Process Development through Solvent Engineering in the Biocatalytic Synthesis of the Heterocyclic Bulk Chemical ε-Caprolactone

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Additional Supporting Information may be found in the online version of this article.

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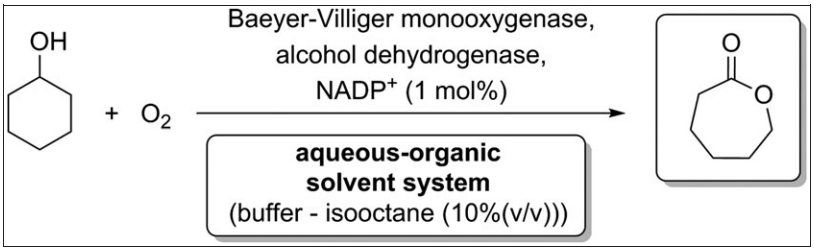
Received July 28, 2015
DOI 10.1002/jhet.2595
Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com).



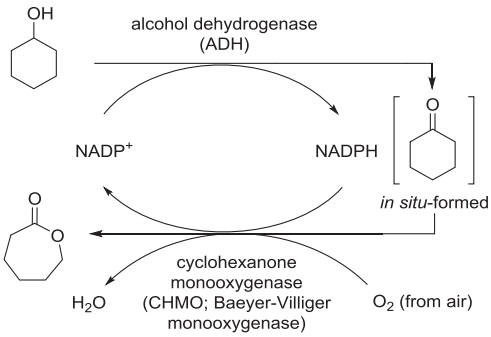
For an alternative synthetic approach toward the heterocyclic industrial chemical ε-caprolactone, which is based on a biocatalytic oxidation of readily available cyclohexanol with air in aqueous media (using an alcohol dehydrogenase and a Baeyer–Villiger monooxygenase as enzyme components), a solvent engineering has been carried out identifying isooctane as a suitable co-solvent. Biotransformations in an aqueous-isooctane biphasic solvent system were found to proceed faster at both investigated substrate concentrations of 40 and 80 m*M*, respectively, compared with the analogous enzymatic reactions in pure aqueous medium. In addition, in all cases quantitative conversions were observed after a reaction time of 23 h when using isolated enzymes. The achievements indicate a high compatibility of isooctane [10%(v/v)] with the enzymes as well as the potential for an *in situ* removal of the organic reaction components, thus decreasing inhibition and/or destabilization effects of these organic components on the enzymes used. In contrast, so far, the use of recombinant whole-cells gave less satisfactory results, which might be due to limitations of the permeation of, for example, the substrate through the cell membrane.

INTRODUCTION

The heterocyclic compounds ε-caprolactam and ε-caprolactone belong to the most important industrial bulk chemicals because of their need as monomers for the manufacture of polymer products [1,2]. With respect to the latter one, its today's favored industrial production technology running on a multi-10.000 tons scale is based on a Baeyer–Villiger oxidation of cyclohexanone utilizing acetic peracid as oxidizing agent [1]. Besides limited selectivity, however, hazardousness of the needed peracid reagent represents a drawback. Recently, jointly with the company Enzymicals AG, we reported a *de novo* approach toward ε-caprolactone, which starts from the industrial basic chemical cyclohexanol and undergoes a direct oxidation toward ε-caprolactone utilizing only molecular oxygen as a reagent in aqueous medium [3]. As a catalyst, two enzymes were used, namely, an alcohol dehydrogenase (ADH) from *Lactobacillus kefir* [4] for the initial dehydrogenation of cyclohexanol to cyclohexanone and a cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* [5,6] for the subsequent Baeyer–Villiger oxidation under formation of ε-caprolactone. It is noteworthy that apart from molecular oxygen (required for the second step), no other co-substrates are required because the cofactor for the first step ($NADP^+$ being transformed to NADPH) is recycled in the second step (by oxidation of NADPH to $NADP^+$ and simultaneous reduction of molecular oxygen to water). This concept, for which also the Bornscheuer group developed independently a similar process [7], is graphically shown in Scheme 1.

Although we could achieve an excellent conversion (>95%) for this biotransformation at substrate concentrations of 20–60 m*M*, elevated substrate concentrations led to decreased conversion [3]. We identified inhibition as well as deactivation effects of the involved organic components (cyclohexanol, cyclohexanone, and ε-caprolactone) as reasons. Recently, Bornscheuer *et al.* found an opportunity to circumvent these inhibition and deactivation effects at elevated substrate and product concentration by a reactive *in situ* product removal through *in situ* oligomerization

Scheme 1. Concept of a direct one-pot transformation of cyclohexanol with air toward ε-caprolactone.



of ε-caprolactone with an additional enzyme [lipase CAL-A (lipase from *Candida antarctica* A)] [8]. As in the past for a range of enzymes in general and other Baeyer–Villiger monooxygenases [9,10], in particular, solvent engineering turned out as a promising tool for process optimization, we became interested in studying the impact of organic water-immiscible solvents on the reaction course also for our ε-caprolactone-forming target process. However, what makes this search challenging is the fact that the CHMO is known as a sensitive enzyme, which, for example, is deactivated rapidly in the presence of a larger amount of water-miscible solvents as demonstrated by Secondo and Fraaije *et al.* recently [9b]. For our purpose, we focused on the use of hydrophobic organic solvents with a high logP value (in particular exceeding logP of 2) because of the following reasons: (i) expected simple phase separation, (ii) the second phase could serve as a reservoir of the starting material cyclohexanol, the intermediate cyclohexanone and the product ε-caprolactone leading to a decreased solubility of these components in the aqueous phase and, thus, suppressing the observed negative impact of these components on the CHMO enzyme, (iii) proven suitability as co-solvents for other enzymes (or expected to be suitable because of positive results with related solvents for such other enzymes) [11], thus making its application also for the CHMO from *A. calcoaceticus* promising. Accordingly, we studied the influence of different organic solvents, and in the following, the results, revealing isooctane as most feasible organic co-solvent among them for this biotransformation, are described.

RESULTS AND DISCUSSION

In our initial solvent screening, we chose ethyl acetate [EtOAc; logP 0.73 (±0.05)] [12], methyl *tert*-butyl ether [logP 0.94 (±0.30)] [12], toluene [logP 2.73 (±0.10)] [12], methylcyclohexane [logP 3.88 (±0.40)] [12], *n*-heptane [logP 4.5 (±0.25)] [12], and isooctane (logP 4.5) [13] as solvent components because of their frequent use in organic synthesis as standard extraction solvents. Furthermore, in terms of sustainability, most of them are listed as "preferred" or "usable" according to the Solvent Guide from the pharmaceutical company Pfizer [within their Green Chemistry/environment, health and safety (EHS) program] [14]. When conducting the biotransformations and determining the concentrations of substrate (cyclohexanol), intermediate (cyclohexanone) and resulting product (ε-caprolactone), several interesting results were obtained (Fig. 1). First,

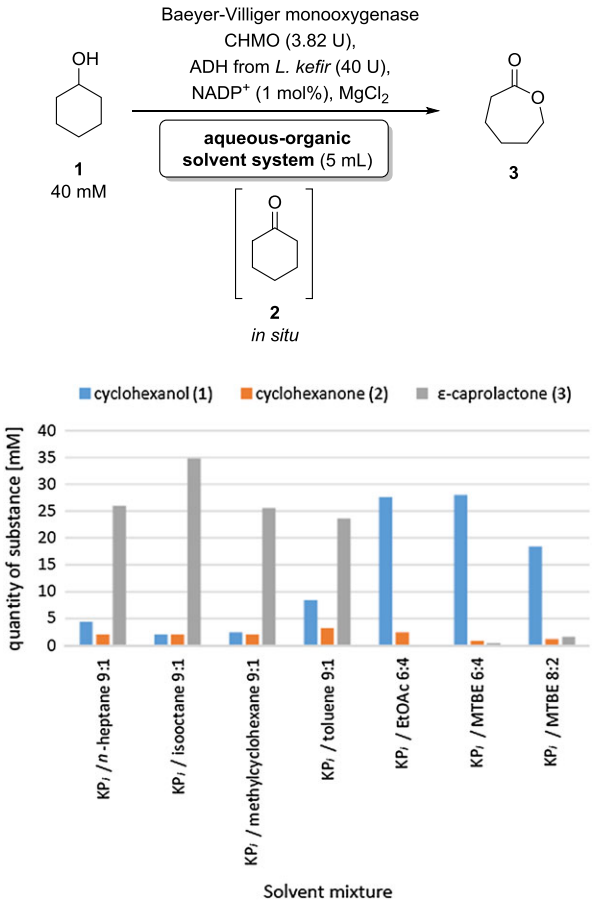


Figure 1. Screening of aqueous-organic reaction media. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com]

whereas most of organic solvents gave a decrease of conversion, it is noteworthy that with isooctane as a highly hydrophobic solvent, a conversion in the same range (and even being slightly superior) to the one achieved when using free buffer was detected. Second, in contrast, some of the solvents such as ethyl acetate and toluene led to a more rapid deactivation. Our hypothesis is that because of the high logP for isooctane, in this case, solubility of the organic solvent in water is low thus leading to a low or negligible deactivation of the enzyme. Nevertheless, it is remarkable that the presence of the second liquid phase does not deactivate the enzyme (as this is true for other cases as observed in the past

[11], e.g., for oxynitrilases and alcohol dehydrogenases). Other highly hydrophobic solvents with the same or similar logP value, such as heptane, gave less satisfactory results. Being surprising on the first glance, this result might also underline that other physicochemical properties play an important role as well. For example, in spite of logP values being in the same range, the solubility of water is significantly less in isooctane (0.56 mg/L, 25°C) compared with heptane (0.05 g/L, 20°C) [15], which might contribute to a higher stability of the enzyme in case of the aqueous-isooctane biphasic system.

Having in hand a promising co-solvent candidate with isooctane, next we got interested in a more detailed insight into the kinetics of this process as well as a comparison of the reaction courses in the presence and absence of this co-solvent isooctane. The reaction courses of these two processes, which were again carried out at a substrate concentration of 40 m*M*, are shown in Figure 2 (for these experiments, the enzyme activities were measured prior to use: alcohol dehydrogenase from *Lactobacillus kefir* (*Lk*-ADH) crude extract/glycerol 1:1 (v/v) 48.08 U/mL; CHMO lyophilized crude extract gave significantly lower activities (0.08 U/mg) than stated for the commercial sample (0.35 U/mg); thus, the amount of added enzyme (in milligram) was accordingly higher). In both cases, a quantitative conversion was reached after a sufficient reaction time (23 h). However, a further interesting insight into the impact of isooctane as a co-solvent one can be gained when looking into the kinetics. Whereas in pure aqueous buffer, initial conversion after 1 h was 8%, followed by 20%, 34%, and 50% after 2, 3, and 4 h, respectively, the conversions in the presence of 10%(v/v) of isooctane were significantly higher at all stages of the reaction course (before reaching a quantitative conversion). After only 1 h reaction time, already 13% conversion was obtained, which rapidly increased to 34%, 58%, and 82% after 2, 3, and 4 h, respectively. These results clearly indicate a beneficial impact when using 10% (v/v) of isooctane as a co-solvent.

In addition, when increasing the substrate concentration up to 80 m*M* while keeping the overall amount of enzyme unchanged, we observed a similar tendency of the reaction course. The kinetic reaction courses of these reactions at 80 m*M* substrate concentration are shown in Figure 3 (for comparison, data for analogous reactions at 40 m*M* substrate concentrations are added in this figure). When conducting the reaction in the presence of 10%(v/v) of isooctane as a co-solvent, reaction proceeds faster, reaching 11%, 27%, 42%, and 60% after 1, 2, 3, and 4 h, respectively. After 23 h reaction time, full conversion was observed. Although full conversion was also observed in the absence of isooctane in pure aqueous buffer, reaction proceeds much slower with 5%, 14%, 22%, and 33% after 1, 2, 3, and 4 h, respectively. It should be added

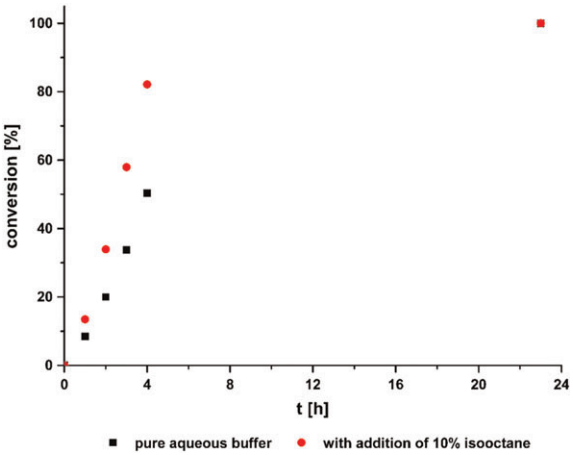


Figure 2. Biotransformations of 40 m*M* cyclohexanol with commercial BVMO enzyme in the absence and presence of isooctane [10%(v/v)] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that preliminary studies showed a similar reaction course when using an increased volume of 30%(v/v) of the co-solvent isooctane (data not shown). From an industrial point of view, recycling of the enzymes (e.g., by direct re-use of the aqueous phase bearing the enzymes) is of economic interest. Thus, studying the impact of isooctane on the long-term stability of the enzymes is a task for prospective experiments.

Next, we were interested about the impact of the "enzyme formulation" on the reaction course, in particular about the influence of the use of the Baeyer–Villiger monooxygenase as isolated enzyme or integrated in a whole-cell catalyst on the reaction course. Thus, we prepared whole-cells of Baeyer–Villiger monooxygenase (7.6 mU/mg_{wcw}), and as a benchmark reaction, we conducted the biotransformation using disrupted cells and the resulting free enzymes (in combination with non-soluble cell materials such as disrupted cell wall pieces). When conducting the reaction with this prepared crude extracts containing cell material, we obtained a slightly lower [compared with the commercial Baeyer-Villiger monooxygenase (BVMO), see Fig. 3] but still satisfactory reaction course, leading to a conversion of 78% after a reaction time of 23 h (Fig. 4; conversion after 1, 2, 3, and 4 h: 4%, 8%, 12%, and 17%). However, when utilizing the whole-cells collected directly from the fermentation broth (and although adding the same amount of external cofactor as in the processes with the "free" enzymes), a dramatic decrease of conversion was observed, and even after 23 h reaction time, only 7% conversion was found (Fig. 4; conversion after 1, 2, 3, and 4 h: 1%, 1%, 2%, and 2%). Thus, permeation processes through the cell wall and the cell membrane, and thus limitation of the transport of substrate into the cell as well as accumulation of substrate, intermediate, and product in the cell (thus causing to an increasing extend inhibition and deactivation effects), might play a role.

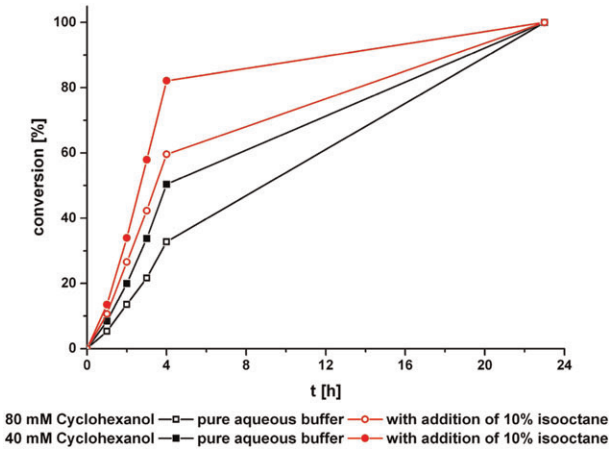


Figure 3. Biotransformations with recombinant BVMO in the presence of isooctane [10%(v/v)] at substrate concentrations of 40 and 80 m*M* (for comparison, the reactions course of the analogous reaction using 40 m*M* substrate concentration as given in Fig. 2 is shown again here in Fig. 3; for reasons of a better clarity, measured points are connected by lines). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

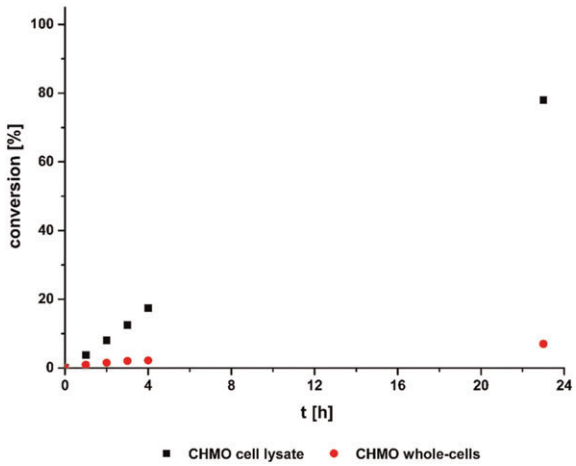


Figure 4. Biotransformations with lysed cells and resting whole-cells bearing the BVMO in recombinant form in the presence of isooctane [10%(v/v)]. CHMO, cyclohexanone monooxygenase. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Thus, currently the favored process option is based on utilization of free enzymes rather than whole cell catalysts, and improvement of whole-cell catalysis represents a task for future work.

CONCLUSION

In this study, we conducted a solvent engineering for our recently developed alternative synthetic approach toward the heterocyclic industrial chemical ε-caprolactone, which is based on a biocatalytic oxidation of readily available cyclohexanol with air in aqueous media using an alcohol dehydrogenase and a Baeyer–Villiger monooxygenase as enzyme components. After identifying isooctane as a suitable co-solvent, biotransformations were conducted in an aqueous-isooctane biphasic solvent system. It is noteworthy that these oxidation processes proceed faster at both investigated substrate concentrations of 40 and 80 m*M*, respectively, compared with the analogous enzymatic reactions in pure aqueous medium. In all cases, quantitative conversions were observed after a reaction time of 23 h. The achievements indicate a high compatibility of isooctane [10%(v/v)] with the enzymes as well as the potential for an *in situ* removal of the organic reaction components, thus decreasing inhibition and/or destabilization effects of these organic components on the enzymes used. Further process development of this novel oxidation process for the synthesis of ε-caprolactone is currently in progress. In contrast, so far the use of recombinant whole-cells gave less satisfactory results, which might be because of limitations of the permeation of, for example, the substrate through the cell membrane.

EXPERIMENTAL

Screening of aqueous-organic reaction media (according to Fig. 1). In a 10-mL flask, the crude extract of CHMO from *A. calcoaceticus* (3.82 U) is dissolved in a solvent mixture and well shaken. Cyclohexanol (**1**, 40 m*M* referring to the overall volume of solvent, consisting of aqueous and organic phase), $MgCl_2$ (1 m*M*), and ADH from *L. kefir* [40 U, crude extract/glycerol 1:1(v/v)] are added. The mixture is stirred for 20 min at room temperature (RT). The reaction is started by the addition of $NADP^+$ (1 mol%, 1.57 mg) and stirred for 24 h at RT. KP_i-buffer (phosphate buffer, pH 7, 50 m*M*) with added organic solvent is used as solvent mixture. The reaction mixture is distributed on seven reaction vessels (Eppendorf AG, Hamburg, Germany) with 0.7 mL each and treated with 0.7 mL DCM (dichloromethane). After 30-min shaking in a Thermomixer (Eppendorf), the two-phase system is separated by centrifugation at 13 000 rpm for 5 min. The lower organic phase is removed, and the remaining aqueous phase and the denatured enzymes are treated with DCM again, and the extraction is repeated two times. The organic phases are collected in a 50-mL volumetric flask, which is filled up to the calibration mark. Three probes are bottled, and the conversion is determined via gas chromatography. For the determination of the conversion via nuclear magnetic resonance (NMR), the solvent of the collected organic phases is evaporated at 900 mbar and 40°C bath temperature. The entire reaction mixture is transferred into an NMR-tube together with a freshly prepared urotropine solution (0.17 M in $CDCl_3$, 0.1 mL) as well as $CDCl_3$ (0.7 mL). The conversion is determined by comparison with the intensity of the singlet peak of the standard urotropine.

Biotransformations in the absence and presence of [10%(v/v)] of isooctane (according to Figs. 2 and 3). The biotransformations were performed in a 10-mL round-bottom flask with 5 mL aqueous reaction volume at 25°C in a tempered water bath on magnet stirrer (IKA-Werke, Staufen, Germany). Cyclohexanol (40 or 80 m*M*) was dissolved in an aliquot of KP_i-buffer (pH 7, 50 m*M*, 200 mg/L $MgCl_2$). In addition, 0.002 mmol $NADP^+$ and CHMO from *A. calcoaceticus* [3.82 U of lyophilized crude extract supplied by Enzymicals AG (Greifswald, Germany); the enzyme activity is referred to cyclohexanone in KP_i-buffer (pH 7, 50 m*M*, 200 mg/L $MgCl_2$) and was measured prior to use spectrophotometrically; because significantly lower activities were measured (0.076 U/mg) than stated for the commercial sample, a higher amount of enzyme (in milligram) has to be added compared with the amount calculated based on the given enzyme activity (0.351 U/mg) of the commercial sample] were dissolved in the KP_i-buffer. ADH from *L. kefir* [40 U crude extract/glycerol 1:1(v/v) recombinantly overexpressed in *Escherichia coli* as previously described [4]; the enzyme activity is referred to acetophenone as reference substrate in KP_i-buffer (pH 7, 50 m*M*, 200 mg/L $MgCl_2$)] was added and filled up to 5 mL aqueous reaction volume with KP_i-buffer. The solution was mixed by pipetting up and down several times, and during the reaction, a 1.5-cm stirring bar mixed the solution by rotating 340 rpm. In the experiments with organic solvent, 0.5 or 1.5 mL isooctane (corresponding to 10%(v/v) and 30%(v/v), respectively) was added. After 1, 2, 3, 4, and 23 h, an aliquot of 0.5 mL of the reaction mixture was taken and extracted three times with 0.5 mL ethyl acetate (with 2 m*M* acetophenone as an external standard) by mixing on a vortexer (Scientific Industries, Bohemia, NY) for 1 min at maximum speed and phase separation for 1 min at 21.500 g in a microcentrifuge (VWR International GmbH, Darmstadt, Germany). The organic phases were combined and filled up to 2 mL with ethyl acetate (2 m*M* acetophenone) in a volumetric flask. After mixing, the liquid was dried with a spatula tip of sodium sulfate (anhydrous). The samples were analyzed by gas chromatography using A GC-2010 (Shimadzu GmbH, Duisburg, Germany) with a BP5MS-column (SGE Analyticals Science, Victoria, Australia) with the following temperature profile: starting with a plateau at 70°C for 3.8 min followed by heating up to 200°C with 40°C/min (SPLI1: 230°C; flow control: linear velocity; linear velocity: 46.9; split ratio 100; injection volume: 1 µL; FID: 230°C). Retention times of the analyzed compounds were as follows: cyclohexanol 3.2 min, cyclohexanone 3.3 min, acetophenone 5.5 min, ε-caprolactone 6.0 min and 6-hydroxyhexanoic acid 6.8 min. The conversion was determined by relative analysis as it has been shown that cyclohexanol and ε-caprolactone were extracted in the identical ratio.

Preparation of whole-cells containing a Baeyer–Villiger monooxygenase in recombinant form. A glycerol stock of *E. coli* BL21(DE3) (New England Biolabs GmbH, Frankfurt am Main, Germany) containing the plasmid encoded gene of the CHMO from *A. calcoaceticus* (GenBank: BAA86293.1 – cloning was described previously [8]) was used for inoculation of 30 mL lysogeny broth (LB) medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl) [16] containing 50 µg/mL kanamycin. Cultivation was performed in a 300-mL shaking flask with baffles at 37°C on an orbital shaker (Infors GmbH, Einsbach, Germany) at 140 rpm overnight. The overnight culture was used as an inoculum to adjust 200 mL terrific broth medium containing 50 µg/mL kanamycin to OD_{600} 0.05. Cultivation was performed in a 1-L shaking flask with baffles at 37°C and 140 rpm. CHMO expression was induced between OD_{600} 0.6 and 0.8 with 0.05 m*M* Isopropyl β-D-thiogalactopyranosid (ITPG). Cultivation was continued at 25°C for 5 to 6 h. Cells were harvested by centrifugation at 4.000 g for 10 min at 4°C and washed twice within KP_i-buffer (pH 7, 50 m*M*, 200 mg/L $MgCl_2$). Without any freeze-thawing step, the obtained cell pellet was adjusted with KP_i-buffer to 250 g_{wcw}/L (wcw = wet cell weight).

Determination of the enzyme activity (of the Baeyer–Villiger monooxygenase and alcohol dehydrogenase, respectively). Enzyme activity was measured (in analogy to standard protocols reported, e.g., in reference [17]) spectrophotometrically at 340 nm by consumption of NADPH through oxidation toward $NADP^+$ with a spectrophotometer V630 (JASCO Germany GmbH, Gross-Umstadt, Germany). Four hundred and forty microliters KP_i-buffer (pH 7, 50 m*M*, 200 mg/L $MgCl_2$) were mixed with 500 µL cyclohexanone (20 m*M* in KP_i-buffer) for CHMO activity determination or with 500 µL acetophenone (22 m*M* in KP_i-buffer) for ADH activity measurement. After performing a blank measurement, 50 µL $NADP^+$ (8 m*M* in KP_i-buffer) were added under rigorous mixing. The reaction was started by the addition of 10 µL enzyme solution under rigorous mixing. The slope (ΔE) of the observed linear range of minimal 60 s was used for activity calculation using the following equation:

$$A = (\Delta E \cdot V \cdot f)/(\varepsilon \cdot v \cdot d)$$

with the enzyme activity A (U/mL), the time depending change in extinction ΔE (1/min), the total volume V (mL), the dilution factor f, the extinction coefficient ε of NADPH at 340 nm [6.300 L/(mol·cm)], and the layer thickness d (cm).

Biotransformations with whole-cells in the presence of [10% (v/v)] of isooctane (according to Fig. 4). The experimental setup was identical to the biotransformations described previously with the exception of using lysed or intact (unfrozen) resting *E. coli* whole-cells over-expressing CHMO from *A. calcoaceticus* instead of commercially

supplied CHMO crude extract. For the determination of the CHMO activity of the whole-cells, first, an aliquot of the whole-cell catalyst was lysed. After cell lysis, the solution was centrifuged at 4.000 g for 10 min at 4°C. An enzyme activity measurement was spectrophotometrically performed with the crude extract of the supernatant as described previously. As the measured activity of free enzyme (U/mL) correlates with the engaged cell concentration (250 g_{wcw}/L), one can examine the volume of the cell suspension that is needed to achieve 3.82 U.

Acknowledgments. The authors thank the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) for generous support within the DBU-network "ChemBioTec" of the funding priority "Biotechnology" (Projects AZ 13234-32; AZ-13268-32).

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