, a toxicity test was performed using 2 mM of substrate or product respectively. Bacterial growth was analyzed over 24 h, but neither (-)-borneol nor 5-exo-hydroxyborneol showed any toxic effect on the cells.
All these results indicate that the best parameters for the conversion of (-)-borneol are a low oxygen level in the media (low amount of side-products), S/P buffer pH 6 as the medium (high product yields) and 30 °C as the conversion temperature (fastest conversion with the highest yield).

Development of a semi-continuous batch system for the production of 5-exo-hydroxyborneol

For the production of larger quantities of 5-exo-hydroxyborneol a semi-continuous batch system was developed, which allowed the reuse of the cells as a catalyst (Figure 2).

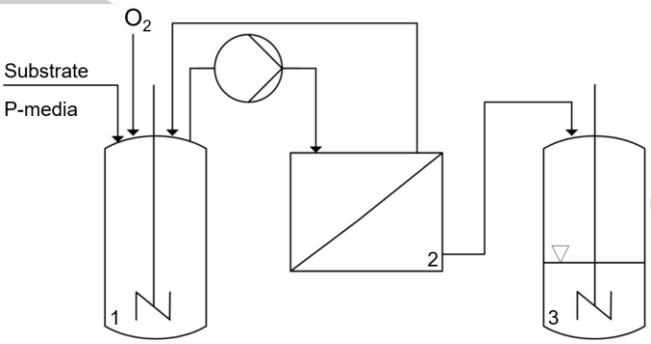

Figure 2. Flow chart of the biotransformation process of (-)-borneol. Biotransformation is performed in a 1.5 L air-flushed stirred tank reactor (1). After complete conversion the product is separated from the cells via cross flow filtration (2) and subsequently extracted with an organic solvent (3), while the cells are returned to the reactor and used for further biotransformations after addition of fresh media and substrate.

After complete substrate conversion was observed, the product was separated from the cells by cross flow filtration and extracted with ethyl acetate. Consequently fresh substrate was added to the cells, which could be reused up to three times (Figure 3).
Substrate conversion was high in every batch process (> 98 %; Table 2), which confirmed that the cells are reusable as a catalyst for the conversion of (-)-borneol. The time to reach full conversion increased from 8 to 40 h, but was even shorter compared to other hydroxylation reactions by P450cam enzymes (e. g. α- and β-ionone whole cell biotransformation with recombinant *E. coli*).[50] Product yields varied slightly with the

addition of fresh substrate, but were similar to those in the preliminary tests and also those reported for other hydroxylation reactions (hydroxylation of limonene by *P. putida* MTCC 1072 cells).[21]

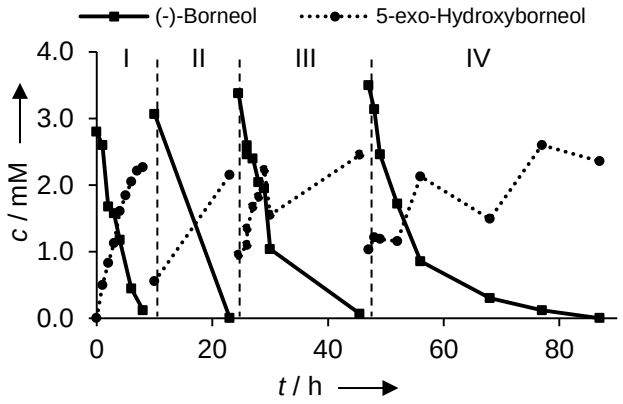

Figure 3. Substrate consumption and product formation during the biotransformation of (-)-borneol with cell recycling. Each dotted vertical line indicates the addition of new substrate. All in all cells could be used four times for the conversion of (-)-borneol (Phase I – IV).

Table 2. Parameters obtained during the biotransformation. *STYs* refer to wet biomass, for calculation see material and methods.

	Phase I	Phase II	Phase III	Phase IV
t [h]	8	13	21	40
Conversion [%]	99.8	99.6	98.0	98.8
Yield [%]	80.9	75.3	81.7	83.3
STY [mg L^{-1} h^{-1}]	48	28	20	11
STY [mg mg^{-1} h^{-1}]	12	6.0	4.0	2.0

By implementing the semi-continuous process, several advantages could be achieved compared to a batch process. The batch process is limited to a substrate concentration of 5 mM, because of the low substrate solubility and after the conversion the product has to be isolated from the cells. With the semi-continuous process it is possible to convert more substrate over time, thereby increasing the total product yield. Additionally, the amount of product per cell could be increased, as the cells can be reused. Furthermore, when cells are present during the extraction with ethyl acetate, an interphase is formed and so extraction after cell removal by cross-flow filtration was more efficient.

In contrast to the preliminary tests no side-product formation was observed, which is most likely due to the controlled lower oxygen level in the media. The preliminary tests were performed in shake flasks, where the oxygen level is supposed to be high. However, in the semi-continuous process, the oxygen level is controlled by an external oxygen supply. By varying the level of oxygen, specific product yields could be increased and best results were obtained at an airflow of 2.75 L (air) L^{-1} (culture volume) min^{-1}. In each cross-flow filtration step, a certain amount of media remained in the reactor, otherwise cell density would increase and cells would be damaged by increased shear stress, and so product yields remained at ~ 80 %. There also could be some loss of substrate during the biotransformation because of the volatile nature of the substrate.

Polymerization of a bio-based borneol polyester

The melting point of 5-exo-hydroxyborneol obtained from the biotransformation was determined (m. p. = 130 – 140 °C) and purity could be increased by recrystallization (> 98 %). To see whether the purified product can be used for polymerization, a proof of concept was done by the polycondensation of 5-exo-hydroxyborneol and succinic acid dimethylester. Succinic acid could be obtained from renewable resources like carbohydrates by fermentation.[51] There are several reports for the biotechnological production of succinic acid by metabolic engineered bacteria[52] and yeasts,[53] which can produce succinic acid at high titers (45 g L^{-1}).[53] Using this monomer for the polycondensation reaction, the resulting biopolymer is a novel all bio-based polyester. Furthermore, this monomer was chosen to produce a polymer, which is somewhat similar to polybutylene terephthalate (PBT), where a C4-alkyl chain and a ring structure are present as well. In the bio-based polyester, the aromatic ring is exchanged by the bicyclic alkyl ring of the terpene monomer and the ester bond now is reversed (Figure 4)

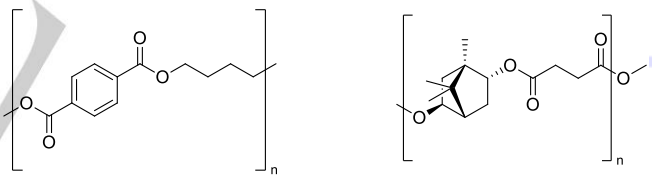

Figure 4. Structure of the commercial polybutylene terephthalate (PBT, left) compared to the terpene-based biopolymer (right).

During the first step of the solvent free polymerization, methanol was released and used as a marker to follow the reaction progress. First, polymerizations were carried out at lower reaction temperatures and times, which resulted in viscous, glutinous reaction mixtures. On increasing reaction temperature and time, an amber-colored, transparent solid was obtained. To get more details about the new material, thermal properties and molecular mass distribution of the biopolymer and an industrial PBT (as reference) were analyzed using differential scanning calorimetry (DSC) (Figure 5) and gel permeation chromatography (GPC) respectively. DSC analysis of the crude biopolymer showed a glass transition temperature (T_g) in the range of 35 – 55 °C. After the removal of residual monomers, the T_g increased to about 70 °C, which is higher than the T_g of PBT (45 – 60 °C). The terpene-based copolymer had a molecular weight in the range of M_w = 2,000 – 4,000 g mol^{-1} (*PDI* = 1.9), which is much lower compared to the industrial PBT

(M_W = 55,000 g mol^{-1}). The difference in the molecular weights might be attributed to the non-optimized polymerization procedure. Polycondensation of modified terpenoids is a rather unexplored area in the wide field of terpene-based polymers. There are a lot of reports, which use other polymerization techniques for terpene-based polymers. Often double bonds are used for the polymerization of terpenes, as a straightforward approach shown for cationic polymerizations reported for the homopolymerization of β-pinene.[54] With this method Satoh et al. could obtain poly(β-pinene) with high molecular weights (up to 50,000 M_N) and a high transparency.[55] Terpenes without double bonds often have to be modified before they are suitable for polymerization. (-)-Menthone for example, carrying a single carbonyl group, can be converted to lactams[56] or lactones,[57] which could be used in ring opening polymerization (ROP) to create polyamides (1.700 – 3.400 g mol^{-1} M_w) and polyesters (up to 90,000 g mol^{-1} M_N) respectively. One of the most common techniques for synthesis of terpene-based polyesters is the combination of ROP with lactones as monomers. For example carvone-derived lactones lead to homopolymers with molecular weights in the range of 700 – 10,500 g mol^{-1} (M_N) by ROP.[58] Compared to this terpene-based polymers, the novel borneol-based biopolymer is an interesting starting point for further investigations, especially using polycondensations as a polymerization technique. Thus, novel bio-based materials could be obtained and by optimizing the polymerization procedure, also the production of high molecular weight borneol-based biopolymers should be possible.

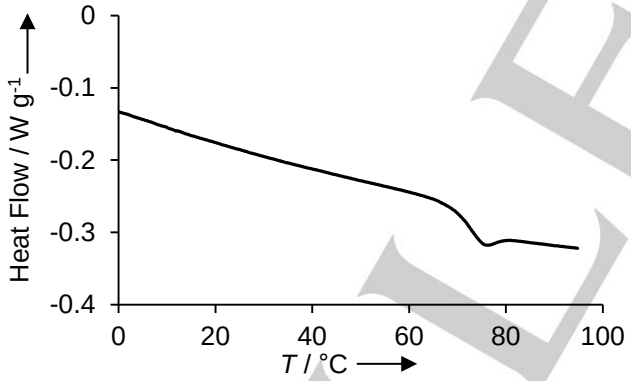

Figure 5. DSC analysis of the terpene-based biopolymer after removal of residual monomers. The second scan of the sample is shown and glass transition temperature was calculated by integration.

Conclusions

Within this study, it was successfully proven that it is possible to hydroxylate (-)-borneol to yield a bifunctional terpene-diol by a whole cell catalytic system. A process was developed, which allowed the semi-continuous production of 5-exo-hydroxyborneol with direct product extraction, in which cells could be reused as catalyst for repeated biotransformations. In a proof of concept the purified product was used for the polymerization with succinic acid dimethylester. The biopolymer had a T_g of around 70 °C and a M_w in a range of 2,000 – 4,000 g mol^{-1}. To increase molecular weights the polymerization procedure has still to be optimized. Therefore a screening on different parameters effecting the polymerization like (i) amount and kind of catalyst, (ii) polymerization temperatures and pressures, (iii) polymerization time, will be performed next. Further investigations on the bio-based borneol polyester, for example determination of impact resistance or ultimate strength, will be performed, even with the low molecular weight polymers, to figure out suitable applications for the material.

Experimental Section

GC-MS

Cell samples were mixed with an equimolar volume of ethyl acetate and centrifuged after vigorous mixing. A sample of the upper organic phase (150°µL) was mixed with ethyl acetate (350 µL) and GC-MS was performed using auto-injector AOC-5000, the Shimadzu GC-2010 Plus and a SGE BPX-column (30 m x 0.25 mm inner diameter). Separation of the substances on the GC-MS was achieved at an oven-temperature of 50 °C followed by a temperature programming from 50 °C to 120 °C (15 °C min^{-1}), then to 170 °C (5 °C min^{-1}), finally to 200 °C (20 °C min^{-1}) and holding this temperature for 10 min under the constant flow of 1.69 mL helium min^{-1}. The resultant chromatograms were analyzed with the Shimadzu GCMSsolution software.

DSC

Thermal properties of polymer materials (e. g. glass transition temperature (T_g)) were recorded with differential scanning calorimetry using the METTLER Toledo DSC 1 Star system. Samples of solid polymer (10 – 15 mg) were heated from 50 °C to 300 °C (25 °C min^{-1}), hold for 2 min and then cooled down to - 20 °C (- 25 °C min^{-1}) and hold for 5 min. Next, the samples were heated to 100 °C (10 °C min^{-1}), then to 400 °C (30 °C min^{-1}) and finally cooled down to 50 °C (- 30 °C min^{-1}). The heating program was performed under constant flow of 50 mL nitrogen min^{-1}. The DSC curves obtained were analyzed with the METTLER STAR^e SW 13.00 software and all T_g values were obtained from the second scan, after removing the thermal history.

GPC

Sample preparation was performed by drying (4 h at 80 °C) the solid polymer samples (5 – 15 mg) under vacuum. Dried samples were dissolved in appropriate volume of HFIP containing NaTFA (0.05 mol L^{-1}). For the calibration curve polymer standards of the ReadyCal-Kit Poly(methyl methacrylate) low were dissolved in HFIP (1.5 mL) containing NaTFA (0.05 mol L^{-1}). Samples and polymer standards were stored for two days at RT protected from light. Samples were then filtered (0.2 µm) and GPC analysis was performed using the SECcurity GPC System (Inline degaser and oven TCC600 (Polymer Standards Service), 1260 Infinity pump, 1260 Infinity auto-sampler, 1260 Infinity RI-detector (Agilent Technologies)), a PSS PFG pre-column (50 mm x 8 mm, particle size 7 µm) and two PSS PFG columns (300 mm x 8 mm, particle size 7 µm, porosity 100 Å and 1000 Å). The GPC curves obtained were analyzed using PSS WinGPC UniChrom software (Polymer Standards Service).

NMR

NMR-spectra were measured on a *JNM-ECA* 400 MHz spectrometer from *JOEL* at 25 °C with the application of standard pulse programs. The chemicals shifts (δ) are reported in ppm with reference to tetramethylsilane. Coupling constants *J* are reported in Hertz (Hz). For determining CH_2-signals, the DEPT135°-technique was used. For correct assignment of the signals, 2D NMR methods, like COSY, HSQC and HMBC. For more information see SI.

Cloning

The P450cam operon was amplified by PCR with genomic DNA from *Pseudomonas putida* ATCC 17453. For cloning the camCAB part of the operon primer P1 and P2 (P1: 5'-CAATAAGAACGAGGTAATGCATGA CGACTGAAACCATACAAAG-3'; P2: 5'-TAATGCGGCCGCGTCCCGG GATTGGCCATTG-3') were used for amplifying the *camC*, *camA* and *camB* genes and adding an overlap sequence at the 3' end of the PCR product. For amplification of the promotor region primer P3 and P4 (P3: 5'-GTACTGAATTCGTTGCCCGGCTCGATCCGAG-3'; P2: 5'- GTATGG TTTCAGTCGTCATGCATTACCTCGTTCTTATTG-3') were used which added an overlap sequence at the 5' end. Both PCR products were then used in an overlap extension PCR yielding camCAB. This PCR product was then digested with EcoRI and NotI and ligated into the vector pBBR122 digested with the same enzymes yielding pBBR122_camCAB. The plasmid was transformed in *E. coli* DH10B and plated on LB plates containing kanamycine (30 mg L^{-1}). Single clones were used for plasmid DNA preparation and sequencing. After the sequencing confirmed the success of cloning the plasmids were transformed in *P. putida* KT2440 by electroporation.

Biotransformation of (-)-borneol

Single *P. putida* clones were used for inoculation of P-media (25 mL; 2 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 1 g L^{-1} $(NH_4)_2SO_4$, 2.5 g L^{-1} glucose, 30 mg L^{-1} kanamycine and 0.4 % trace metal solution (TMS)). The TMS contained 10 g L^{-1} MgO, 2 g L^{-1} $CaCO_3$, 5.6 g L^{-1} $FeSO_4x7H_2O$, 1.44 g L^{-1} $ZnSO_4x7H_2O$, 0.85 g L^{-1} $MnSO_4xH_2O$, 0.25 g L^{-1} $CuSO_4x5H_2O$, 0.28 g L^{-1} $CoSO_4x7H_2O$, 0.062 g L^{-1} H_3BO_3 and 50 mL L^{-1} HCl. The cells were incubated in Erlenmeyer flasks overnight (30 °C, 150 rpm). This pre culture was then used to inoculate 1 L fresh media with an OD_{600} of 0.2. The cells were then further incubated (30 °C, 150 rpm) for 24 h. Afterwards (-)-borneol (3 – 5 mM) was dissolved in dodecane or ethyl acetate and added to the cell broth. Cells were further incubated (30 °C, 150 rpm), samples were taken at different time intervals and analyzed by GC-MS.

Optimization of the biotransformation

P. putida cells containing the pBBR122_camCAB plasmid were incubated (2 d, 30 °C, 120 rpm) in shake flasks. To check whether different buffers at different pH affect conversion, cells were harvested by centrifugation (4,000 xg, 30 min, 4 °C), washed twice with the corresponding buffer (pH 6.0 – 8.0) and set to an OD_{600} of 1.0. All buffers (20 mM) contained equal amounts of glucose (2.5 g L^{-1}) and TMS (0.4 %) as the P-media. Cells were treated with (-)-borneol (1 mM), further incubated (30 °C, 120 rpm) and samples for GC-MS were taken after 0 h and 6 h. For the conversion at different temperatures (T = 23 °C – 40 °C), cells were produced as above and also incubated (30 °C, 120 rpm) with (-)-borneol (1 mM) at different temperatures (23, 30, 37, 40 °C). Samples for GC-MS were taken at regular time intervals. The conversion in a two phase system was performed using *n*-hexane and *n*-dodecane as solvents (each 20 % (v/v)) containing (-)-borneol (50 mM). Cells were incubated (30 °C, 120 rpm), samples for GC-MS were taken at different time intervals and space-time yields (*STY*) were calculated (Equation 1 and 2).

[Eq. (1)] $$STY\ [g\ L^{-1}\ h^{-1}] = \frac{m\ (\text{obtained product [g]})}{V\ (\text{obtained Volume [L]})\ t\ (\text{time [h]})}$$

[Eq. (2)] $$STY\ [g\ g^{-1}\ h^{-1}] = \frac{c\ (\text{obtained product [g L}^{-1}\text{]})}{m\ (\text{wet biomass [g L}^{-1}\text{]})\ t\ (\text{time [h]})}$$

For the toxicity tests of substrate, product or the corresponding solvent, the cells were incubated (30 °C and 120 rpm) with (-)-borneol, 5-exo-hydroxyborneol (each 2 mM) or ethyl acetate either for 48 h and OD_{600} values were measured with the Ultraspec 10 cell densitiy meter (Amersham Biosciences) at different time intervals and compared to a control.

Production of the diol compound

For the biotransformation of borneol to the corresponding diol, cells were produced as described above and (-)-borneol was used as the substrate. The OD_{600} of the main culture was set to 1.0 and 1 L of the culture broth was then transferred to a stirred tank reactor with a total volume 1.5 L. The broth was incubated (30 °C, 215 rpm, air flow of 2.75 L L^{-1} min^{-1}) and (-)-borneol (3 – 5 mM) was added as substrate. Samples withdrawn at regular time intervals were analyzed by GC-MS. After full conversion of (-)-borneol, the wet biomass weight was determined and the product was separated from the cells by cross flow filtration with a Vivaflow 200 membrane (MWCO = 30 kDa, Sartorius). In addition, the reactor was filled up with fresh media and substrate. This step was repeated three more times. The aqueous product was extracted with the same amount of ethyl acetate and the organic solvent was removed by evaporation.

Purification of the diol compound by recrystallization

To each gram of product, 6.2 g of ethyl acetate were added and heated to 77 °C. After 2 –3 min the clear reaction mixture was allowed to cool down to RT, whereby white crystals precipitated. The suspension was incubated for 24 h at RT, following an incubation of another 24 h at 4 °C. After an additional incubation for 30 h at – 20 °C the suspension was vacuum filtered and white crystals were obtained. The resulting filtrate was purified once more with the same procedure.

Polymerization

For the transesterification of succinic acid dimethyl ester (1.29 g, 8.8 mmol) with 5-exo-hydroxyborneol (1.05 g, 6.2 mmol) in the presence of 0.5 mol-% tin(II) acetate (7.1 mg, 0.029 mmol, on 5-exo-hydroxyborneol), a two-necked round bottom flask equipped with a magnetic glass stirrer and a reflux condenser with a distillation bridge were used and heated to 105 °C for 16 h. The educts were added under an atmosphere of nitrogen and transesterification was conducted solvent-free for 2 h at 190 °C under a nitrogen atmosphere. In the second step the reaction mixture was heated to 245 °C and under reduced pressure (p = 90 mbar), the excess of succinic acid dimethyl ester was removed. After 4 h, the reaction mixture was cooled down to RT.

For the removal of residual monomers polymer samples (200 – 250 mg) were pulverized and then mixed with methanol (10 mL) for 16 h at RT. After filtration, followed by a washing step with methanol (5 mL), the solvent was removed by evaporation and the filter was dried.

Acknowledgements

The authors are grateful for the financial support by the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) (PhD grant AZ: 20015/382). The authors also thank Claudia Falcke, Marion Wölbing and Paul N. Stockmann for scientific conversations and help with DSC, GPC and NMR analysis.

Keywords: Biocatalysis • Biotransformations • Copolymerization • Polymers • Terpenoids

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10.1002/cssc.201701146

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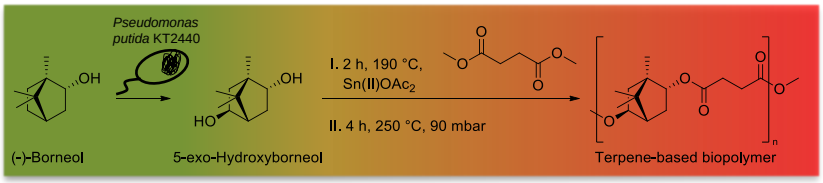

A new monomer for terpene-based biopolymers was produced by whole cell biotransformation. *Pseudomonas putida* KT2440 was used as a host for the hydroxylation of (-)-borneol by P450cam enzymes. A semi-continuous batch system was developed to yield 5-exo-hydroxyborneol in a final concentration of 0.54 g L^{-1}. In a proof of principle, the purified product was then used to create novel terpene-based polyesters, which were further characterized by DSC and GPC analysis.

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