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Direct biocatalytic one-pot-transformation of cyclohexanol with molecular oxygen into ε-caprolactone

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ABSTRACT

The development of a biocatalytic process concept for ε-caprolactone, which directly converts cyclohexanol as an easily available industrial raw material into the desired ε-caprolactone in a one-pot fashion while only requiring air as sole reagent, is reported. The desired product ε-caprolactone was obtained with 94–97% conversion when operating at a substrate concentration in the range of 20–60 mM. At higher substrate concentrations, however, a significant drop of conversion was found. Subsequent detailed studies on the impact of the starting material, intermediate and product components revealed a significant inhibition and partial deactivation of the BVMO by the product ε-caprolactone (in particular at higher concentrations) as well as an inhibition of the BVMO by cyclohexanol and cyclohexanone.

1. Introduction

The industrial chemical ε-caprolactone (Scheme 1) represents an important compound particularly used as a monomer for different polymer productions, *e.g.*, the manufacture of poly-ε-caprolactone in ring-opening polymerizations [1,2]. Industrial applications of ε-caprolactone can be found in the area of adhesives, colorants, coating materials and polyurethanes. The production capacity of ε-caprolactone is at an annual multi-10.000 tons scale. Industrially, ε-caprolactone is produced by means of the so-called UCC-process [2] starting from cyclohexanone, thus being an example for a classic chemical oxidation on large production scale. However, instead of molecular oxygen as an oxidation agent, stoichiometric amounts of peracetic acid (or alternatively highly concentrated H_2O_2 and acetic acid) are required in this large-scale process for the manufacture of ε-caprolactone. Besides the economical drawback (compared with air as a cheaper alternative) the reagent peracetic acid represents a chemical being problematic and challenging also from the perspective of toxicity, ecology and safety. In addition, the selectivity of this oxidation process does not exceed the range of 85–90% [2].

Due to these drawbacks of the existing industrial process and the option to use air in enzymatic oxidations, we became interested in developing a biocatalytic process for synthesizing the target molecule ε-caprolactone. In contrast to earlier work, which starts from cyclohexanone [3–10] and requires a further (organic) co-substrate for *in situ*-cofactor recycling besides molecular oxygen, however, we were interested in a direct oxidation process starting from cyclohexanol under the sole use of molecular oxygen (in form of air) as the oxidation reagent. As cyclohexanone, also cyclohexanol represents an industrial chemical readily available on large scale [2]. In the set up of such a process concept for ε-caprolactone we have been very much inspired by an early work of Willetts et al. [11] who demonstrated the suitability of such a combination of a Baeyer–Villiger monooxygenase (BVMO) and an alcohol dehydrogenase (ADH) using solely air as reagent for the one-pot transformation of the chiral alcohols *endo*-bicyclo[2.2.1]heptan-2-ol and *endo*-bicyclo[3.2.0]hept-2-en-6-ol stereoselectively into the corresponding lactones [11]. Since besides air no further co-substrate is required, this concept appeared to be particularly attractive for the synthesis of the bulk chemical ε-caprolactone in terms of both economy and sustainability. Accordingly, our envisaged

Abbreviations: ADH, alcohol dehydrogenase; BVMO, Baeyer–Villiger monooxygenase; UCC, Union Carbide Corporation; LK-ADH, alcohol dehydrogenase from *Lactobacillus kefir*.

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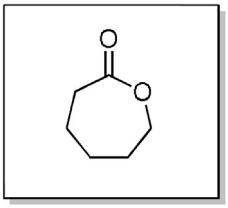


Scheme 1. Structure of ε-caprolactone.

process concept for ε-caprolactone consists of two combined biocatalytic oxidation steps conducted in the following manner (Scheme 2): starting from cyclohexanol as a substrate the first oxidation reaction is catalyzed by an ADH and furnishes cyclohexanone, while converting the (oxidized) cofactor NAD(P)$^+$ into NAD(P)H. Cyclohexanone as a key intermediate is then directly transformed *in situ* into the desired ε-caprolactone in a second oxidation reaction. This second reaction is catalyzed by a BVMO and requires oxygen as co-substrate and the (reduced) cofactor form NAD(P)H. Thus, coupling these two oxidation reactions in a one-pot process enables the direct transformation of cyclohexanol into ε-caprolactone without the need for additional (organic) co-substrates for *in situ*-cofactor regeneration (as required when conducting each of these oxidation reactions in a separate fashion). The only component besides air as oxidation agent is the substrate cyclohexanol as an easily available industrial raw material. In the following, we report our studies and results on such a biocatalytic process concept for ε-caprolactone.

2. Materials and methods

2.1. Synthesis of ε-caprolactone via biocatalytic double oxidation of cyclohexanol with air

In a 10 mL round-bottom flask 3.82 U of the Baeyer–Villiger monooxygenase from *Acinetobacter calcoaceticus* recombinantly overexpressed in *E. coli* (commercial preparation from Enzymicals AG; ECS-MO-1; 0.153 U/mg) [12] was dissolved in 5 mL of sodium phosphate buffer (pH 7.0, 50 mM). Subsequently 0.1–0.5 mmol of cyclohexanol, 1 mg of $MgCl_2$ and 40 U of an alcohol dehydrogenase from *Lactobacillus kefir* recombinantly overexpressed in *E. coli* (LK-ADH; crude cell extract, diluted with glycerol (1:1); 978 U/mL; activity determined using acetophenone as a reference substrate) [13,14] were added. The mixture was stirred for about 20 min at room temperature and then the reaction was started through addition of the cofactor NADP$^+$ (0.002 mmol). The reaction mixture was stirred for 24 h at room temperature in the closed round-bottom flask. Then, the reaction mixture with a volume of *ca.* 5 mL was separated in five parts of 1 mL, and each of them was transferred into an empty 2 mL-Eppendorf vial. After addition of 1 mL of methylene chloride to each of the aqueous phases in the vials, the aqueous phases were extracted by agitating the two-phase systems for 30 min in a thermomixer apparatus (apparatus: Eppendorf Thermomixer Comfort Type 5355; Eppendorf, Hamburg, Germany; 500 rpm, 25 °C). Subsequently, the supernatant (aqueous) phases were removed using a syringe with a long needle and transferred into five new, empty 2 mL-Eppendorf vials. Then, once again 1 mL of methylene chloride was added to each of the aqueous phases and a

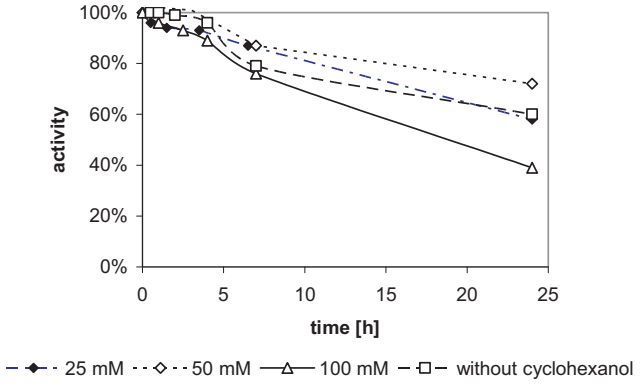
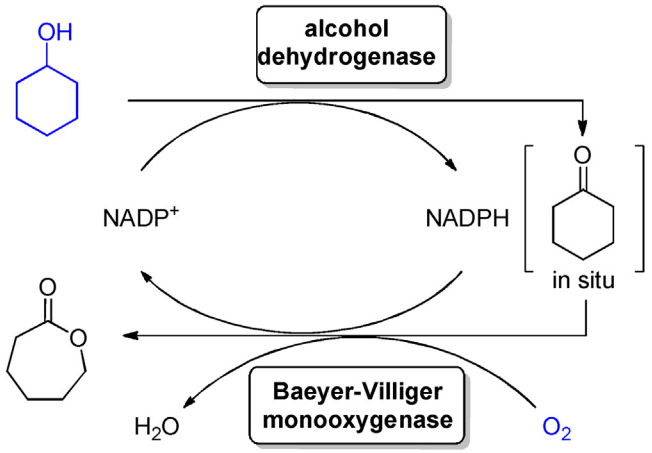


Fig. 1. Stability of the BVMO in the presence of cyclohexanol.



Scheme 2. Biocatalytic double oxidation of cyclohexanol with air as sole reagent as a synthetic approach towards ε-caprolactone (blue color: required substrate/reagent). (For interpretation of the references to color in this scheme caption, the reader is referred to the web version of the article.)

further extraction was carried out as described above. Subsequently, this extraction procedure was repeated a third time (with exception of separation the phases after agitation in a thermomixer apparatus). Finally, the 2 × 5 vials with organic phases resulting from the first and second extraction stage as well as the 5 vials containing both aqueous and organic phases resulting from the third extraction stages were centrifuged for about 30 min (13,000 rpm, 25 °C). The organic layers from all these vials (consisting of 15× *ca.* 1 mL) were combined by transferring them into a 25 mL round-bottom flask, and the solvent was carefully removed in vacuum (900 mbar). The subsequent determination of the absolute amount of the ε-caprolactone was carried out by ^{1}H NMR spectroscopic analysis of the crude product in combination with the obtained weigh-out quantity. The ^{1}H NMR spectra were recorded using a Bruker Avance 300 or Bruker Avance 400 NMR spectrometer.

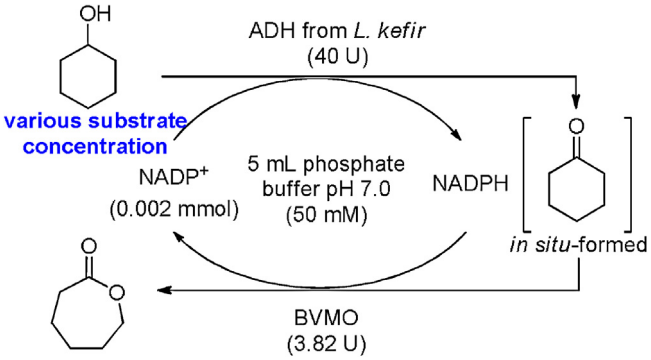
2.2. Spectrophotometric determination of the inhibition of the BVMO by cyclohexanone (substrate inhibition) and ε-caprolactone (product inhibition), respectively

The enzyme activity has been determined spectrophotometrically at a wavelength of 340 nm by measuring the consumption of NADPH through oxidation towards NADP$^+$ with cyclohexanone as a substrate. For studying the inhibition of cyclohexanone (substrate inhibition) and ε-caprolactone (product inhibition), respectively, these components were used at different concentrations. The molar extinction coefficient ε_{340} was 6.3 mM^{-1} cm^{-1}. At first in a 1 mL-cuvette sodium phosphate buffer (pH 7.0, 50 mM), a solution of cyclohexanone (300 mM) in sodium phosphate buffer (pH 7.0, 50 mM) and a solution of the Baeyer–Villiger monooxygenase (ECS-MO-1) in sodium phosphate buffer (pH 7.0, 50 mM) are mixed. For the study of the inhibition effect of cyclohexanone (substrate inhibition), the stock solution of cyclohexanone (300 mM) is added in such a volume, leading to a concentration of cyclohexanone in the range of 10 mM to 100 mM (see also Fig. 2). For the study of the inhibition effect of ε-caprolactone (product inhibition), the concentration of cyclohexanone is adjusted to 25 mM, and the stock solution of ε-caprolactone is added in such a volume, leading to a concentration of ε-caprolactone in the range of 25–400 mM (see also Fig. 4). After preparation of these solutions for the study of the substrate and product inhibition, respectively, subsequently after 5 min the reaction is started through addition of 50 μL of a solution of NADPH (8 mM) in sodium phosphate puffer (pH 7.0, 50 mM) and subsequently the consumption of NADPH is measured spectrophotometrically.

2.3. Spectrophotometric determination of the stability of the BVMO in the absence and presence of additives

The enzyme activity has been determined spectrophotometrically at a wavelength of 340 nm by measuring the consumption of NADPH through oxidation towards NADP$^+$ using cyclohexanone as a substrate and in the presence of an additive (ε-caprolactone, cyclohexanol or cyclohexanone). The molar extinction coefficient ε_{340} was 6.3 mM^{-1} cm^{-1}. At first in a 1 mL-cuvette 725 μL sodium phosphate buffer (pH 7.0, 50 mM), 125 μL of a solution of cyclohexanone (200 mM) in sodium phosphate buffer (pH 7.0, 50 mM) and 100 μL of a solution containing Baeyer–Villiger monooxygenase in sodium phosphate buffer (pH 7.0, 50 mM) and optionally cyclohexanol or cyclohexanone (25–100 mM) or ε-caprolactone (0.1–2.0 M) as an additive, which has been stirred before for a certain period of time (up to 24 h, see Figs. 1, 3 and 5, respectively), are mixed. Subsequently, after 5 min the reaction is started through addition of 50 μL of a solution of NADPH (8 mM) in

Table 1
Process development of the synthesis of ε-caprolactone *via* double oxidation (according to the process concept shown in Scheme 2).



Entry[a]	ADH [U]	BVMO [U]	*c* (substrate) [mM]	Conversion[b] [%]	Product conc. [g/L]
1	40	3.82	20	97 (99)[c]	2.20 (2.25)
2	40	3.82	40	97	4.41
3	40	3.82	60	94	6.45
4	40	3.82	80	36[d]	3.21
5	40	3.82	100	24[d]	2.72

[a] For detailed reaction conditions and the experimental protocol, see Section 2.
[b] The conversion is defined here as conversion related to the formation of the product ε-caprolactone.
[c] In parentheses the result of a repeating experiment is given.
[d] In this experiment also an additional accumulation of 2–3% of cyclohexanone was observed.

sodium phosphate buffer (pH 7.0, 50 mM) and subsequently the consumption of NADPH is measured spectrophotometrically.

3. Results and discussion

First, enzymes being capable to catalyze the two biotransformations required for this biocatalytic "double oxidation" one-pot process concept for ε-caprolactone (as outlined above in the introduction section and according to Scheme 2) were chosen for our synthetic work. For the initial oxidation of cyclohexanol to cyclohexanone a recombinant ADH from *Lactobacillus kefir* [13,14] (LK-ADH, as a crude extract) has been widely reported to have a broad substrate range comprising also cyclic molecules and turned out to be synthetically very suitable for the desired oxidation of cyclohexanol. For the transformation of cyclohexanone into ε-caprolactone we applied a commercially available BVMO from *Acinetobacter calcoaceticus* recombinantly overexpressed in *E. coli* (commercial preparation from Enzymicals AG; ECS-MO-1) due to its known capability to catalyze the Baeyer–Villiger oxidation of cyclohexanone [12]. When combining both biotransformations into a one-pot process according to the process set-up shown in Scheme 2, we found that already the initial synthetic experiments conducted at a cyclohexanol concentration of 20 mM proceeded successfully [15]. In the presence of air and without any other "external" co-substrate, the desired ε-caprolactone was directly formed with excellent conversion of 97% (Table 1, entry 1). Repeating this experiment gave 99% conversion, thus confirming the high conversion of the initial experiment. So, this biotransformation represents a proof of concept for the synthesis of ε-caprolactone by means of this biocatalytic one-pot process based on a double oxidation starting from cyclohexanol.

Based on these promising results, subsequently process development work has been started, and first the impact of an increased substrate concentration was studied. Notably, even at increased substrate concentrations of 40 and 60 mM of cyclohexanol, respectively, high conversions of 97% and 94% have been obtained after 24 h at room temperature (Table 1, entries 2 and 3). In addition, product formation reached 6.45 g/L when operating at a substrate concentration of 60 mM (Table 1, entry 3). However, further increasing the substrate concentration up to 100 mM of cyclohexanol led to a significant decrease of both conversion and formed amount of product. So, at a substrate concentration of 80 mM of cyclohexanol conversion dropped to 36%, and a further decreased conversion of only 24% was observed when operating with a substrate concentration of 100 mM of cyclohexanol (Table 1, entries 4–5). Thus, when conducting the biotransformation at 100 mM substrate concentration product formation was only 2.72 g/L, thus being less than half compared with the product formation when operating at a 60 mM substrate concentration (Table 1, entries 3 and 5).

In order to find an explanation for the observed significant drop of conversion when using higher substrate concentrations, we studied the impact of the substrate (cyclohexanol), intermediate (cyclohexanone) and product (ε-caprolactone) with respect to inhibition and stability of the BVMO. The BVMO was considered as the more critical enzyme component (compared to the LK-ADH) due to the known high stability of the LK-ADH in the presence of hydrophobic organic molecules at high concentrations [14]. Whereas for our stability experiments we studied the impact of all three organic components involved in the process (namely cyclohexanol, cyclohexanone and ε-caprolactone), in terms of the inhibition investigations we focused in particular on understanding the impact of ε-caprolactone since both the substrate cyclohexanol and intermediate cyclohexanone can be easily adjusted to low, less critical concentration levels by dosage, and suitable ratio of enzyme activities for the initial and second step.

First, as a benchmark and reference experiment the residual activity of the BVMO in the absence of any of the components mentioned above was measured. When stirring this enzyme in a phosphate buffer of pH 7 (50 mM) for 24 h at room temperature, a remaining activity of 60% was found (Fig. 1).

To start with the impact of the starting material cyclohexanol on the BVMO in terms of stability and residual activity, a remaining activity of below 40% was found after 24 h when treating the BVMO in buffer with cyclohexanol at a concentration of 100 mM (Fig. 1). In contrast, lower concentrations of cyclohexanol of 25 mM and 50 mM led to a remaining activity of the BVMO being in the same time range as observed in the absence of additives. Thus, in general a negative impact of cyclohexanol on enzyme stability could only be observed at an elevated cyclohexanol concentration of 100 mM.

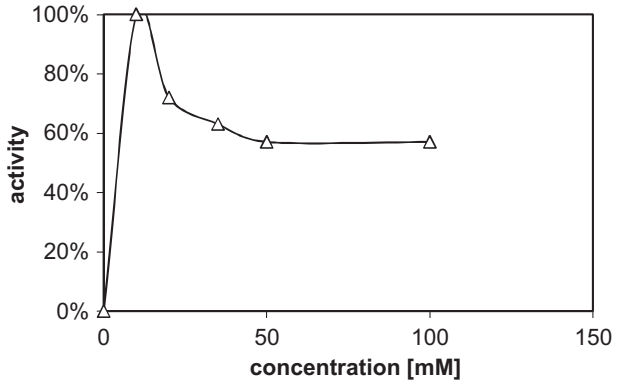


Fig. 2. Inhibition of the BVMO by the substrate cyclohexanone.

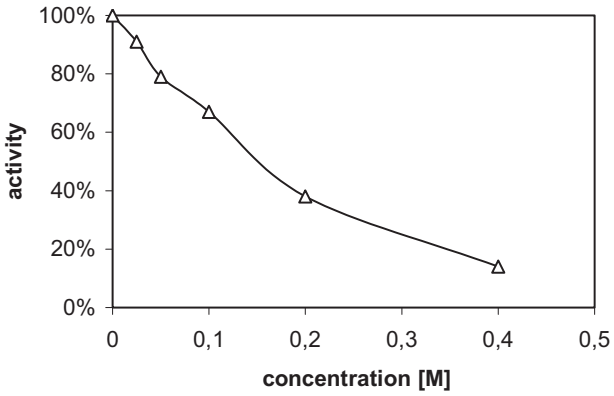


Fig. 4. Inhibition of the BVMO by ε-caprolactone.

This result is encouraging for future process development since cyclohexanol as a substrate can be easily feeded to the reaction mixture, thus keeping the substrate (cyclohexanol) concentration at sub-critical levels.

Next inhibition of the BVMO caused by cyclohexanone was studied. The highest activity of the BVMO was achieved at a substrate concentration of about 12 mM of cyclohexanone (Fig. 2). Beyond this concentration, decreased activities are observed corresponding to a significant substrate inhibition.

Our study on the impact of cyclohexanone in terms of stability of the BVMO revealed low activities of less than 40% after 24 h already in the presence of a cyclohexanone concentration of 50 mM (Fig. 3). At an increased cyclohexanone concentration of 100 mM the remaining activity of the BVMO further dropped to only 21%.

However, since cyclohexanone represents an intermediate, which is assumed to be present in the reaction medium only at low stationary concentrations (due to its subsequent *in situ*-oxidation to ε-caprolactone), the impact of this compound on the observed decreased conversion at higher substrate concentrations can be expected to be negligible. Furthermore, for future process development cyclohexanone concentration might be adjusted to a range of *ca.* 12 mM due to the highest enzyme activity observed at this concentration and the relatively low deactivation rate at this cyclohexanone concentration.

Subsequently, the influence of the product ε-caprolactone on both activity and stability of the BVMO was investigated in detail. With respect to our study on the inhibition of the BVMO by ε-caprolactone, at a concentration of 0.4 M of ε-caprolactone only a very low remaining activity is observed, thus indicating a strong product inhibition effect. Even at an ε-caprolactone (product) concentration of 0.1 M, already a significant inhibition effect with a remaining activity below 70% was observed. The corresponding results for the inhibition of the BVMO by ε-caprolactone are graphically summarized in Fig. 4. Thus, these experiments revealed both a significant inhibition and deactivation of the BVMO by ε-caprolactone (in particular at higher concentrations of ε-caprolactone) and this can be assumed to represent a further reason for the observed unsatisfactory conversion at elevated substrate concentrations (according to the results shown in Table 1).

Besides inhibition studies we evaluated the stability of the BVMO within a duration of 24 h at concentrations of ε-caprolactone of 0.1, 0.25, 0.5 and 1.0 M. The obtained results on the stability of the BVMO in dependency on the concentration of ε-caprolactone are summarized in Fig. 5. Accordingly, at a ε-caprolactone concentration of 0.1 M the remaining activity of the BVMO exceeds 50% after 24 h whereas almost a complete loss of activity after the same time was observed for the BVMO in the presence of ε-caprolactone at a concentration of 0.25 M. Notably, at higher concentrations of ε-caprolactone of 0.5 M and 1.0 M, no enzyme activity was observed already in the initial measurements. Thus, these results show that the presence of the desired product ε-caprolactone is critical in terms of both inhibition and stability of the BVMO, in particular at an elevated concentration of 0.25 M. For future process development work these results indicate the need to identify BVMOs being less inhibited and deactivated by ε-caprolactone as well as the need for an efficient *in situ*-product removal of ε-caprolactone.

Thus, in summary these results on inhibition and deactivation of the used BVMO through starting material, intermediate and product, respectively, indicate a significant inhibition as well as a partial deactivation of the BVMO at high (ε-caprolactone) product

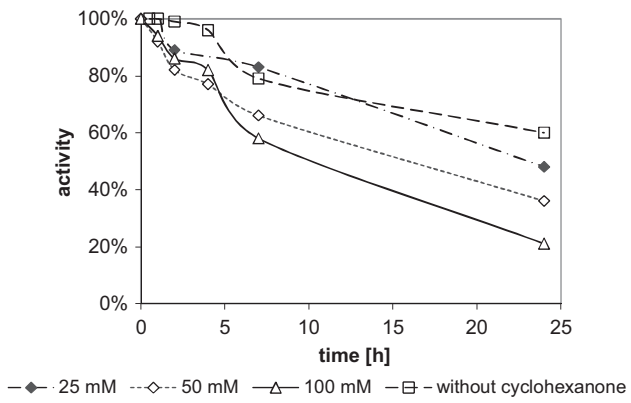


Fig. 3. Stability of the BVMO in the presence of cyclohexanone.

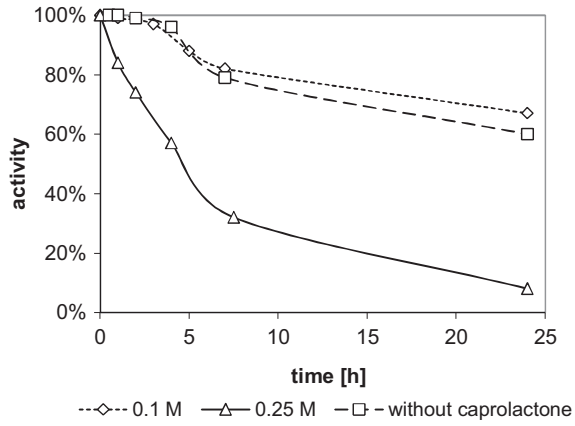


Fig. 5. Stability of the BVMO in the presence of ε-caprolactone.

concentrations as a pronounced effect. In addition, inhibition of the BVMO by cyclohexanol and cyclohexanone plays a role in this biotransformation process.

4. Conclusions

In conclusion, we reported the development of a biocatalytic process concept for ε-caprolactone, which directly converts cyclohexanol as an easily available industrial raw material into the desired ε-caprolactone in a one-pot fashion while only requiring air as sole reagent. The desired ε-caprolactone was obtained with 94–97% conversion when operating at a substrate concentration in the range of 20–60 mM. At higher substrate concentrations, however, a significant drop of conversion was found. Subsequent detailed studies on the impact of the starting material (cyclohexanol), intermediate (cyclohexanone) and product (ε-caprolactone) on the BVMO revealed a significant inhibition and partial deactivation of the BVMO by the product ε-caprolactone (in particular at elevated concentrations) as well as an inhibition of the BVMO by cyclohexanol and cyclohexanone. Thus, future work will address the issue of this observed undesired effect of a strong product inhibition and deactivation of the BVMO and as options to suppress this effect we will consider a protein engineering of the BVMO as well as process engineering concepts. Furthermore, optimization of the NADPH concentration and a detailed process development is also planned for the future.

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