Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Chapter 6
Modular biocatalytic cascades for the production of nylon-6 monomer from cyclohexanol

This chapter is based on the following planned publication:
6.1 Introduction

The synthesis of polymers (plastics) historically relies on oil as a feedstock.\(^1\) In recent years, the scientific community has been focusing on increasing the utilization of bio-based feedstocks and further lowering the necessary energy input by employing enzymes.\(^2\)\(^-\)\(^4\) Polyamides are a class of plastics that serves an important role in the everyday life, of which nylon-6 and nylon-6,6 are the most profound examples (Scheme 6.1). However, the environmental issues associated with their synthesis make them interesting targets for the development of biocatalytic synthetic processes. For example, biocatalytic syntheses of nylon-12\(^5\)\(^,\)\(^6\) and nylon-9\(^7\) monomers were reported. However, it is true that nylon-6 and nylon-6,6 comprise the majority of the polyamide market and therefore, it is logical that they attract the most attention.\(^8\)-\(^\)\(^13\) There is a significant amount of recent publications dealing with the available feedstocks and means of process optimization. Moreover, two important papers describing the biocatalytic synthesis of nylon-6 monomer have been published. In the first publication, the collaboration lead by DSM resulted in a study focused on fermentative production of nylon-6 monomer starting from glucose. In this work, they successfully demonstrated the possibilities arising from metabolic engineering.\(^14\) In another previous contribution, Kroutil’s group reported an inspiring cascade that enables the transformation of cyclohexanol (1) to 6-aminohexanoic acid (6) through a number of intermediates, namely cyclohexanone (2), caprolactone (3), 6-hydroxy-, 6-oxo- and 6-aminohexanoic acid methylesters.\(^15\) The process was initially intended for the use of unprotected carboxylic acids. However, they discovered an inhibition of the enzyme responsible for the oxidation of 6-hydroxyhexanoic acid (4) to 6-oxohexanoic acid (5), which was due to the presence of the carboxylic moiety. The final process design relied on two independent modules starting from cyclohexanone—the main feedstock for chemical
manufacture of caprolactam. Naturally, we were intrigued by the multi-enzymatic process and we attempted to improve its atom-economy by substituting the second module—that involved a primary alcohol dehydrogenase (prim-ADH), a ω-transaminase (ω-TA), a pyruvate dehydrogenase and an esterase—with an arguably more efficient “hydrogen-borrowing” system (i.e., more properly “hydride-borrowing”, H-B) that comprises a prim-ADH and an amine dehydrogenase (AmDH). The elegance of the H-B approach stems from the internal recycling of the redox equivalents as it was already described in the Chapter 2. Regarding the first module, the production of 3 or derivatives thereof has been vigorously investigated over the last decade. Several remarkable publications demonstrated the maturity of the system that consists of a secondary alcohol dehydrogenase (sec-ADH) and Bayer-Villiger monooxygenase (BVMO), which enable the oxidation of 1 to 2 and subsequent formation of 3 using molecular oxygen. The connection of the two modules is enabled by a hydrolase that catalyzes the transformation of 3 to 4 (Scheme 6.2).

Scheme 6.2. Two-module biocatalytic cascade for the synthesis of 6-aminohexanoic acid from cyclohexanol.
Upon studying the literature, we chose three enzymes that were reported to be active with the involved substrates: sec-ADH from *Lactobacillus brevis* (LbADH), BVMO acting on cyclohexanol from *Acinetobacter* species (AcCHMO), and monoterpane hydrolase acting on caprolactone from *Rhodococcus erythropolis* (ReLac). Moreover, Fraaije’s group has engineered an interesting fusion enzyme consisting of TbADH and TmCHMO (TbTm-Fus) that were genetically linked together. TbTm-Fus exhibited TON values as high as 20000. Fraaije’s group kindly provided us with the plasmid harboring the gene fusion. However, at the time of this study, there were no reports about any prim-ADH and amine dehydrogenase that would catalyze the oxidation of 4 as well as the reductive amination of 5, respectively. Therefore, those were the challenges of our investigation.

### 6.2 Results and discussion

#### 6.2.1 Module 1 and related experiments

The previous research into the combination of ADHs and BVMOs provided insight in the proper choice of the initial biocatalysts. Initial activity testing revealed sufficient activities for both enzymes, after their expression and purification. Initially, we investigated the influence of increasing the concentration of either LbADH or AcCHMO on the final conversion. However, we always obtained ca. 60% conversion when using an enzyme loading between 20 and 40 μM (Figure 6.1a and 6.1b). Furthermore, we tested the performance of the ReLac. The experiment revealed that the hydrolysis of 3 is also largely spontaneous (Figure 6.1c). In conclusion, we decided not to test the behavior of the Module 1 further. Conversely, we decided to compare the system comprising LbADH, AcCHMO and ReLac with the system comprising TbTm-Fus and ReLac. Interestingly, in our hands, the discrete LbADH/AcCHMO and fused TbTm systems exhibited a similar performance at 1 mM loading of the NADP⁺. However, the difference of the performance became evident when 0.2 mM NADP⁺ was used (Figure 6.1d). We attribute the observation to the fusion system being possibly more susceptible to inhibition by the substrate and/or products. The investigation into
ammonium citrate as a reaction buffer was prompted by the observation described in section 6.2.3.

### 6.2.2 Analytical problems related to the cascade

The initial experiments involving the investigation of Module 1 were analyzed using GC-FID. The reaction mixture was acidified to a pH of ca. 4 using 3 M HCl and subsequently extracted with EtOAC containing 40 mM toluene as an internal standard. The protocol enabled almost quantitative extraction of 1 and 2. However, extraction of 3, 4 and 5 was problematic. Moreover, we faced a problem of extraction and quantification of 6.
In general, we were not able to devise a GC-based analytical protocol that would work in a satisfactory extent. The analytical approach used by Sattler and colleagues\textsuperscript{15} was based on previously published method for simultaneous methylation of amino and carboxylic acid moieties using ethyl chloroformate and 4-dimethylaminopyridine in methanol. Indeed, we were able to reproduce the derivatization reaction, but the detected amounts of methylated products were only useful for qualitative analysis. More specifically, we have encountered substantial loss of target compounds during the sample preparation. We have tried several work-flow set-ups in the lab, albeit without any success. Therefore, we decided to try reverse-phase (RP) HPLC to circumvent the derivatization step. Moreover, we had to couple the RP-HPLC system with a refractive index detector (RI) due to the lack of any chromophore in the compounds of Module 2 (and the overall cascade in general). Accordingly, we had to use an isocratic elution since RI detection works on the basis of the difference of refraction between the sample and a reference cell that contains the mobile phase. A suitable column for this task was a RP column from Shimadzu (Shimpack GIST C18-AQ). Notably, this particular column was the only one at our disposal that was able to retain compound 6. Therefore, this allowed us to develop an analytical method that secured the retention of all of the Module 2 components with sufficient resolution. The mobile phase allowing us the separation consisted of degassed and filtered MiliQ water with 4% MeOH and 0.1% trifluoroacetic acid, at 1 ml min\textsuperscript{-1} flow and at 40 °C. Moreover, we could quantify the concentration of 4, 5 and 6 using calibration curves. Furthermore, we could separate and quantify all of the six components of the cascade (Figure 6.2).
6.2.3 Finding suitable catalysts for module 2

Since there were no suitable catalysts reported in the literature for H-B alcohol amination, we investigated two possible targets, namely prim-ADH from *Geobacillus stearothermophilus* (ht-BsADH)\(^{26}\) and the ε-lysine dehydrogenase (LysEDH) from the same source.\(^ {27}\) Initial tests of the activity of the enzymes with reference substrates confirmed that we indeed had active catalysts in our hands. For the enhancement of the desired substrate scope (work done in collaboration with Dr. M. F. Masman), we evaluated the possibility of engineering the ht-BsADH and LysEDH. The crystal structure of ht-BsADH has been known since 2004. However, available PDB entries did not contain the cofactor, which was critical in order to identify residues in contact with the substrate during the catalytic cycle. However, surprisingly, we observed the formation of 5 when we performed the oxidation of 4 using ht-BsADH together with nicotinamide adenine dinucleotide oxidase from *Streptococcus mutans* (SmNOx)\(^ {28}\) for cofactor regeneration. Next, we focused on LysEDH. Unfortunately, the crystal structure of the enzyme was not available and initial crystallization efforts were unsuccessful (note: crystallization was successful later on during my PhD research and it was reported in Chapter 5). Therefore, we constructed a homology model of LysEDH containing the cofactor. As mentioned above also, the generation of the homology model and its validation by X-ray crystallography is described in Chapter 5. In general, we identified the following putative α-amino acid binding residues: H181, Y238 and T240 (Figure 6.3) using the molecular docking of L-lysine into the active site of the

![Figure 6.2. RI-chromatogram: distribution of analytes using RP-HPLC.](image-url)
enzyme. We argued that altering these polar residues should result in better accommodation of substrate 5 in the active site, since the difference between ligand 6 and L-lysine lies in the additional α-amino moiety of the latter only (Figure 6.3). It is noteworthy that 5 is not commercially available. Therefore, Ms. M. L. Corrado synthesized the compound during this work. Notably, the wild-type LysEDH already accepted substrate 5. Nonetheless, we were intrigued whether we could identify a better-performing variant. Therefore, a number of variants were created by performing an alanine-scan.

Figure 6.3. Binding of the α-amino moiety of L-lysine inside the active site of LysEDH_WT. 6-aminohexanoic acid is depicted in blue, the C-N bond of α-amino moiety of L-lysine is shown in magenta and LysEDH_WT is shown in grey. Black lines depict the hydrogen bonding of the α-amino moiety of L-lysine to the residues of the active site: H181, Y238 and T240.
6.2.4 Module 2 and related experiments

The first step in the study of Module 2 was to identify whether ht-BsADH tolerates an ammonium-containing buffer. We chose five different buffer systems: ammonium formate, ammonium acetate, ammonium citrate, ammonium phosphate, and ammonium chloride together with K-phosphate buffer as a reference system. Initially, we wanted to run the reactions at 0.5 M concentration of ammonium species. However, we figured out that 100 mM 4 inside 1 M stock solution of the buffer significantly changes the pH from 8.6 to ca. pH 7.3. Therefore, we had to run the reactions at 1 M concentration of ammonium species. Interestingly, we also discovered that the oxidation proceeds at very slow rate in buffers containing formate and acetate ions. We speculate that these small organic acids can cause the inhibition of ht-BsADH in a same way as was reported for 4. On the other hand, the highest conversion was observed in ammonium citrate buffer (Figure 6.4) and it was notably comparable with the

![Figure 6.4. Catalytic performance of ht-BsADH in different ammonium containing buffers.](image)
conversion obtained in K-phosphate buffer. Therefore, we successfully found an ammonium-containing buffer system that can support ht-BsADH.

The second step in the study of Module 2 was the identification of the best performing alanine scan variants. Therefore, we decided to compare the catalytic activity as well as the thermostability of the individual variants, and clear trends were observed. Compared with the wild type enzyme, the presence of the mutation T240A led to higher conversion while the H181A mutation did not. On the contrary, the mutation Y238A resulted in significantly lower performance. Interestingly, the presence of T240A could not counteract the negative effect of Y238A in variants containing multiple mutations. Therefore, variants containing the Y238A mutation were not considered any further (Figure 6.5). Next, we performed the analysis of the melting point. Our results confirmed the importance of the Y238 residue for the stability and activity of the enzyme, since the presence of Y238A mutation resulted again in a negative result. More specifically, we observed diminished thermostability in variants containing single mutation Y238A and double mutation Y238A_T240A. Interestingly, we observed that the mutation
H181A increases the thermostability of the LysEDH. Moreover, unlike in the case of the catalytic performance, the presence of the beneficial mutation H181A could counteract the negative effect of Y238A. Therefore, the assumption that we can disregard variants containing Y238A mutation from further experiments was validated.

Before performing the detailed investigation on the four remaining variants, we decided to test whether we can combine the ht-BsADH and LysEDH_WT into the functioning

<table>
<thead>
<tr>
<th>Entry</th>
<th>ht-BsADH [μM]</th>
<th>LysEDH_T240A [μM]</th>
<th>SmNOX [μM]</th>
<th>NAD⁺ [mM]</th>
<th>Type of substrate [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>90</td>
<td>-</td>
<td>1</td>
<td>50 (4)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>90</td>
<td>-</td>
<td>5</td>
<td>50 (4)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>90</td>
<td>-</td>
<td>1</td>
<td>10 (4)</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>90</td>
<td>-</td>
<td>1</td>
<td>50 (6)</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>1</td>
<td>50 (4)</td>
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<tr>
<td>6</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>5</td>
<td>50 (4)</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>-</td>
<td>10</td>
<td>1</td>
<td>50 (4)</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>1</td>
<td>50 (6)</td>
</tr>
</tbody>
</table>

Table 6.1. List of conditions investigated in troubleshooting of Module 2.

Figure 6.6. Troubleshooting of module 2: compound distribution. Entry indicates the experimental conditions illustrated in Table 6.1. Due to the reversibility of the H-B reaction, substrate was either 6-hydroxyhexanoic acid (4) or 6-aminohexanoic acid (6).
Module 2. However, we could not observe any formation of 5 from 4, let alone 6. Therefore, we decided to investigate different reaction set-ups in order to elucidate the problem (Table 6.1, Figure 6.6).

Our reactions were usually performed using 10 μM of ht-BsADH, 90 μM of LysEDH_variant, 50 mM 4 as a substrate and 1 mM NAD⁺. We decided to investigate different loading of individual components. However, from all of the investigated conditions, we could detect 6 only in two instances. More specifically, the increased cofactor concentration (5 mM NAD⁺) resulted in 0.4% of 6 whereas the decreased concentration of substrate

<table>
<thead>
<tr>
<th>Entry</th>
<th>ht-BsADH [μM]</th>
<th>LysEDH_H181A [μM]</th>
<th>NADH [μM]</th>
<th>Substrate 5 [mM]</th>
</tr>
</thead>
<tbody>
<tr>
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<td>50</td>
<td>50</td>
<td>5</td>
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</tr>
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<td>50</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 6.7. Preferred reaction pathway: reduction of 5 to 4 vs. reductive amination of 5 to 6 – compound distribution. Entry relates to the reaction conditions illustrated in Table 6.2.
(4; 10 mM) resulted in 0.7% of 6. These particular results suggested us that the problem might have been an insufficient formation of 5 (thus, indicating an issue with ht-BsADH) or the poor formation of 6 caused by insufficient amount of 5 (thus, indicating and issue with LysEDH_variant). To better understand the origin of these limitations, we carried out a set of reactions starting from 5, wherein the NADH (1 or 5 mM) would be supplied instead of NAD\(^+\) thereby resulting in two possible reaction outcomes: i) reduction of the 5 to 4 (reduction of an aldehyde to the alcohol) or ii) reductive amination of 5 to 6 (conversion from the aldehyde to amine). Notably, it appears that when 50 mM substrate is used, the outcome of the reaction is dependent on the ADH-AmDH ratio. Unless there was significantly less ADH than AmDH (1:9 ratio to be precise), reduction of aldehyde to alcohol was preferred over reductive amination (Table 6.2, Figure 6.7). Moreover, we observed a few discrepancies that are difficult to explain: the amount of the product did not correspond exactly to the available cofactor. The total concentration of the components was lower than the concentration of substrate that was added to the reaction. The discrepancy might be explained by the known higher reactivity of aldehydes compared with ketones. The aldehyde moiety of 5 can interact with the amino groups of the proteins in solution in an unspecific manner, thus lowering the actual concentration of aldehyde substrate that is available for the reaction and detectable by HPLC analysis. Similar situations were observed in other research by our group such as alcohols amination in vivo catalyzed by ADH and AmDH, and aldehydes oxidation catalyzed by aldehyde dehydrogenases.\(^{29,30}\) We can exclude evaporation of the compounds during the reaction due to their physicochemical properties. It is noteworthy that the discrepancy related to the apparent reaction mass balance was not observed when the compounds 4 or 6 were involved as substrates or used in blank reactions. In general, we realized that the initial design of Module 2 is attractive but still very challenging at the moment.
The problems with the performance of the hydrogen-borrowing and alcohol-aminating Module 2 prompted us to think about a viable alternative. In Chapter 1, the class of alcohol oxidases has been introduced. These enzymes facilitate the oxidation of alcohols to the corresponding carbonyl compounds while utilizing molecular oxygen as a final electron acceptor. The option of utilizing an alcohol oxidase in the “cascade to nylon” has been previously considered by us. However, none of the available alcohol oxidases met our needs.

Figure 6.8. Comparison of the best ε-LysDH variants in combination with AcCO6.
oxidases could catalyze the oxidation of 4 to 5. Moreover, to the best of our knowledge, there were no reports about such an oxidase either. In 2019, Turner's group engineered a primary alcohol oxidase (based on the choline oxidase from *Arthrobacter chlorophenolicus*)\textsuperscript{31} that exhibits an exceptionally broad substrate scope. The authors also reported that 4 is accepted by the final variant (AcCO6) albeit with a moderate specific activity. Nevertheless, this discovery gave us the possibility to re-design Module 2 (Scheme 6.3). The oxidation would then be catalyzed by the AcCO6 and the reductive amination would be performed by the LysEDH\_variant. Since the new design does not foresee an internal recycling of the NAD coenzyme, we decided to utilize the formate dehydrogenase from *Candida boindii* (CbFDH)\textsuperscript{32} in the same manner as described in Chapter 3. Since the hydrogen peroxide—formed as co-product by the AcCO6—is known to be detrimental for the stability of the enzymes, we decided to add a catalase to enable its disproportionation. We successfully expressed AcCO6 in *E. coli*, purified it using metal-affinity chromatography, and we subsequently tested the performance of re-designed Module 2. Thus, AcCO6 was combined with the four best variants of LysEDH (Figure 6.8). The experiment revealed that the presence of catalase boosted the conversion of 4 to 5 by more than 10%. Moreover, we noticed that LysEDH\_T240 was the most active from all of the alanine scan variants. Notably, LysEDH\_WT performed almost as well as LysEDH\_T240 in this particular experiment. This was the first time that we could run Module 2 affording a significant analytical yield (43.7%), thus equal to 21.8 mM of product titer.
6.2.6 Combination of Modules 1 and 2

Upon successful alteration of the second module, we attempted to combine Module 1 with Module 2 concurrently in one pot (Scheme 6.4). Initially, we turned our attention to the LysEDH variants. We performed reactions containing all of the reaction components at the following concentrations: 15 μM TbTm-Fus, 10 μM Relac, 25 μM AcCO6, 50 μM LysEDH_WT or LysEDH_T240A, 10 μM CbFDH, 1.66 μM catalase, 1 mM NAD+, 1 mM NADP+ and 25 or 50 mM substrate in 1 M ammonium formate buffer pH 9. In the initial experiments, TbTm-Fus was used for Module 1 even though we previously described that the discrete Lb-ADH and AcCHMO better tolerates ammonium species in the reaction. The test was also accomplished to obtain data on the possible utilization of fusion enzymes in future. Moreover, the performance of TbTmFus at 1 mM concentration of NADP+ was comparable to the separate enzymes. In relation to the amine dehydrogenase used, the results revealed a slightly better performance of LysEDH_T240A in terms of analytical (Figure 6.9a). Nevertheless, we
have decided to continue further with LysEDH_T240A, in order to maximize the analytical yield. Furthermore, we wanted to assure that the initial assessment of the performance of Module 1 was correct. Therefore, we decided to compare the performance of discrete ADH-BVMO and TbTm fusion of Module 1 again. Therefore, we performed new experiments, this time with the 50 μM LysEDH_T240A in all conditions combined with 20 μM LbADH and 20 μM AcCHMO or 20 μM Tb-Tmfus combined with 10 μM ReLac. Moreover, we have investigated the performance at two different substrate concentration (25 or 50 mM 1) in order to get better insight. The results revealed quite a large difference when using the TbTm-Fus or the discrete LbADH and AcCHMO. More specifically, there was no substrate 1 left at 25 mM substrate concentration when discrete LbADH and AcCHMO were used. At 50 mM substrate 1 concentration, the cascade afforded 34% yield when TbTm-Fus was used, and 67% yield when discrete LbADH and AcCHMO were used (Figure 6.9b). In summary, since the used of discrete ADH and BVMO in Module 1 still exhibited the higher efficiency in the cascade from 1 (Figure 6.10), we decided to continue with these enzymes.
Next, we tried to optimize the performance of the whole system by testing the pH dependency. The results of the experiment performed at 50 mM concentration of 1 revealed that the Module 1 works well as the conversion reached above 60% throughout the whole range of tested pH. However, we have observed accumulation of 4 along with poor analytical yield of 6 (1.5–2.7%) (Figure 6.11). We attributed this fact to the possible inhibition of AcCO$_6$ effected by the substrate or Module 1’s intermediates. Therefore, we investigated that by running the Module 2 independently at 25 mM concentration of 4 in presence of 25 mM of either 1, or 2 or 3. The set-up was supposed to mimic the situation when the 50% of the substrate has been already converted to 4. Under such conditions, we observed lower conversion for Module 2 in presence of 25 mM 2. However, this does not really explain the accumulation of 4 in the overall cascade because 2 cannot exceed 1 mM concentration as it always relates...
to the NADP⁺/NADPH concentration applied in Module 1. Therefore, we started to look for a different solution.

Figure 6.11. Compound distribution observed during the investigation into the pH-dependency of the full cascade.
6.2.7 Optimization of Module 2 using the system consisting of AcCO6 and Ch1-AmDH

To better understand the behavior of AcCO6, we decided to use an alternative AmDH. In particular, we decided to use a combination of AcCO6 and Ch1-AmDH rather than with LysEDH (Scheme 6.5). The idea was to investigate the optimum pH, temperature and substrate loading, since these parameters were not extensively investigated in the original publication. 1-Hexanol (7a) was used as substrate and resulting hexanal (7b) is readily transformed into 1-aminohexane (7c) by Ch1-AmDH.

Additionally, we decided to adjust the concentration of AcCO6 because its specific activity with 7a was reported to be 571 mU mg⁻¹, which is almost 100-fold higher than the specific activity reported for 4 (6.8 mU mg⁻¹).³¹ According to the observed pH optimum (Figure 6.12a), the system performs better at more alkaline pH at which conversion of 7a and analytical yield of 7c rose. Furthermore, testing of the optimal substrate concentration revealed that the 5 μM AcCO6, 50 μM Ch1-AmDH, 11 μM CbFDH and 0.17 μM catalase were sufficient to provide 99% analytical yield at 20 mM loading, 89% analytical yield at 40 mM loading and 61% analytical yield at 60 mM loading (Figure 6.12b). Remarkably, lowering the temperature from 30 °C to 20 °C resulted in 10% increase of the conversion (62%) at 60 mM loading of 7a (Figure 6.12c).

Moreover, we have never detected the 7b intermediate. Therefore, we assume that the amount of AcCO6 could have been the limiting factor at this stage. If this is the case, the increase of the concentration of AcCO6 in the reaction mixture will result in elevated
conversion levels. Additionally, the broad substrate scope of AcCO6 suggests its combination with AmDHs as a viable option for the further development of biocatalytic cascades. The initial exploitation of this interesting enzyme was already reported by Turner's group,\textsuperscript{33} and described in Chapter 1 of this thesis.

Figure 6.12. Investigation of reaction parameters of AcCO6 using an alternative AmDH by varying: a) pH; b) substrate loading and c) temperature.
6.2.8 Different reactions set-ups

Finally, we decided to investigate three possible set-ups of the whole system, namely: 

i) one-pot approach;  

ii) one-pot fed-batch approach wherein substrate aliquots would be added after a certain time; and  

iii) one-pot two-step approach that would rely on temporal separation of Modules 1 and 2. The one-pot cascade has been previously performed. However, we performed the experiment this time with either lower loading of substrate (30 mM compared with 50 mM) or higher loading of AcCO6 (57 μM compared to 25 μM). We observed no accumulation of 4 at 30 mM concentration of 1, and increased performance (in terms of product titer as well as depletion of the substrate) of the whole system with increased loading of AcCO6 (Figure 6.13). The fed-batch set-up was investigated by the step-wise addition of 10 mM aliquot of 1 every two hours until reaching the maximum of 40 mM (reaction times 2–10 h). It appears that the first module might be less efficient. We argue this based on the lack of further

Figure 6.13. Compound distribution observed during the investigation into the single batch setup with varying concentration of AcCO6 and substrate loading.
depletion (or a very slow one) of starting material after addition of the second aliquot of substrate. However, since the concentration of the observed intermediate remained more or less constant and it was accompanied by a slow build-up of product, we argued that Module 2 maintained (at least partly) its activity (Figure 6.14).

The temporal separation of Modules 1 and 2 resulted in the highest analytical yield of 6 that we have observed up to that point (Figure 6.15). The temporal separation enabled to circumvent the accumulation of 4. Interestingly, 1 was depleted only to 50% level, regardless of the initial substrate concentration. This observation is particularly interesting since it is consistent with the other experiments that were performed in this series. The inability of the Module 1 enzymes to enable quantitative conversion at 30 or 50 mM substrate loading is indeed a problem and, together with the observed lowering of the activity after 2 hours of reaction time, it constitutes a significant drawback. We can attribute this behavior to some inhibitory effect from the substrate or intermediates, and/or a diminished stability under the required reaction conditions. Luckily, Module 2 operated well, albeit its efficiency can and must be improved. The
current major limitation is the low overall reaction rate, since the specific activity of AcCO6 was reported to be only 6.8 mU mg\(^{-1}\). Furthermore, we have never achieved full conversion using LysEDH variants. Overall, it appears that the biocatalytic cascade affording 6-aminohexanoic acid works as proof-of-principle although not at the level of efficiency that is mandatory for the synthesis of a bulk chemical. However, the results presented in this section show several alternatives, thereby representing a groundwork for development of a synthetically applicable biocatalytic process.
6.3 Conclusion

The initially envisioned one-pot modular multi-enzymatic cascade for the synthesis of linear nylon-6 monomer (i.e., 6-aminohexanoic acid) proved to be very challenging to realize. However, we have re-designed the second module by substituting the initially employed alcohol dehydrogenase (ht-BsADH) with an alcohol oxidase (AcCO6). The substitution was prompted by the inability of ht-BsADH to oxidize the 6-hydroxyhexanoic acid at sufficient extent when combined with AmDH in hydride-borrowing fashion. During the development of the system, we investigated two different possibilities of running the module 1 (singular fused biocatalyst TbTm-fus vs. the discrete LbADH and AcCHMO). Moreover, we performed an investigation into a LysEDH from G. stearothermophilus. The small library of variants lead to minimal improvement of performance, but we could observe the critical importance of the Y238 residue in the active site.

The subsequent combination of modules 1 and 2 yielded further insights into the limitations associated with combining multiple enzymes into single process such as pH optima, stability, substrate- and intermediate-based inhibition phenomena, insufficient activity, etc. In the end, we circumvented the compatibility issues by the temporal separation of modules 1 and 2. This suggests that the enzymes currently used in the system might be more suitable for a flow-based application using immobilized enzymes. To conclude, the system constitutes an interesting addition to the family of long linear biocatalytic cascades and suggests a route towards more sustainable synthesis of polyamides.
6.4 Experimental

6.4.1 Expression and purification of enzymes

6.4.1.1 General protocol for expression in pET-based plasmids

For recombinant expression of the target proteins, overnight culture was prepared by inoculation of 20 mL of LB-media containing kanamycin (50 μg ml\(^{-1}\)) with a glycerol stock of *E. coli* containing plasmid with target protein and the overnight culture was incubated overnight at 37 °C, 170 rpm. Subsequently, main culture was prepared as follows. 800 ml of LB medium with kanamycin was inoculated with 15 ml of an overnight culture of *E. coli* cells harboring the plasmid with the gene of interest. The main culture was grown at 37 °C until an OD\(_{600}\) (optical density measured at 600 nm wavelength) reached value of 0.6–0.9. At that point, the expression was induced by the addition of IPTG (0.5 mM final concentration). Expression was carried out overnight and after harvesting of the cells (4 °C, 3400 g, 15 min), the cell pellet was washed with 0.9% NaCl solution, centrifuged at the same conditions and frozen at -20°C. Expression levels were checked by SDS-PAGE of the pre- and after-incubation samples. Obtained cells were used for purification of the protein with affinity chromatography.

6.4.1.2 Expression of TbTm-fus

For recombinant expression of TbTm-fus, pBAD plasmid containing the gene (which was kindly given by prof. Marco Fraaije) was cloned into BL21 DE3 expression strain. For the expression, 800 ml of LB medium with ampicillin (75 μg ml\(^{-1}\)) was inoculated with 15 ml of an overnight culture (prepared as described in 6.4.1.1) of *E. coli* cells harboring pBAD with TbTm-fus. Expression was started immediately by the addition of 0.02% arabinose (sterilized by filtration). Culture was grown at 25 °C for 7 h and, after harvesting of the cells (4 °C, 3400 g, 15 min), the cell pellet was washed with 0.9% NaCl solution, centrifuged at the same conditions and frozen at -20°C. Expression level was checked by SDS-PAGE of the pre- and after-incubation samples.
6.4.1.3 General protocol for purification using His-tag system

Frozen cell pellets containing overexpressed protein were thawed and re-suspended in lysis buffer (50 mM KH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8). The suspension was subsequently sonicated for 15 minutes (10 s on, 15 s off, 45% amp) until the viscosity returned back to normal. Sonicated sample was then centrifuged for 1 h at approx. 35000 g, 4°C. Supernatant was collected, filtrated through 0.45 μm filter (Whatman) and loaded onto HisTrapHP column (GE Healthcare) charged with Ni$^{2+}$. After loading of the lysate, column was washed with washing buffer (50 mM KH$_2$PO$_4$, 300 mM NaCl, 25 mM imidazole, pH 8) and the protein was eluted with elution buffer (50 mM KH$_2$PO$_4$, 300 mM NaCl, 300 mM imidazole, pH 8). Elution fractions containing protein were pooled and dialyzed overnight at 4°C against a 50 mM KPi buffer. The following day, the enzyme was concentrated using Vivaspin (Milipore); the concentration was determined by measuring either the absorbance at 280 nm considering the molar extinction coefficient of the target protein, or the FAD absorption around 440 nm. Subsequently, the enzyme was flash frozen in liquid nitrogen and stored at -80°C. Purity of the proteins was checked by the SDS-PAGE with 7 μg of the protein loaded into each lane.

6.4.1.4 General protocol for purification using GST-tag system

Frozen cell pellets containing overexpressed protein were thawed, re-suspended in GST-binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). The suspension was subsequently sonicated for 15 minutes (10 s on, 15 s off, 45% amp) until the viscosity returned back to normal. Sonicated sample was then centrifuged for 1 h at approx. 35000 g, 4°C. Supernatant was collected, filtrated through 0.45 μm filter (Whatman) and loaded onto GST-trap column (GE Healthcare). After loading of the lysate, column was washed with GST-binding buffer and the protein was eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8). Elution fractions containing protein were pooled and dialyzed overnight at 4°C against 50 mM Tris-HCl buffer. The following day, the enzyme was concentrated using Vivaspin (Milipore); the concentration was determined by measuring either the absorbance at 280 nm considering the molar extinction coefficient of the target protein, or the FAD
absorption around 440 nm. Subsequently, the enzyme was flash frozen in liquid nitrogen and stored at -80 °C. Purity of the proteins was checked by the SDS-PAGE with 7 μg of the protein loaded into each lane.

### 6.4.1.5 Overview of purified proteins

Table 6.3 includes an overview of proteins used in the experiments.

Table 6.3. Overview of purified proteins, Exp. T., expression temperature; Pur. Add., Purification additive; n. a., not applicable.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vector</th>
<th>Exp. T [°C]</th>
<th>IPTG [mM]</th>
<th>Tag</th>
<th>Pur. Add. [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LbADH</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>Gst</td>
<td>Mg²⁺ (1)</td>
</tr>
<tr>
<td>AcCHMO</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>Gst</td>
<td>FAD (0.1)</td>
</tr>
<tr>
<td>TbTm-fus</td>
<td>pBAD</td>
<td>37</td>
<td>n. a.</td>
<td>His</td>
<td>FAD (0.1)</td>
</tr>
<tr>
<td>ReLac</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>ht-BsADH</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_WT</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_H181A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_Y238A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_T240A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_Y238A, T240A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_H181A, Y238A, T240A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>LysEDH_H181A, T240A</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>AcCO6</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>FAD (0.1)</td>
</tr>
<tr>
<td>CbFDH</td>
<td>pET-28b</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>-</td>
</tr>
<tr>
<td>SmNOX</td>
<td>pET-21a</td>
<td>25</td>
<td>0.5</td>
<td>His</td>
<td>FAD (0.1)</td>
</tr>
</tbody>
</table>

### 6.4.2 Optimization reactions involving module 1

#### 6.4.2.1 Amount of LbADH

Investigation of the influence of increasing LbADH concentration was performed as follows. Reaction mixture contained 1 M ammonium formate pH 8.7 (2 M stock solution) as a buffer of choice, 1 mM NADP⁺ (10 mM stock in water), 20–40 μM LbADH, 20 μM AcCHMO, 50 mM cyclohexanol as a substrate in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of substrate and incubated at
30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 120 μl of 3 N HCl. Extraction was performed using EtOAc with 40 mM toluene as internal standard twice (300 and 350 μl). Combined organic phases were dried over MgSO₄ and analyzed on GC-FID according to the section 6.4.8 and the results are listed in Table 6.4.

Table 6.4. Influence of LbADH loading on conversion of 1 and 2 to 3 and 4 in ammonium formate buffer (1 M, pH 8.7) with LbADH (20–40 μM), AcCHMO (20 μM), NADP⁺ (1 mM), 1 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>LbADH [mM]</th>
<th>Conversion [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>30</td>
<td>67</td>
</tr>
<tr>
<td>40</td>
<td>71</td>
</tr>
<tr>
<td>50</td>
<td>67</td>
</tr>
</tbody>
</table>

6.4.2.2 Amount of AcCHMO

Investigation on the influence of increasing AcCHMO concentration was performed as follows. Reaction mixture contained 1 M ammonium formate pH 8.7 (2 M stock solution) as a buffer of choice, 1 mM NADP⁺ (10 mM stock in water), 20 μM LbADH, 20–40 μM AcCHMO, 50 mM cyclohexanol as a substrate in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 120 μl of 3 N HCl. Extraction was performed using EtOAc with 40 mM toluene as internal standard twice (300 and 350 μl). Combined organic phases were dried over MgSO₄ and analyzed on GC-FID according to the section 6.4.8 and the results are listed in Table 6.5.

Table 6.5. Influence of AcCHMO loading on conversion of 1 and 2 to 3 and 4 in ammonium formate buffer (1 M, pH 8.7) with LbADH (20 μM), AcCHMO (20–40 μM), NADP⁺ (1 mM), 1 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>AcCHMO [mM]</th>
<th>Conversion [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>64</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>30</td>
<td>62</td>
</tr>
</tbody>
</table>

219
6.4.2.3 Amount of ReLac

Investigation on the activity of different charges and preparations of lactonase was performed as follows. Reaction mixture contained 50 mM Tris-HCl pH 9 as a buffer of choice, 1 mM NADP⁺ (10 mM stock in water), 20 μM LbADH, 20 μM AcCHMO, 5–20 μM ReLac, 50 mM cyclohexanol as a substrate in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 120 μl of 3 N HCl. Extraction was performed using EtOAc with 40 mM toluene as internal standard (650 μl). Combined organic phases were dried over MgSO₄ and analyzed on GC-FID according to the section 6.4.8 and the results are listed in Table 6.6.

Table 6.6. Influence of ReLac loading on conversion of 1 and 2 to 3 and 4 in ammonium formate buffer (1 M, pH 8.7) with LbADH (20 µM), AcCHMO (20 µM), ReLac (5–20 µM) NADP⁺ (1 mM), 1 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>AsCHMO [mM]</th>
<th>Conversion [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>66</td>
</tr>
<tr>
<td>40</td>
<td>67</td>
</tr>
</tbody>
</table>

6.4.2.4 Comparison of fusion and discrete enzyme systems

Activity comparison concerning fully discrete module 1 system (LbADH, AcCHOM and ReLac) and fusion module 1 system (TbTm-fus and ReLac) was performed as follows. Reaction was performed in three different buffers: 200 mM KPi pH 8, 1 M ammonium formate pH 8.6 or 1 M ammonium citrate pH 8.6 containing 0.2 mM NADP⁺ (10 mM stock in water), 15 μM LbADH together with 15 μM AcCHMO or 15 μM TbTm-Fus, 10 μM ReLac and 25 mM cyclohexanol as a substrate in a total volume of 0.5 ml in 1.5 ml
Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 120 μl of 3 N HCl. Extraction was performed using EtOAc with 40 mM toluene as internal standard (650 μl). Combined organic phases were dried over MgSO₄ and analyzed on GC-FID according to the section 6.4.8 and the results are listed in Table 6.7.

Table 6.7. Comparison of TbTm-fus and LbADH/AcCHMO containing module 1 in different buffers with TbTm-fus (15 μM) or LbADH (15 μM), AcCHMO (15 μM) and ReLac (15 μM) NADP⁺ (1 mM), 1 (25 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TbTm-fus + ReLac</th>
<th>LbADH + AcCHMO + ReLac</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM KPi pH 8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 M Amm form pH 8.6</td>
<td>66</td>
<td>100</td>
</tr>
<tr>
<td>1 M Amm cit pH 8.6</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>blank</td>
<td>29</td>
<td>12</td>
</tr>
</tbody>
</table>

6.4.3 Optimization of reactions involving module 2

6.4.3.1 ht-BsADH in ammonium buffers

Investigation on the activity of ht-BsADH in different buffers was performed as follows. Reaction mixture contained 1 M ammonium based buffer pH 8.6–8.7 (2 M stock solution containing 100 mM 6-hydroxyhexanoic acid, anions: formate, acetate, phosphate, citrate and chloride) or 0.5 M K-phosphate buffer pH 8 (1 M stock solution containing 100 mM 6-hydroxyhexanoic acid) as a buffer of choice, 1 mM NAD⁺ (50 mM stock in water), 50 μM ht-BsADH, 10 μM SmNOₓ, 50 mM 6-hydroxyhexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.9 and the results are listed in Table 6.8.
Table 6.8. Influence of ammonium containing buffers (1 M, pH 8.6–8.7) on activity of ht-BsADH. ht-BsADH (50 µM), SmNOx (10 µM), NAD⁺ (1 mM), 4 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Analytical yield of 5 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M K-phosphate pH 8</td>
<td>69.5 ± 0.8</td>
</tr>
<tr>
<td>1 M Ammonium formate pH 8.6</td>
<td>7.1 ± 0.0</td>
</tr>
<tr>
<td>1 M Ammonium acetate pH 8.6</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>1 M Ammonium phosphate pH 8.7</td>
<td>23.1 ± 0.1</td>
</tr>
<tr>
<td>1 M Ammonium citrate pH 8.6</td>
<td>67.2 ± 0.4</td>
</tr>
<tr>
<td>1 M Ammonium chloride pH 8.7</td>
<td>7.2 ± 0.0</td>
</tr>
</tbody>
</table>

6.4.3.2 Characterization of the alanine scan variants of LysEDH: activity

Investigation on the activity of LysEDH variants in different buffers was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution containing 100 mM 6-oxohexanoic acid) as a buffer of choice, 1 mM NAD⁺ (50 mM stock in water), 50 µM LysEDH_variant, 15 µM CbFDH, 50 mM 6-oxohexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 µl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 µl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.9.

Table 6.9. Activity test of LysEDH variants in ammonium formate buffer (1 M, pH 8.6) with LysEDH_variant (50 µM), CbFDH (15 µM), NAD⁺ (1 mM), 5 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>LysEDH_variant</th>
<th>Analytical yield of 6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>74.1 ± 0.2</td>
</tr>
<tr>
<td>H181A</td>
<td>54.0 ± 0.0</td>
</tr>
<tr>
<td>Y238A</td>
<td>66.4 ± 0.3</td>
</tr>
<tr>
<td>T240A</td>
<td>67.2 ± 0.2</td>
</tr>
<tr>
<td>Y238A, T240A</td>
<td>40.2 ± 0.4</td>
</tr>
<tr>
<td>H181A,Y238A</td>
<td>29.3 ± 0.1</td>
</tr>
<tr>
<td>H181A,T240A</td>
<td>59.2 ± 0.1</td>
</tr>
<tr>
<td>H181A, Y238A, T240A</td>
<td>34.8 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>
6.4.3.3 Characterization of the alanine scan variants of LysEDH: thermostability

Thermostability of LysEDH variants was measured using differential scanning fluorometry using a Biorad-750 QPCR machine. The analysis was performed in 1 M ammonium formate pH 8.6 in a 96-well plate. The single well reaction mixture (20 μl) contained SYPRO orange, 1 μg of enzyme and the buffer. Data were collected as a continuous standard melt curve (20–90 °C, hold 1 min at 20 °C, 1% increment, hold 1 min at 90 °C). ROX was used as a reporter. Results are summarized in Table 6.10.

Table 6.10. Thermostability of the LysEDH variants in ammonium formate buffer (1 M, pH 8.6) n = 3.

<table>
<thead>
<tr>
<th>LysEDH_variant</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>69.0 ± 0.2</td>
</tr>
<tr>
<td>H181A</td>
<td>71.9 ± 0.1</td>
</tr>
<tr>
<td>Y238A</td>
<td>66.5 ± 0.2</td>
</tr>
<tr>
<td>T240A</td>
<td>68.6 ± 0.1</td>
</tr>
<tr>
<td>Y238A, T240A</td>
<td>66.0 ± 0.2</td>
</tr>
<tr>
<td>H181A, Y238A</td>
<td>70.3 ± 0.1</td>
</tr>
<tr>
<td>H181A, T240A</td>
<td>70.7 ± 0.1</td>
</tr>
<tr>
<td>H181A, Y238A, T240A</td>
<td>69.8 ± 0.1</td>
</tr>
</tbody>
</table>

6.4.3.4 Initial combination of Module 2

Initial attempts to combine ht-BsADH and LysEDH variants were performed as follows. Reaction mixture contained 1 M ammonium citrate buffer pH 8.6 (2 M stock solution containing 100 mM 6-hydroxyhexanoic acid) as a buffer of choice, 1 mM NAD$^+$ (50 mM stock in water), 50 μM ht-BsADH, 50 μM LysEDH_variant (WT–7), 50 mM 6-hydroxyhexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.11.
Table 6.11. Combination of ht-BsADH and LysEDH variants in ammonium formate buffer (1 M, pH 8.6) on with ht-BsADH (50 µM) LysEDH_variant (50 µM), NAD⁺ (1 mM), 4 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>LysEDH variant</th>
<th>Analytical yield of 6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0</td>
</tr>
<tr>
<td>H181A</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>Y238A</td>
<td>0</td>
</tr>
<tr>
<td>T240A</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Y238A, T240A</td>
<td>0</td>
</tr>
<tr>
<td>H181A,Y238A</td>
<td>0</td>
</tr>
<tr>
<td>H181A,T240A</td>
<td>0</td>
</tr>
<tr>
<td>H181A, Y238A, T240A</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>ht-BsADH control</td>
<td>0</td>
</tr>
</tbody>
</table>

6.4.3.5 Troubleshooting of Module 2

Investigation on the possible limitations concerning module 2, comprising ht-BsADH and LysEDH variants, was performed as follows. Reaction mixture contained 1 M ammonium citrate buffer pH 8.6 (2 M stock solution containing 100 mM 6-oxohexanoic acid) as a buffer of choice, 1 or 5 mM NAD⁺ (50 mM stock in water), 10 or 50 µM ht-BsADH, 50 or 90 µM LysEDH_T240A, 10 µM SmNOX, 10 or 50 mM 6-hydroxyhexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube (investigated combinations are detailed in Table 6.12). Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 µl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 µl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.12.

Table 6.12. List of conditions investigated during the troubleshooting of module 2 in ammonium formate buffer (1 M, pH 8.6) with ht-BsADH (10 or 50 µM) LysEDH_T240A (50 or 90 µM), NAD⁺ (1 or 5 mM), 4 (10 or 50 mM), 6 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Entry</th>
<th>ht-BsADH [µM]</th>
<th>LysEDH_T240A [µM]</th>
<th>SmNOX [µM]</th>
<th>NAD⁺ [mM]</th>
<th>substrate [mM]</th>
<th>Analytical yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>90</td>
<td>-</td>
<td>1</td>
<td>50 (4)</td>
<td>0 (6)</td>
</tr>
</tbody>
</table>
6.4.3.6 Preferred reaction pathway

Investigation on the preferred reaction pathway of module 2, comprising ht-BsADh and LysEDH variants, was performed as follows. Reaction mixture contained 1 M ammonium citrate buffer pH 8.6 (2 M stock solution containing 100 mM 6-oxohexanoic acid) as a buffer of choice, 1 or 5 mM NADH (50 mM stock in water), 10, 50 or 90 μM ht-BsADh, 10, 50 or 90 μM LysEDH_H181A, 10 μM CbFDH, 10 or 50 mM 6-oxohexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube (investigated combinations are detailed in Table 6.13).

Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.13.

Table 6.13. List of conditions investigated during study on the preferred reaction pathway in ammonium formate buffer (1 M, pH 8.6) with ht-BsADh (10–90 μM) LysEDH_H181A (10–90 μM), NAD⁺ (1 or 5 mM), 5 (10 or 50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Entry</th>
<th>ht-BsADH [μM]</th>
<th>LysEDH_H181A [μM]</th>
<th>NADH [μM]</th>
<th>Substrate [mM]</th>
<th>Compound [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>50</td>
<td>5</td>
<td>50</td>
<td>3.2 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>10</td>
<td>5</td>
<td>50</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>90</td>
<td>5</td>
<td>50</td>
<td>5.9 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>50</td>
<td>5</td>
<td>10</td>
<td>4.9 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>50</td>
<td>1</td>
<td>50</td>
<td>2.6 ± 0.1</td>
</tr>
</tbody>
</table>
6.4.4 Re-design of module 2

6.4.4.1 Activity test of AcCO6

Activity of AcCO6 in different buffers was determined in a series of reactions containing purified AcCO6 as follows. Reaction mixture contained 1 M ammonium formate pH 8.6 (2 M stock solution containing 100 mM 6-hydroxyhexanoic acid) as a buffer of choice, 1 mM NAD\(^+\) (50 mM stock in water), 25 µM AcCO6, 50 µM LysEDH_H181A, 14 µM CbFDH, 10 or 50 mM 6-hydroxyhexanoic acid or 6-oxohexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 µl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 µl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.14.

### Table 6.14. List of conditions investigated during the activity test of AcCO6 in ammonium formate buffer (1 M, pH 8.6) with AcCO6 (25 µM) LysEDH_H181A (50 µM), CbFDH (14 µM), NAD\(^+\) (1 mM), 4 (10 or 50 mM), 5 (10 mM) T = 30 °C, t = 24 h, n = 2

<table>
<thead>
<tr>
<th>Entry</th>
<th>AcCO6 [µM]</th>
<th>LysEDH_H181A [µM]</th>
<th>CbFDH [µM]</th>
<th>substrate [mM]</th>
<th>Conversion [%]</th>
<th>Analytical yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>50 (4)</td>
<td>0.0</td>
<td>0.0 (6)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>50</td>
<td>14</td>
<td>10 (5)</td>
<td>74.5</td>
<td>68.7 ± 0.0 (6)</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>50</td>
<td>14</td>
<td>50 (4)</td>
<td>42.5</td>
<td>12.9 ± 0.2 (6)</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>10 (4)</td>
<td>81.1</td>
<td>81.1 ± 0.0 (5)</td>
</tr>
</tbody>
</table>
6.4.4.2 AcCO6 and the LysEDH variants

Comparison of the activity of LysEDH variants when combined with AcCO6 was performed as follows. Reaction mixture contained 1 M ammonium formate pH 8.6 (2 M stock solution containing 100 mM 6-hydroxyhexanoic acid) as a buffer of choice, 1 mM NAD⁺ (50 mM stock in water), 25 μM AcCO6, 50 μM LysEDH_variant, 10 μM CbFDH, 0 or 0.1 mg ml⁻¹ catalase and 50 mM 6-hydroxyhexanoic acid as a substrate (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.15.

Table 6.15. List of conditions investigated during activity test of AcCO6 in ammonium formate buffer (1 M, pH 8.6) with AcCO6 (25 μM), LysEDH_variant (50 μM), CbFDH (10 μM), NAD⁺ (1 mM), 6 (50 mM), T = 30 °C, t = 24 h, n = 2. *, catalase present.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcCO6</td>
<td>52.7</td>
<td>n. d.</td>
</tr>
<tr>
<td>AcCO6 + Catalase</td>
<td>61.7</td>
<td>n. d.</td>
</tr>
<tr>
<td>AcCO6 + LysEDH*</td>
<td>86.5</td>
<td>43.2 ± 0.8</td>
</tr>
<tr>
<td>AcCO6 + LysEDH_H181A*</td>
<td>86.0</td>
<td>29.1 ± 0.1</td>
</tr>
<tr>
<td>AcCO6 + LysEDH_T240A*</td>
<td>86.8</td>
<td>43.6 ± 0.7</td>
</tr>
<tr>
<td>AcCO6 + LysEDH_H181A_T240A*</td>
<td>81.3</td>
<td>32.8 ± 0.4</td>
</tr>
<tr>
<td>blank*</td>
<td>0</td>
<td>100 ± 0.0</td>
</tr>
</tbody>
</table>

6.4.5 Combination of Modules 1 and 2

6.4.5.1 Comparison of two best LysEDH variants in the whole cascade

Initial combination of all of the enzymes into a functioning multi-enzymatic cascade was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution) as a buffer of choice, 15 μM TbTm-fus, 10 μM ReLac 1 mM NADP⁺ and 1 mM NAD⁺ (both as 50 mM stock solutions in water), 25 μM AcCO6, 50 μM LysEDH_T240A, 20 μM CbFDH, 0.1 mg ml⁻¹ of catalase and 25 or 50 mM
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cyclohexanol in a total volume of 0.5 ml in 1.5 ml Eppendorf tube (investigated combinations are detailed in Table 6.16 together with the results). Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 µl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 µl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8.

Table 6.16. List of conditions investigated during preliminary investigation of the whole cascade in ammonium formate buffer (1 M, pH 8.6) with TbTm-fus (15 µM), ReLac (10 µM), AcCO6 (25 µM), LysEDH_variant (50 µM), CbFDH (20 µM), NAD⁺ (1 mM), 1 (25 or 50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Sample (LysEDH_variant; Substrate [mM])</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysEDH (25)</td>
<td>60.4</td>
<td>14.6 ± 2.8</td>
</tr>
<tr>
<td>LysEDH (50)</td>
<td>51.1</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>LysEDH_T240A (25)</td>
<td>86.0</td>
<td>20.0 ± 0.0</td>
</tr>
<tr>
<td>LysEDH_T240A (50)</td>
<td>85.5</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>blank (25)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>blank (50)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

6.4.5.2 Behavior of different module 1 set-up inside a whole cascade

Comparison of two different set-ups of module 1 inside a multi-enzymatic cascade was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution) as a buffer of choice, 20 µM LbADH and 20 µM AcCHMO or 20 µM TbTm-fus, 10 µM ReLac, 1 mM NADP⁺ and 1 mM NAD⁺ (both as 50 mM stock solutions in water), 25 µM AcCO6, 50 µM LysEDH_T240A, 13 µM CbFDH, 0.1 mg ml⁻¹ of catalase and 25 or 50 mM cyclohexanol in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 µl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 µl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.17.
Table 6.17 List of conditions investigated during two different set-ups of module 1 in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM) and AcCHMO (20 μM) or TbTm-fus (20 μM), ReLac (10 μM), AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD+ (1 mM), 1 (25 or 50 mM), T = 30 °C, t = 24 h, n = 2.* 20% loss of substrate observed.

<table>
<thead>
<tr>
<th>Module 1 (substrate loading [mM])</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TbTm-fus (25 mM)</td>
<td>76.5</td>
<td>35.8 ± 0.3</td>
</tr>
<tr>
<td>TbTm-fus (50 mM)</td>
<td>33.7</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>LbADH + AcCHMO (25 mM)</td>
<td>100</td>
<td>8.3 ± 1.1</td>
</tr>
<tr>
<td>LbADH + AcCHMO (50 mM)</td>
<td>65.7</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>blank (25 mM)</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>blank (50 mM)</td>
<td>0*</td>
<td>0</td>
</tr>
</tbody>
</table>

6.4.5.3 Whole cascade in different pH

Comparison of the two different set-ups of module 1 inside a multi-enzymatic cascade was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8–9 (2 M stock solution) as a buffer of choice, 20 μM LbADH, 20 μM AcCHMO, 10 μM ReLac, 1 mM NADP+ and 1 mM NAD+ (both as 50 mM stock solutions in water), 25 μM AcCO6, 50 μM LysEDH_T240A, 13 μM CbFDH, 0.1 mg ml⁻¹ of catalase and 25 or 50 mM cyclohexanol in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8 and the results are listed in Table 6.18.

Table 6.18. Investigation onto the pH optimum of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8–9) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD+ (1 mM), 1 (50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>pH</th>
<th>Compound [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>8.05</td>
<td>19.7 ± 0.4</td>
</tr>
<tr>
<td>8.25</td>
<td>19.7 ± 0.0</td>
</tr>
<tr>
<td>8.5</td>
<td>18.3 ± 0.3</td>
</tr>
<tr>
<td>8.76</td>
<td>16.7 ± 0.3</td>
</tr>
</tbody>
</table>
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6.4.5.4 Module 2 in presence of module 1 intermediates

Influence of module 1 intermediates on the performance of module 2 was studied as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8–9 (2 M stock solution containing 50 mM 6-hydroxyhexanoic acid) as a buffer of choice, 1 mM NAD$^+$ (both as 50 mM stock solutions in water), 25 μM AcCO$_6$, 50 μM LysEDH_T240A, 13 μM CbFDH, 0.1 mg ml$^{-1}$ of catalase and 25 mM 6-hydroxyhexanoic acid (added together with the buffer) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Furthermore, the reaction mixture contained 25 mM cyclohexanol, or cyclohexanone or caprolactone added in form of DMSO stock solution (investigated combinations are detailed in Table 6.19 together with the results). Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8.

Table 6.19. Investigation into the behavior of module 2 in presence of module 1’s intermediates in ammonium formate buffer (1 M, pH 8.6) with AcCO$_6$ (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD$^+$ (1 mM), 4 (25 mM), 1, 2 or 3 (25 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound [mM]</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Module 2</td>
<td>-</td>
<td>100</td>
<td>70.4 ± 0.0</td>
</tr>
<tr>
<td>Module 2 + Intermediate</td>
<td>1 (25)</td>
<td>99.1</td>
<td>69.9 ± 0.0</td>
</tr>
<tr>
<td>Module 2 + Intermediate</td>
<td>2 (25)</td>
<td>55.9</td>
<td>51.6 ± 0.0</td>
</tr>
<tr>
<td>Module 2 + Intermediate</td>
<td>3 (25)</td>
<td>63.3</td>
<td>91.5 ± 1.7</td>
</tr>
</tbody>
</table>

### pH and Compound [mM]

<table>
<thead>
<tr>
<th>pH</th>
<th>Compound [mM]</th>
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</thead>
<tbody>
<tr>
<td>9.01</td>
<td>18.9 ± 0.5</td>
</tr>
<tr>
<td>8.5 (blank)</td>
<td>47.3 ± 0.4</td>
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</tbody>
</table>
6.4.6 Optimization of module 2 reaction conditions using AcCO6 and Ch1-AmDH

6.4.6.1 General reaction using AcCO6 and Ch1-AmDH in tandem

Investigation into the optimal reaction conditions for AcCO6/Ch1-AmDH tandem was performed using the following general protocol. Reaction mixture contained 1 M ammonium formate pH 8.6 (2 M stock solution) as a buffer of choice, 1 mM NAD\(^+\) (50 mM stock in water), 5 μM AcCO6, 50 μM Ch1-AmDH, 11 μM CbFDH, 0.1 mg ml\(^{-1}\) catalase and 20 mM 1-hexanol as a substrate (added as a DMSO stock) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 70 μl of 10 M KOH. Extraction was performed using EtOAc with 20 mM toluene as internal standard (twice, 300 and 350 μl of EtOAc, equaling 650 μl). Combined organic phases were dried over MgSO\(_4\) and analysed on GC-FID according to the section 6.4.8.

Results detailing the pH optimum, substrate loading and temperature optimum are described in Tables 6.20–22.

Table 6.20. Investigation into the behavior of module 2 at different pH, in ammonium formate buffer (1 M, pH 8–9) with AcCO6 (5 μM), Ch1-AmDH (50 μM), CbFDH (11 μM), NAD\(^+\) (1 mM), 7a (20 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>pH</th>
<th>Analytical yield of 7c [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.05</td>
<td>69.4 ± 4.7</td>
</tr>
<tr>
<td>8.25</td>
<td>75.5 ± 2.3</td>
</tr>
<tr>
<td>8.5</td>
<td>73.0 ± 0.1</td>
</tr>
<tr>
<td>8.76</td>
<td>74.3 ± 3.8</td>
</tr>
<tr>
<td>9.01</td>
<td>73.4 ± 1.4</td>
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<tr>
<td>8.50 (blank)</td>
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</tr>
</tbody>
</table>
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Table 6.21. Investigation into the behavior of module 2 at different substrate loading, in ammonium formate buffer (1 M, pH 8.6) with AcCO6 (5 μM), Ch1-AmDH (50 μM), CbFDH (11 μM), NAD⁺ (1 mM), 7a (20 - 100 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Substrate loading</th>
<th>Analytical yield of 7c [%]</th>
</tr>
</thead>
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<tr>
<td>20 mM (blank)</td>
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<tr>
<td>20 mM</td>
<td>99</td>
</tr>
<tr>
<td>40 mM</td>
<td>88.6 ± 0.0</td>
</tr>
<tr>
<td>60 mM</td>
<td>61.2 ± 0.1</td>
</tr>
<tr>
<td>80 mM</td>
<td>43.7 ± 0.0</td>
</tr>
<tr>
<td>100 mM</td>
<td>36.7 ± 0.0</td>
</tr>
</tbody>
</table>

Table 6.22. Investigation into the behavior of module 2 at different temperature in ammonium formate buffer (1 M, pH 8.6) with AcCO6 (5 μM), Ch1-AmDH (50 μM), CbFDH (11 μM), NAD⁺ (1 mM), 7a (60 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Analytical yield of 7c [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C</td>
<td>76.3 ± 0.1</td>
</tr>
<tr>
<td>30 °C</td>
<td>68.2 ± 0.0</td>
</tr>
<tr>
<td>40 °C</td>
<td>56.9 ± 0.0</td>
</tr>
<tr>
<td>50 °C</td>
<td>39.0 ± 0.0</td>
</tr>
<tr>
<td>30 °C (blank)</td>
<td>0</td>
</tr>
</tbody>
</table>

6.4.7 Different reaction set-ups

6.4.7.1 One-pot system at varied concentration of AcCO6 and substrate

Investigation into the single batch setup was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution) as a buffer of choice, 20 μM LbADH, 20 μM AcCHMO, 10 μM ReLac, 1 mM NADP⁺ and 1 mM NAD⁺ (both as 50 mM stock solutions in water), 25 μM or 57 μM AcCO6, 50 μM LysEDH_T240A, 13 μM CbFDH, 0.1 mg ml⁻¹ of catalase and 30 or 50 mM cyclohexanol (added as DMSO stock solution) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm, for 24 h. After the reaction time was finished, reactions were quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl
aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8. Observed conversions and analytical yields are listed in Table 6.23 and the compound distribution is described in Table 6.24.

Table 6.23. Investigation into the batch set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), AcCO6 (25-57 μM), LysEDH_variant (50 μM), CbFDH (20 μM), NAD+ (1 mM), 1 (30 or 50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 uM AcCO6 (30 mM)</td>
<td>47.6 ± 1.4</td>
<td>30.3 ± 0.9</td>
</tr>
<tr>
<td>25 uM AcCO6 (50 mM)</td>
<td>48.1 ± 0.1</td>
<td>8.4 ± 1.0</td>
</tr>
<tr>
<td>57 uM AcCO6 (50 mM)</td>
<td>52.8 ± 0.1</td>
<td>12.4 ± 0.7</td>
</tr>
</tbody>
</table>

Table 6.24. Final compound distribution of the batch set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), AcCO6 (25 - 57 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD+ (1 mM), 1 (30 or 50 mM), T = 30 °C, t = 24 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 uM AcCO6 (30 mM)</td>
<td>15.7 ± 0.4</td>
<td>0.0</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>25 uM AcCO6 (50 mM)</td>
<td>26.0 ± 0.1</td>
<td>0.0</td>
<td>13.5 ± 1.3</td>
<td>0.4 ± 0.2</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>57 uM AcCO6 (50 mM)</td>
<td>23.6 ± 0.0</td>
<td>0.0</td>
<td>14.3 ± 0.7</td>
<td>0.0</td>
<td>6.2 ± 0.3</td>
</tr>
</tbody>
</table>

6.4.7.2 Fed-Batch system

Investigation on the fed-batch set-up was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution) as a buffer of choice, 20 μM LbADH, 20 μM AcCHMO, 10 μM ReLac, 1 mM NADP+ and 1 mM NAD+ (both as 50 mM stock solutions in water), 25 μM AcCO6, 50 μM LysEDH_T240A, 13 μM CbFDH, 0.1 mg ml⁻¹ of catalase and 10 mM cyclohexanol (added as DMSO stock solution) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube prepared in five pairs. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm; additional aliquots of cyclohexanol (10 mM) were added every two hours. Reference samples were taken after 2, 4, 6 and 8 hours. One set of reactions was incubated with 10 mM cyclohexanol for 4 hours. After the reaction time was finished, reactions were
quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8. Observed conversions and analytical yields are listed in Table 6.25 and the compound distribution is described in Table 6.26.

Table 25. Investigation on the fed-batch set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD⁺ (1 mM), 1 (10–40 mM), T = 30 °C, t = 2–8 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (10 mM)</td>
<td>47.0 ± 0.5</td>
<td>14.3 ± 0.2</td>
</tr>
<tr>
<td>4 (10 mM)</td>
<td>46.1 ± 0.1</td>
<td>25.2 ± 0.6</td>
</tr>
<tr>
<td>4 (10+10 mM)</td>
<td>25.3 ± 1.7</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>6 (10+10+10 mM)</td>
<td>19.2 ± 0.3</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>8 (10+10+10+10 mM)</td>
<td>17.2 ± 0.3</td>
<td>9.2 ± 0.0</td>
</tr>
</tbody>
</table>

Table 26. Compound distribution observed in the fed-batch set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD⁺ (1 mM), 1 (10–40 mM), T = 30 °C, t = 2–8 h, n = 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2 (10 mM)</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td>4 (10 mM)</td>
<td>5.4 ± 0.0</td>
</tr>
<tr>
<td>4 (10+10 mM)</td>
<td>14.9 ± 0.3</td>
</tr>
<tr>
<td>6 (10+10+10 mM)</td>
<td>24.3 ± 0.1</td>
</tr>
<tr>
<td>8 (10+10+10+10 mM)</td>
<td>33.1 ± 0.1</td>
</tr>
</tbody>
</table>

6.4.7.3 One-pot two-step cascade

Investigation on the fed-batch set-up was performed as follows. Reaction mixture contained 1 M ammonium formate buffer pH 8.6 (2 M stock solution) as a buffer of choice, 20 μM LbADH, 20 μM AcCHMO, 10 μM ReLac, 1 mM NAD⁺ (50 mM stock solutions in water) and 10 or 30 mM cyclohexanol (added as DMSO stock solution) in a total volume of 0.5 ml in 1.5 ml Eppendorf tube. Reactions were started by the addition of a substrate and incubated at 30 °C, 170 rpm. After 24 h, reaction mixture was centrifuged (ca. 17000 g, 1 min, 4 °C) and the supernatant was transferred into 2 ml Eppendorf tube containing 25 μM AcCO6, 50 μM LysEDH_T240A, 13 μM CbFDH and
0.1 mg ml⁻¹ of catalase in 1 M ammonium formate buffer pH 8.6. The combined reaction was incubated for additional 24 h at 30 °C, 170 rpm. The reactions were subsequently quenched by addition of 170 μl of 3 N HCl. Samples were centrifuged (ca. 17000 g, 4 °C, 5 min) and 500 μl aliquot was taken for the analysis. Analysis was performed using RP-HPLC according to section 6.4.8. Observed conversions and analytical yields are listed in Table 6.27 and the compound distribution is described in Table 6.28.

Table 27. Investigation on the time-separated set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), 1 (30–50 mM), T = 30 °C, t = 24 h. AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD⁺ (1 mM) added later, T = 30 °C, t = 24 h, n = 2. Final concentration 15–30 mM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conversion [%]</th>
<th>Analytical yield of 6 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>module 1 (30 mM initial, 30 mM final)</td>
<td>46.6 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>module 1+2 (30 mM initial, 15 mM final)</td>
<td>49.9 ± 0.7</td>
<td>29.2 ± 0.4</td>
</tr>
<tr>
<td>module 1+2 (50 mM initial, 25 mM final)</td>
<td>50.7 ± 0.1</td>
<td>30.3 ± 0.0</td>
</tr>
<tr>
<td>module 2 (50 mM initial, 25 mM final)</td>
<td>93.7 ± 0.0</td>
<td>56.0 ± 0.2</td>
</tr>
</tbody>
</table>

Table 28. Compound distribution observed during the Investigation on the time-separated set-up of the cascade to nylon-6 monomer in ammonium formate buffer (1 M, pH 8.6) with LbADH (20 μM), AcCHMO (20 μM), ReLac (10 μM), 1 (30–50 mM), T = 30 °C, t = 24 h. AcCO6 (25 μM), LysEDH_T240A (50 μM), CbFDH (20 μM), NAD⁺ (1 mM) added later, T = 30 °C, t = 24 h, n = 2. Final concentration 15–30 mM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>module 1 (30 mM initial, 30 mM final)</td>
<td>16.0 ± 0.2</td>
</tr>
<tr>
<td>module 1+2 (30 mM initial, 15 mM final)</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>module 1+2 (50 mM initial, 25 mM final)</td>
<td>12.3 ± 0.0</td>
</tr>
<tr>
<td>module 2 (50 mM initial, 25 mM final)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

### 6.4.8 Analytics

**GC-FID**

GC-FID analysis was performed on an Agilent 7890B chromatograph using H₂ as carrier gas.
**Column:** Agilent HP-5 (30 m, 320 μm, 0.25 μm), Agilent J&W DB1701 (30 m, 250 μm, 0.25 μm).

**Method A:** T injector 250 °C; constant flow 1.8 ml min⁻¹; temperature program: 60 °C; 5 °C min⁻¹ to 100 °C, hold 0 min; 25 °C min⁻¹ to 300 °C, hold 3 min.

**Method B:** T injector 250 °C; constant pressure 6.9 psi; temperature program: 60 °C, hold 6.5 min; 20 °C min⁻¹ to 100 °C, hold 1 min; 20 °C min⁻¹ to 280 °C, hold 1 min.

Table 29. Retention times of compounds analyzed via GC-FID.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Column</th>
<th>Method</th>
<th>Split ratio</th>
<th>Retention time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyclohexanol</td>
<td>HP-5</td>
<td>A</td>
<td>20</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>Cyclohexanone</td>
<td>HP-5</td>
<td>A</td>
<td>20</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>Caprolactone</td>
<td>HP-5</td>
<td>A</td>
<td>20</td>
<td>9.6</td>
</tr>
<tr>
<td>4</td>
<td>6-Hydroxyhexanoic acid</td>
<td>HP-5</td>
<td>A</td>
<td>20</td>
<td>11.1</td>
</tr>
<tr>
<td>7a</td>
<td>1-hexanol</td>
<td>DB1701-30m</td>
<td>B</td>
<td>20</td>
<td>8.3</td>
</tr>
<tr>
<td>7b</td>
<td>hexanal</td>
<td>DB1701-30m</td>
<td>B</td>
<td>20</td>
<td>7.6</td>
</tr>
<tr>
<td>7c</td>
<td>1-aminohexane</td>
<td>DB1701-30m</td>
<td>B</td>
<td>20</td>
<td>5.9</td>
</tr>
</tbody>
</table>

**HPLC**

RP-HPLC measurements were performed on a Prominence-i LC 2030C 3D (Shimadzu) with a Shimadzu Shim-pack GIST 5 μm C18 AQ (150 mm length, 4.6 mm inner diameter) column.

**Method D:** Flow: 1 ml min⁻¹; oven temperature: 40 °C; isocratic flow 25 min (65 minutes for the whole system).

**Detector:** Refractive index

**Mobile phase:** MiliQ + 4% MeOH + 0.1% TFA; filtered and degassed.
Table 30. Retention times of the compounds analyzed by HPLC. n.a., not applicable, hydrolyzed during the work-up.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Retention time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyclohexanol</td>
<td>54.2</td>
</tr>
<tr>
<td>2</td>
<td>Cyclohexanone</td>
<td>41.6</td>
</tr>
<tr>
<td>3</td>
<td>Caprolactone</td>
<td>n. a.</td>
</tr>
<tr>
<td>4</td>
<td>6-Hydroxyhexanoic acid</td>
<td>18.2</td>
</tr>
<tr>
<td>5</td>
<td>6-Oxohexanoic acid</td>
<td>15.3</td>
</tr>
<tr>
<td>6</td>
<td>6-Aminohexanoic acid</td>
<td>4.9</td>
</tr>
</tbody>
</table>
6.5 References