Application and Protein Engineering of Oxidoreductases

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vorgelegt von
Hauke Wulf
geboren am 3. Juni 1982
in Hamburg

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Dekan: Prof. Dr. Klaus Fesser
1. Gutachter: Prof. Dr. Uwe T. Bornscheuer
2. Gutachter: Prof. Dr. Marco W. Fraaije

INDEX

INDEX.......................................................................................................................................................... 1

ABBREVIATIONS ........................................................................................................................................ 2

1 AIM OF THE THESIS AND ARTICLE OUTLINES .................................................................................. 3

Article I:  Asymmetric synthesis of D-glyceric acid by an alditol oxidase and directed evolution for
enhanced oxidative activity towards glycerol ............................................................................................... 3
Article II:  Kinetic resolution of glyceraldehyde using an aldehyde dehydrogenase from Deinococcus
geothermalius DSM 11300 combined with electrochemical cofactor recycling ........................................... 3
Article III: Protein engineering of a thermostable polyol dehydrogenase .................................................... 4
Article IV: A self-sufficient Baeyer-Villiger biocatalysis system for the synthesis of ε-caprolactone from
cyclohexanol .................................................................................................................................................... 4

2 BACKGROUND ........................................................................................................................................ 5

2.1 GLYCEROL AS BIO-BASED FEEDSTOCK AND RELATED VALUE ADDED PRODUCTS............................... 5
2.2 INTRODUCTION OF THE APPLIED OXIDOREDUCTASES AND TECHNIQUES ........................................... 6

Alditol oxidase and enzyme mutagenesis ..................................................................................................... 6
Aldehyde Dehydrogenase and electrolytic cofactor recycling ...................................................................... 6
Engineering of enzyme thermostability and polyol dehydrogenases ............................................................ 7

2.3 ε-CAPROLACTONE AND CYCLOHEXANONE MOOXYGENASE ................................................................. 8

3 RESULTS .................................................................................................................................................. 10

3.1 ENGINEERING OF AN ALDITOL OXIDASE AND OXIDATION OF GLYCEROL ............................................. 10
3.2 DERACEMIZATION OF GLYCALDEHYDE USING ELECTROLYTIC COFACTOR RECYCLING ......................... 13
3.3 ENGINEERING OF A THERMOSABLE POLYOXIDOREDUCTASE ................................................................. 17
3.4 PRODUCTION OF ε-CAPROLACTONE WITH CLOSED LOOP RECYCLING .................................................. 22

4 CONCLUDING REMARKS ....................................................................................................................... 25

5 LITERATURE ............................................................................................................................................ 26

6 ARTICLES AND AUTHOR CONTRIBUTIONS .......................................................................................... 31

ERKLÄRUNG ............................................................................................................................................... 66

LEBENSLAUF ............................................................................................................................................. 67

DANKSAGUNG .......................................................................................................................................... 69
ABBREVIATIONS

%ee enantiomeric excess
°C degree Celsius
ABTS 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate)
ALDH aldehyde dehydrogenase
AldO alditol dehydrogenase
Å Ångström
bicine N,N-Bis(2-hydroxyethyl)-glycin
BVMO Baeyer-Villiger monooxygenase
CHL cyclohexanol
CHMO cyclohexanone monooxygenase
CHO cyclohexanone
*D. geothermalis* Deinococcus geothermalis
Da/kDa dalton/kilodalton
DNA deoxyribonucleic acid
*E. coli* Escherichia coli
ePCR error-prone PCR
ε-CL ε-caprolactone
FAD flavin adenine dinucleotide
g gram
GA glyceric acid
GroES/GroEL molecular chaperones
His<sub>6</sub>-tag hexahistidine affinity tagging sequence
HPLC high performance liquid chromatography
I liter
k<sub>cat</sub> turnover number (s<sup>-1</sup>)
K<sub>m</sub> Michaelis-Menten constant (mM)
M/mM concentration (mol·l<sup>-1</sup>)/(mmol·l<sup>-1</sup>)
ml milliliter
NAD nicotinamide adenine dinucleotide
NADP nicotinamide adenine dinucleotide phosphate
PCL poly-ε-caprolactone
PCR polymerase chain reaction
PDH polyol dehydrogenase
pH decimal cologarithm of hydrogen ion activity
r correlation coefficient
*R. sphaeroides* Rhodobacter sphaeroides
s seconds
SDR short chain dehydrogenase/reductase
t/a tons per year
T<sub>50</sub> temperature were 50% residual activity remains after incubation for 60 min
T<sub>m</sub> melting point temperature
tris tris-(hydroxymethyl)-aminomethane
U units conversion (µmol·min<sup>-1</sup>)
U·mg<sup>-1</sup> specific activity
WT wild-type
1 AIM OF THE THESIS AND ARTICLE OUTLINES

The area of biotransformation has a great potential to facilitate the growth of a sustainable chemical industry. This work aims to contribute to this field by developing biocatalysis strategies and applications with new oxidative and thermostable enzymes.

The focus of the first two articles was the engineering and application of enzymes for the conversion of the bio-based resources glycerol and its oxidation product glyceraldehyde for the production of the value added product glyceraldehyde. Article III focuses on the cloning, exploration and engineering of a polyol dehydrogenase, which later on was used as cofactor recycling system in order to produce ε-caprolactone from cyclohexanol as presented in article IV. The following paragraphs will give a short outline of each article.

ARTICLE I: ASYMMETRIC SYNTHESIS OF D-GLYCERIC ACID BY AN ALDITOL OXIDASE AND DIRECTED EVOLUTION FOR ENHANCED OXIDATIVE ACTIVITY TOWARDS GLYCEROL.

The alditol oxidase of Streptomyces coelicolor A3(2) (AldO) was used to catalyze the oxidation of glycerol to glyceraldehyde and glyceraldehyde. The enantioselectivity for the FAD-dependent glycerol oxidation was elucidated and different strategies were used to enhance the substrate specificity towards glycerol. Directed evolution by error-prone PCR led to an AldO double mutant with 1.5-fold improved activity for glycerol. Further improvement of activity was achieved by combination of mutations, leading to a quadruple mutant with 2.4-fold higher specific activity towards glycerol compared to the wild-type enzyme. In small-scale biotransformation concentrations up to 2.0 g·l⁻¹ D-glyceric acid could be reached using whole cells. Investigation of the effects of the introduced mutations led to a further identification of essential amino acids with respect to enzyme functionality and structural stability.

ARTICLE II: KINETIC RESOLUTION OF GLYCERALDEHYDE USING AN ALDEHYDE DEHYDROGENASE FROM DEINOCOCCUS GEOThERMALIS DSM 11300 COMBINED WITH ELECTROCHEMICAL COFACTOR RECYCLING.

Two aldehyde dehydrogenases (ALDH) from Escherichia coli BL21 and Deinococcus geotherm alis were cloned, characterized and evaluated according to their applicability for a biocatalysis setup with electrolytic cofactor recycling. Both ALDHs turned out to have a similar substrate scope and favor short to medium chain aldehydes and both oxidize glyceraldehyde to D-glyceric acid. The ALDH variant of D. geotherm alis shows higher specific activity towards
glyceraldehyde and has an elevated optimum temperature compared to the BL21 enzyme. Due to the higher specific activity of the ALDH of *D. geothermalis*, this enzyme was used to conduct a kinetic resolution of glyceraldehyde with electrolytic NAD$^+$ recycling at a glassy carbon foam electrode with ABTS as redox mediator yielding in 1.8 g·l$^{-1}$ glyceric acid.

**ARTICLE III: PROTEIN ENGINEERING OF A THERMOSTABLE POLYOL DEHYDROGENASE.**


The new enzyme polyol dehydrogenase PDH-11300 from *D. geothermalis* was extensively characterized regarding its temperature optimum and thermostability. A peptide stretch responsible for substrate recognition from the PDH-11300 was substituted by this particular stretch of a homolog enzyme, the galactitol dehydrogenase from *Rhodobacter sphaeroides* (PDH-158), resulting in a chimeric enzyme (PDH-loop). The substrate scopes were determined and basically the chimeric enzyme represented the average of both wild-type enzymes. A rather unexpected finding was the notably increased $T_{50}^{60}$, by 7°C to 55.3°C, and an increased specific activity against cyclohexanol. Finally, the cofactor specificity was successfully altered from NADH to NADPH by an Asp55Asn mutation, which is located at the NAD$^+$ binding cleft, without influencing the catalytic properties of the dehydrogenase.

**ARTICLE IV: A SELF-SUFFICIENT BAUDRAY-VILLIGER BIOCATALYSIS SYSTEM FOR THE SYNTHESIS OF ε-CAPROLACTONE FROM CYCLOHEXANOL.**


The application of the engineered PDH-loopN mutant [1] *(Article III)* for the production of ε-caprolactone from cyclohexanol was investigated in a co-immobilization approach with the cyclohexanone monoxygenase from *Acinetobacter calcoaceticus*. Biotransformation with solubilized enzymes led to an isolated yield of 55% pure ε-caprolactone with no residual cyclohexanol to be detected. During the immobilization experiments a higher enzyme ratio in favor of the CHMO led to higher reaction velocities. Similarly, the addition of soluble fresh CHMO during reuse of co-immobilization batches significantly increased the activity identifying the CHMO as the bottleneck in this reaction setup.
2 BACKGROUND

2.1 GLYCEROL AS BIO-BASED FEEDSTOCK AND RELATED VALUE ADDED PRODUCTS

Glycerol is a renewable commodity descending mainly and in large quantities from the production of biodiesel. Biodiesel is the name for the fatty acid methyl esters of rapeseed or other oilplants that meet the specifications summarized in DIN EN 14214. During the industrial fabrication of biodiesel, 10-14% of the raw oil mass is turned into glycerol [2-4]. Alkalines (hydroxides, carbonates and alkoxides), acids (sulfuric-, sulfonic or hydrochloric acid), or lipases are common catalysts for the transesterification reaction [5]. One goal of the work was the biocatalytic regioselective oxidation of glycerol forming glyceraldehyde or glycemic acid (GA). Turning the glycerol into such value added compounds would be of great value and provides a commercial benefit for the biodiesel producing industry. It has the potential to stabilize the biodiesel price and thus increases the planning reliability for the biofuel industry and related areas.

Glycerol can be turned into various value added products. Glyceraldehyde is the chiral product of the oxidation at the primary alcohol group. GA can be formed by subsequent oxidation of glyceraldehyde (Figure 1). Standard chemical oxidations (e.g. Jones [6], Dess-Martin [7], or Swern [8]) lack regio- and enantioselectivity and lead to unwanted side products. Selective oxidation at the primary hydroxyl groups using molecular oxygen from air can involve palladium, platinum, gold or metal oxides in classic heterogenic catalysis with up to 92% selectivity [9-11]. Industrial production of glyceraldehyde involves hydration of glycidaldehyde based on acrolein [12].

Glyceraldehyde and GA are the targeted compounds in Articles I and II. Both compounds could serve as valuable chiral building blocks in chemical synthesis. However, since glyceraldehyde and GA represent rare fine chemicals, they have not yet been used in industrial synthesis routes. Few scientific applications are published, e.g. glyceraldehyde can serve as structural motif in 4-(dihydroxyalkyl)-β-lactams [13, 14]. GA is known as a liver stimulant in dogs and has effects on the acetaldehyde detoxification in rats [15, 16]. GA is a building block in glycerate phospholipid analogues. Acyl glycerate derivatives have an “inverse ester” function at the carboxylic group of the GA compared to natural phospholipids that circumvents the hydrolysis by phospholipase PLA1 [17]. GA furthermore can be used as a novel monomer for biobased polymers [18]. Recently, GA has been converted into dilinoleoyl- and dipalmitoyl-D-glycemic acid for elucidation of its applicability in skin care products [19, 20].
2.2 INTRODUCTION OF THE APPLIED OXIDOREDUCTASES AND TECHNIQUES

ALDITOL OXIDASE AND ENZYME MUTAGENESIS

Biocatalytic oxidation of polyols and hence the biooxidation of glycerol can be done either using polyol dehydrogenases or oxidases. Alditol oxidase (AldO) from the actinomycete *Streptomyces coelicolor* A3(2) is a soluble monomeric flavoenzyme containing one flavin adenine dinucleotide (FAD) molecule covalently attached to a histidine residue [21]. The enzyme catalyzes the regioselective oxidation of several alditols (polyols) at the terminal C-atom to the corresponding aldehyde. At the same time FAD is reduced and reoxidized in a second reaction while the electrons are transferred to molecular oxygen forming H₂O₂ [22]. It was proposed that substrates oxidized at the primary alcohol group are subsequently further oxidized to the corresponding carboxylic acid (Figure 1, [23]).

![Reaction scheme of AldO-catalyzed oxidation of glycerol forming glyceraldehyde and subsequently glyceralic acid. During the two step reaction two moles of hydrogen peroxide are released](image)

Figure 1: Reaction scheme of AldO-catalyzed oxidation of glycerol forming glyceraldehyde and subsequently glyceralic acid. During the two step reaction two moles of hydrogen peroxide are released.

Article I focused on the elucidation of the reaction products of the glycerol oxidation, their chiral composition, as well as alditol oxidase improvement by directed evolution and site directed mutagenesis. Directed evolution [24] describes the process of improving an enzyme feature without the need to have a detailed knowledge of the enzymes structure function relationship. It involves screening and/or selection for an evolved beneficial property e.g. by application of a high-throughput enzyme activity tests based on either microtiter-plates or selective or color-changing media on agar plates. Error-prone polymerase chain reaction (epPCR) was applied in Article I and is a common mutation method to randomly introduce point mutations [25]. The engineering of enzymes by site-directed mutagenesis requires certain knowledge about the enzyme structure and mechanism. That information can be obtained directly analyzing a crystal structure, by building a structure homology model or by aligning highly homologous protein sequences. Site-directed mutagenesis represents the introduction of point mutations by using mutagenic primers [26], and is achieved by using specified degenerate primer pairs.

ALDEHYDE DEHYDROGENASE AND ELECTROLYTIC COFACTOR RECYCLING

The aldehyde dehydrogenases investigated during this work belong to the enzyme class EC 1.2.1.3 catalyzing the irreversible oxidation of a wide variety of endo- and exogenous aldehydes to their corresponding carboxylic acids. The protein YdcW of *Escherichia coli* belongs to the superfamily of aldehyde dehydrogenases (ALDHs), and was annotated as betaine aldehyde dehydrogenase (EC 1.2.1.8). According to the current kinetic studies the enzyme can be more precisely denoted as medium chain aldehyde dehydrogenase (EC 1.2.1.3) [27, 28]. Like many other ALDHs the crystal structure of the protein reveals a homotetrameric quar-
ternary structure with four catalytic sites and one nicotinamide adenine dinucleotide (NAD\(^+\)) bound to each subunit. The subunits consist of a catalytic domain, a cofactor binding domain, and an arm-like oligomerisation domain including the C-terminus. The enzyme Dgeo 1120 is an ALDH from the radiation resistant and thermophile organism *Deinococcus geothermalis* (type strain DSM 11300) [29, 30] and was annotated as succinic-semialdehyde dehydrogenase. The reaction mechanism of human ALDHs has been identified as an ordered sequential mechanism: (i) NAD\(^+\) cofactor and aldehyde binding to the enzyme, (ii) nucleophilic attack by the sulfur of a catalytic cysteine residue and (iii) transfer of the hydride to the C4 of the nicotinamide moiety. The conformational change of the NADH then favors the activation of a water molecule, which attacks the substrate-enzyme-thioester bond and the corresponding carboxylic acid is released prior to the NADH [31-33].

The ALDHs used within this work depend on NAD\(^+\) as electron acceptor. Preparative scale biotransformations using dehydrogenases generally require cofactor recycling systems in order to establish a cost-efficient process. This work focused on the biotransformation of glyceraldehyde to glyceric acid, mediated by an electrochemical cofactor recycling system (Figure 2). Unfortunately, the oxidation of NADH at carbon electrodes in neutral or alkaline buffers requires a high overpotential, which leads to non-specific side products and enzyme deactivation. To circumvent the high potentials, 2,2'-azinobis(3-ethylbenothiazoline-6-sulfonate) (ABTS) can be used as redox mediator. ABTS spontaneously oxidizes NADH and can be re-reduced at glass-like carbon electrodes at lower potentials than NADH [34].

![Scheme of the ALDH catalyzed oxidation of glyceraldehyde to glyceric acid. NAD\(^+\) is simultaneously reduced to NADH, which in turn is recycled by ABTS.](image_url)

**Engineeering of enzyme thermostability and polyol dehydrogenases**

Biocatalysts for industrial processes have to compete with established pure chemical processes and need to match both, chemical and economical demands. Especially thermostable enzymes are highly useful since they exhibit stability over a broad temperature range. This feature is essential if the advantages of elevated reaction temperatures, i.e. better substrate solubility, reduced medium viscosity and a lowered risk of microbial contaminations, should be exploited [35].

The known structural reason for thermostability in enzymes is a high structural rigidity. Improvements of the structural rigidity can be achieved by enhancing molecular interactions like salt bridges, H-bonds and π–π effects [36, 37]. A major contribution to the engineering
of enzyme thermostability was the development of the ‘B-factor iterative test’ (B-FIT, [38]), which is based on the observation that certain residues in protein crystal structures cannot be fully resolved due to a high flexibility of these side chains. The B-value is a measure for this flexibility and can be easily retrieved from pdb-files of protein structure data by means of the program PyMol or the B-FIT software and can consequently help to guide the design of more thermostable variants of enzymes by protein engineering [38, 39].

Eijssink et al. suggested three ways to access enzymes with improved thermostability: (1) isolating enzyme variants from organisms living in extreme environments, (2) rational-based mutagenesis considering all accessible structural information towards enzyme structure stabilization, and (3) directed evolution with random mutagenesis followed by screening or selection rounds [35]. In this work on polyol dehydrogenases (PDH) approaches (1) and (2) were applied for the engineering of a PDH towards higher thermostability.

The two polyol dehydrogenases (PDH) that were investigated for this purpose originate from *Rhodobacter sphaeroides* DSM 158 (PDH-158) and from *Deinococcus geothermalis* Dgeo 2865 (PDH-11300). Polyol dehydrogenases comprise a large family of oxidoreductases active towards di- or polyhydroxylated compounds. Both enzymes are classified as short chain dehydrogenase/reductases (SDR) and share 51% sequence identity. The galactitol dehydrogenase from *Rhodobacter sphaeroides* D is a polyol dehydrogenase evolved from a chemostat culture [40]. The crystal structure of this enzyme (pdb-code: 2wzd) was used for homology modeling, since no structure for PDH-158 is available. The PDH-158 differs in five amino acid residues from the galactitol dehydrogenase from *R. sphaeroides* D. The differing positions are A45G, Q53E, A61S, E79A and E208G (the galactitol dehydrogenase residue is named first).

### 2.3 ε-CAPROLACTONE AND CYCLOHEXANONE MONOOXYGENASE

One part of this thesis focused on the development of a new synthesis route to ε-caprolactone (ε-CL). ε-CL was formerly of great industrial relevance and has regained importance due to biomedical applications. The former industrial process was using ε-CL as intermediate during the production of ε-caprolactam for the nylon fabrication (20,000 t/a). ε-CL is produced starting from cyclohexanone (CHO) using the Baeyer-Villiger oxidation with acetic acid as byproduct [41]. Today the major purpose for ε-CL (40–60,000 t/a in 2003 [42]) is the ring-opening polymerization forming the thermoplastic poly-ε-caprolactone (PCL). PCL is of commercial relevance due to its application as biodegradable plastic. Several examples were published using PCL in blends, compounds or as grafting polymer in combination with polylactides or other biodegradable polymers [43, 44]. Due to its good bio-absorption properties PCL is used in suture material blends (e.g. Caprosyn™) and research is carried out towards PCL application as 3D scaffolds for tissue engineering via rapid prototyping [45, 46].

During the ε-CL formation the carbonylic group of CHO undergoes a nucleophilic attack by the hydroxyllic group of a peroxycarboxylic acid. This introduction of oxygen adjacent to a
The natural counterpart of the Baeyer-Villiger reaction catalyzed by a Baeyer-Villiger monooxygenase (BVMO) was first described while elucidating the cyclohexanol metabolism of Acinetobacter species [49]. The responsible enzyme, cyclohexane monooxygenases (CHMO), is characterized as mixed function oxidase [50] and belongs to the enzyme class EC 1.14.13.22. The cofactor dependency to reduced nicotine adenine dinucleotide (NADPH) and flavin adenine dinucleotide (FAD) classifies this enzyme as type-1-BVMO [51]. The catalysis cycle of the CHMO starts with NADPH to enter the binding site and reduce the FAD moiety through a hydride shift. Oxygen is bound to the complex and electrophilically attacks the FADH'. The peroxy group attacks the carbonyl group of the CHO substrate leading to the Criegee-intermediate. The rearrangement of this intermediate leads to the ε-CL and a hydroxylated form of the isoalloxantin residue, C4a hydroxyflavine. The elimination of water and the dissociation of the lactone and the NADP⁺ complete the catalytic cycle (Figure 3) [52, 53].

Subsequent regeneration of the NADPH cofactor is crucial in order to operate an economic synthesis process. Recycling systems for BVMO reactions use either whole cells systems or isolated NADP-reducing enzymes. Both approaches have their advantages and drawbacks. As the biggest advantage, whole cells make enzyme purification and additional recycling enzymes needless. Isolated enzymes can be advantageous because of higher activities per mass unit and faster diffusion rates to active sites [52, 54, 55]. One possibility to set up a recycling scheme is the closed loop system [56]. In closed loop systems the advantage is that the product of the first NADPH generating reaction serves as substrate for the second NADPH consuming reaction. Such a process has a high level of efficiency since no additional substrate is required for the cofactor regeneration and consequently no by-product will interfere the isolation of the actual desired product.
3 RESULTS

3.1 ENGINEERING OF AN ALDITOL OXIDASE AND OXIDATION OF GLYCEROL

The work on the alditol oxidase (AldO) started resting upon the findings of van Hellemont et al., who reported an alditol oxidase from *Streptomyces coelicolor* A3(2), which readily accepts glycerol as a substrate, but with poor catalytic efficiency compared to its preferred substrate sorbitol [23]. The recombinantly expressed His-tagged AldO wild-type enzyme (AldO-WT) with 2.17 U·mg⁻¹ is most active towards sorbitol. An activity of 0.26 U·mg⁻¹ (12% of the activity for sorbitol) was observed towards glycerol. Towards glyceraldehyde the enzyme activity was still 70% of the glycerol specific activity.

Our three goals were to improve the AldO-activity towards glycerol, the application of AldO for the formation of glyceraldehyde and glyceric acid as well as the designation of the chirality of the oxidation product.

Mutant libraries for the generation of improved AldO mutants were established by error-prone polymerase chain reaction (epPCR) as well as site-directed mutagenesis. The epPCR approach led to a library of 8,000 clones with a mutation rate of 1-4 per gene. 1,600 of these clones were active during pre-screening with a colorimetric agar plate assay. The positive clones were screened more precisely by cultivation in auto-induction medium in deep-well plates. Those clones that showed a cell density comparable to the *E. coli* strain bearing the wild-type AldO (AldO-WT) and exhibited activity against glycerol where further examined in shake-flask experiments. The evolved enzymes bearing the mutations V133M, G399R and V125M/A244T have increased activity against glycerol (Figure 4, red). The enzymes contain

![Figure 4: Identified beneficial AldO mutants. Mutants evolved during the first round of epPCR are shown in red. Mutants designed by site-directed mutagenesis combining the (red) random mutations identified by the epPCR approach are shown in blue.](image-url)
results

substitutions predominantly within the FAD-binding domain. The mutant AldO-V125M/A244T has the amino acid substitutions V125M and A244T and has a 1.55-fold increased activity against glycerol. A second round of epPCR with AldO-V125M/A244T as template yielded in 2,700 mutants, but no further improved enzyme was identified.

The mutations obtained in the random mutagenesis approach were subsequently combined by site-directed mutagenesis (Figure 4, blue). Three mutants with improved activity were identified. Introducing the mutation V133M into AldO-V125M/A244T led to a 1.6-fold improvement of activity, combination of the mutants AldO-V125M/A244T and AldO-G399R led to a 2-fold increase in activity (always compared to AldO-WT activity).

The combination of four mutations, V133M, G399R as well as the initial mutations V125M and A244T led to a 2.4-fold increased specific activity and hence to the best enzyme created during this study. The mutations A64V, G236D or G399W were found with improved activity in the epPCR approach. However they had no effect, or even decreasing activity if combined with any of the mutations mentioned above.

In order to identify further hotspots for a rational design approach, glycerol was docked in silico into the known AldO crystal structure (pdb-code: 2vft, [57]). The structure model revealed polar contacts between glycerol and E320, R322 and H274 (Figure 5). Since E320 shares three polar contacts with glycerol at all of its hydroxylic groups, we assumed that due to this residue the glycerol cannot move deep enough into the binding pocket. Residues E320, Q288 and T345 were subjected to a saturation mutagenesis. The positions Q288 and T345 were chosen in order to guide the glycerol closer to the FAD and K375. Three different libraries (Q288 and E320, Q288 and T345, and all three simultaneously) were constructed using the NNK codon for the selected positions. Screening of three mutant libraries failed to identify any mutation that gave improved activity towards glycerol oxidation. Moreover, the mutations where mainly found in inactive mutants. All investigated single and double mutants revealed a poor expression.
The temperature optimum for both of the two selected mutants AldO-V125M/A244T and AldO-V125M/A244T/V133M/G399R as well as AldO-WT is 50°C and the residual activity at 20°C is still 60%. All three enzymes retained 70% activity after incubation at 50°C for 30 min and they lost their activity after 30 min at 60°C. The pH optimum of both mutants and the WT was between pH 8.0–9.0, while no activity could be detected below pH 5. All three enzymes retained 75% of their initial activity after 30 min incubation across a pH range from 7.0 to 11.0 in the presence of glycerol.

The improvement of the enzyme is mainly explained by the decrease of the Michaelis-Menten constant $K_m$. The best mutant has only a slightly better turnover number than the WT, but the $K_m$ is lowered to 40% of the wild-type $K_m$ (Table 1). Since no mutation in the active site and therefore no direct change in the substrate binding was evident, the increased specific activity must be explained by long ranging structural effects. The mutations V125M and V133M introduce sterically more demanding amino acids at the intersection of a loop region and an α-helix (in case of V125) and two disordered loops (in case of V133). This could have an impact on the tertiary structure of both enzymes. Especially in the region around V133 a significant move of the strand opposite of the mutation was observed during molecular dynamic simulations using YASARA [58].

### Table 1: Overview of the kinetic parameters of the AldO wild-type enzyme and two of the best mutants.

<table>
<thead>
<tr>
<th>AldO enzyme</th>
<th>$K_m$</th>
<th>$v_{max}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>266</td>
<td>0.12</td>
<td>5.65</td>
<td>2.13</td>
</tr>
<tr>
<td>V125M/A244T</td>
<td>157</td>
<td>0.11</td>
<td>4.96</td>
<td>3.16</td>
</tr>
<tr>
<td>V125M/A244T/V133M/G399R</td>
<td>111</td>
<td>0.12</td>
<td>5.71</td>
<td>5.14</td>
</tr>
</tbody>
</table>

The A244T mutation of both improved variants and the G399R mutation in the most reactive variant are located on the surface of the protein. Surface mutations would not be expected to influence the shape of the active site, but could have strong effects on solubility and folding. We believe that the improved activity resulted from both, a decrease in $K_m$ and from a higher amount of correctly and more actively folded enzyme in *E. coli* C43 (DE3) compared to AldO-WT.

Molecular dynamics simulation unveiled an additional potential explanation for improved mutant activities. The difference between the AldO mutants is based on a slight general change in the overall structure. The most important effect is a different binding pattern of glycerol. The distance between the hydride accepting N5 atom of FAD and glycerol C1 decreases from 3.84 Å in the AldO-WT to 3.74 Å in AldO-V125M/A244T and even 3.42 Å in AldO-V125M/A244T/V133M/G399R (Figure 6). Considering the hydride shift distance as major important impact on stereoselectivity, one could predict that all of the three enzymes favor the oxidation of the pro-R carbon. This in fact was verified experimentally. We observed that the increased activities of the AldO mutants correlate with the decreased dis-
istance between the pro-R C1 and FAD N5 of the calculated structures, and that the product of glycerol oxidation is always the (R)-enantiomer.

The reaction with glycerol using either purified AldO-WT, AldO-V125M/A244T or AldO-V125M/A244T/V133M/G399R confirmed that the final oxidation product of glycerol is glyceraldehyde for all three variants. The oxidation of 30 mM glycerol with the WT yielded 15.7 mM GA and 3 mM glyceraldehyde after 40 h. Chiral HPLC showed that the final product of the two-step glycerol oxidation was D-(R)-glyceraldehyde with >99%ee. All three enzymes showed the same enantioselectivity, i.e. oxidation of the alcohol group of the pro-R carbon atom. Biotransformation with whole E. coli cells resulted in 19 mM D-glyceraldehyde (2.0 g·l⁻¹) with 99.6% ee after 60 h at a conversion of 6.3%. We have to admit, though, that the productivity of our system cannot compete with recently published systems like the formation of D-glyceraldehyde using whole cells like Acetobacter or Gluconobacter sp. [59, 60].

We showed that directed evolution and combination of best mutants led to the identification of improved variants, the best having 2.4-fold higher specific activity against glycerol. However further efforts are needed to make this oxidase suitable enough for applications in biocatalysis for glyceraldehyde production or for in situ generation of hydrogen peroxide as laundry bleach. The distance to K375 could explain the low conversion of glycerol compared to sorbitol since K375 is supposed to be part of the catalytic mechanism [57]. The results indicate that the positions Q288, E320 and T345 within the binding site are crucial to the structural integrity of the enzyme.

3.2 Deracemization of Glyceraldehyde using Electrolytic Cofactor Recycling

As the chemical glyceraldehyde synthesis results in a racemic mixture of D,L-glyceraldehyde, a kinetic resolution is inevitable in order to obtain pure glyceraldehyde or glyceraldehyde. Here,
an enzymatic method for the kinetic resolution using aldehyde dehydrogenase coupled to electrolytic cofactor regeneration is presented.

Two enzymes, ALDH-BL21 from *E. coli* and ALDH-11300 from *D. geothermalis* could be expressed in active form with and without His6-tag. Both enzymes showed the highest activity at pH 8.5 in Tris-HCl. At pH 5.5 the enzymes showed residual activities of 10% and a complete loss of activity took place at pH 4.0 (Figure 7-A,B). ALDH-BL21 has a temperature optimum of 35°C. ALDH-11300 has a temperature optimum of 45°C and is hence exactly situated at the border between the mesophilic and thermophilic enzymes (Figure 7-C). ALDH-BL21 is more stable than ALD-11300 during storage at 0°C. Storage of purified enzymes at −20°C resulted in complete loss of activity, but freeze-dried ALDH-11300 retains 80% of its activity towards glyceraldehyde.

The cloned ALDH-BL21 has a specific activity of 0.6 U·mg\(^{-1}\) against glyceraldehyde and differs in one amino acid (V197I) from the *E. coli* K12 enzyme, which previously has been described by Gruez et al. [28]. The cloned DNA sequence of the gene *ydcW* differed in one nucleotide (A994G) to the database entry B21_01412. This difference leads to the mutation S332G at the protein level in ALDH-BL21. The newly characterized ALDH-11300 has a much higher specific activity of 3.5 U·mg\(^{-1}\) towards glyceraldehyde. ALDH-BL21 had only 2% residual activity using NADP\(^+\) as cofactor, ALDH-11300 showed 15% activity using NADP\(^+\) instead of NAD\(^+\). The biocatalytic reduction of glyceraldehyde and glyceric acid was investigated too. However, no activity was observed with both enzymes towards either glyceraldehyde or glyceric acid using the reduced cofactor NADH. Both aldehyde dehydrogenases were not active towards 1-propanol, 1,2-propanediol and glycerol as substrates using NAD\(^+\) as substrate. These findings indicate the chemoselectivity of these enzymes towards the oxidation of a terminal carbonyl group.

ALDH-11300 catalyzed the oxidation of isobutyraldehyde with highest turnover rates (2.9 s\(^{-1}\)). The second and third best turnover rates were observed with glyceraldehyde (2.75 s\(^{-1}\)) and butyraldehyde (2.61 s\(^{-1}\)). The \(K_m\) for glyceraldehyde, however, was the second highest (2.5 mM). These findings indicate that the ALDH-11300 favors substrates with a
RESULTS

chain length of 3-4 and hydroxylated aldehydes. In accordance to the findings of Gruez et al. ALDH-BL21 shows increased turnovers as the chain length of the substrate increases. In contrast to the findings for the K12-ALDH [28], the best substrate for ALDH-BL21 according to $k_{cat}$-values is the C4 substrate butyraldehyde. ALDH-BL21 oxidized glyceraldehyde with a turnover of 0.86 s$^{-1}$.

Since ALDH-11300 showed tenfold better catalytic efficiency for glyceraldehyde conversion than ALDH-BL21 ($k_{cat}/K_m$ 1.1 vs. 0.1), a kinetic resolution of racemic glyceraldehyde was conducted with ALDH-11300. The recycling of the cofactor NADH was carried out by electrolytic reoxidation using a glass-like carbon (glassy-carbon) working electrode via ABTS as mediator. The working electrode consisted of glassy-carbon foam to provide for a large surface area for the re-oxidation of ABTS and was cylindrically surrounded by a platinum counter electrode.

During cyclic voltammetry (CV) measurements we could affirm, that no conflicting reactions like oxidation of glyceraldehyde or GA directly on the electrode are about to interfere the electrolytic cofactor recycling. The first redox system of ABTS (ca. 500 mV) is reversible and important for the NADH reoxidation (Figure 8, plot d). The second redox system (850 mV) is irreversible and must be avoided as otherwise ABTS is eliminated from the equilibrium. The addition of enzyme and GA shifts the current peak of the recycling system towards a higher potential, which may be due to blocking of catalytic active sites at the electrode (Figure 8, plot a,b,c). The increase of the overpotential of the recycling system requires the application of a higher potential as would be required for isolated ABTS mediated NADH reoxidation. The potential for the biotransformation should be at least 650 mV vs. Ag/AgCl, hence for biotransformation experiments 700 mV where applied.

Biotransformations using the electrolytic recycling surprisingly differed in the extent of the conversion and the measured transferred charge ($q_m$). Two exemplary biotransformations, A and B, are compared in Figure 9. Starting from 45 mM glyceraldehyde, the concentration of
GA in biotransformation A increased by 16.4 mM while the concentration of glyceraldehyde decreased by 14.5 mM. In biotransformation B the product GA increased by 7.9 mM while glyceraldehyde dropped by 12.7 mM (starting from 45 mM as well). In the beginning of the reactions the $q_m$ was lower than the the theoretically transferred charge ($q_t$). To the end of the reaction the proportion changed and $q_m$ was higher than $q_t$. The difference between $q_m$ and $q_t$ increased with the progression of the reaction time. Since subsequent oxidation of substrate or product at the anode was ruled out by CV measurements, unselective oxidation was not an explanation for neither the differing $q_t$ and $q_m$ nor for the divergent concentrations. The large surface and the high number of pores and cavities of the carbon foam electrode were identified as explanation. Glyceraldehyde and GA diffuse into the pores, and hence evade the chromatographic quantitation. Washing steps of the working electrode after the biotransformation revealed that GA and glyceraldehyde where retained by the electrode and indeed remained in the cavities. This probably led to the systematic error in the determination of the extent of the biotransformation. With ongoing reaction time the enzyme most likely became oxidized at the electrode as described by Manjón et al. for glucose dehydrogenase [61]. This fact presumably accounts for the uncompleted conversion of glyceraldehyde. The enantiomeric excess of the resulting D-GA decreased during both biotransformations, yet no direct oxidation of glyceraldehyde at the electrode could be observed at the applied potential. At the first measurement, the enantiomeric excess of both reactions was still 98.5 % (A) and >99.9%ee (B) but ended up in only 91.8 and 87.9%ee.

In order to investigate the applicability for the kinetic resolution of glyceraldehyde two enzymes converting glyceraldehyde were compared. It was shown that the newly described enzyme from *D. geothermalis* has a mesothermophilic temperature profile making it applicable at around 45°C, nevertheless the ALDH from *E. coli* was more stable upon storage. A new experimental design for the kinetic resolution of glyceraldehyde using electrolytic cofactor recycling could be established. However the reproducible biotransformation due to the complex architecture of the recycling system was challenging and differed strongly in the extent of the conversion from 17 to 36%.

**Figure 9:** Biotransformation experiments A and B differ in the prolonged reaction time and greater number of sample points in biotransformation B. $q_m$ and $q_t$ refer to the left axis, concentrations of glyceraldehyde (GLA), GA and %ee to the two left axes.

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**RESULTS**

3 Results

![Graph A: Biotransformation A](image1)

![Graph B: Biotransformation B](image2)
3.3 ENGINEERING OF A THERMOSTABLE POLYOL DEHYDROGENASE

Polyol dehydrogenases were initially studied in order to evaluate their ability to convert glycerol. When it turned out that the activities were to low, the focus was broadened to include studies and engineering of the temperature stability, substrate spectra and the cofactor dependency.

Two polyol dehydrogenases were investigated during this work. The first one is PDH-158 and originates from *Rhodobacter sphaeroides* DSM 158. The second enzyme is named PDH-11300 and originates from *Deinococcus geothermalis*. It was beforehand unnamed and is encoded by the Dgeo 2865 gene. Both enzymes belong to the enzyme class of short chain dehydrogenase/reductases and share 51% sequence identity. A highly homologue enzyme of PDH-158 is GatDH from *R. sphaeroides* D (pdb-code: 2wdz).

The inspection of the crystal structure of GatDH revealed a loop region (195–203 aa, EMTLKM RER, Figure 10) containing amino acids with high B-values. This particular stretch was also found in the sequence of PDH-158 by means of homology modeling. As it was described for GatDH, this loop is located next to the active site and is a flexible substrate binding loop which fits to several different substrates [62]. Based on this knowledge a chimeric enzyme was constructed introducing this loop of PDH-158 into the protein scaffold of PDH-11300. In the alignment of the amino acid sequence of PDH-158 with the PDH-11300 sequence, this loop matched the amino acid positions 196–204 of PDH-11300 (PLTRRGLET). These amino acid residues of PDH-11300 were substituted by the loop of PDH-158 by primer extension PCR to construct a chimeric enzyme, PDH-loop.

Regarding the temperature optima, PDH-11300 and the mutant PDH-loop showed the highest activity at 45°C. At 55°C a significant loss of activity was observed for PDH-11300. Mutant PDH-loop still had 64% activity at 60°C whereas the wild-type was not active any more. While testing the temperature stability, PDH-11300 revealed no loss in activity during 6 h of incubation at 40°C. During incubation at 45°C a linear decrease in activity was observed (12.5% residual activity after 6 h). The PDH-158 activity already decreased during incubation at 30°C (Figure 11-A,B). The mutant PDH-loop was stable for 6 h of incubation even at a temperature of 50°C (Figure 11-C). The comparison of the melting points ($T_m$) of the enzymes resulted in a similar ranking of the thermostability (Table 2). The PDH-158

![Figure 10: Localization of the stretch showing the high B-values (from red: high B-value to blue low B-value) at the top of the substrate binding site of one monomer of the tetrameric galactitol dehydrogenase of *Rhodobacter sphaeroides* D (pdb-code: 2wdz).](image)
Figure 11: Activity plots of PDH-158 (A), PDH-11300 (B) and PDH-loop (C) after incubation up to 6 h at the given temperatures for elucidation of the thermostability of the enzymes.

possessed the lowest $T_m$ (43.2°C), whereas PDH-11300 and PDH-loop showed a 5.2°C (48.4°C) and 10.4°C (53.6°C) higher melting point, respectively. The thermostability of PDH-11300 facilitated an easy purification step by heat treatment. Incubation at 50°C for 10–30 min yielded in a 1.7-fold higher purity without loss of activity. Regarding the pH profile, PDH-11300 showed the optimum in bicine buffer at pH 9.0. PDH-loop was still active in basic pH-range above 9.0 and showed highest activity at pH 11 in glycine-NaOH buffer (140% compared to bicine buffer at pH 9.0).

In the homology model of PDH-11300 four residues of the substrate binding pocket were identified that differed from the PDH-158 structure. This part of the binding pocket of PDH-158 displays a highly non-polar surface whereas this pocket of PDH-11300 shows a more polar surface, which is attributed to the four differing amino acids. These four residues therefore could represent determinants of substrate specificity and activity. To verify this assumption, four mutations (V97A, N99L, Q157A and N161M) were introduced to create mutants of PDH-11300 bearing a nonpolar binding pocket, resembling that of PDH-158. Based on the structural analysis five different mutant enzymes were constructed with PDH-loop as template, expressed and purified (Table 2).

The $T_m$ of the active site mutants revealed no mutant with a melting point lower than PDH-11300. The PDH-loopQ157A and PDH-loopV97A/N99L variants showed no decreased melting point, but the mutant PDH-loopN99L had an increase of the $T_m$ by 3°C. A combination of the double mutant and mutation Q157A gave a $T_m$ reduction by 5°C for the PDH-loopV97A/N99L/Q157A triple mutant (Table 2).

The cofactor dependency of PDH-11300 and mutants is strictly linked to NAD$. In order to broaden the cofactor specificity of PDH-loop, a further point mutation was introduced in order to relax the cofactor specificity of the enzyme. The bases of the NAD(H) dependence of SDRs and hence the incapability to use NADP(H) as cofactor is mostly due to ionic repulsion of certain residues in the cofactor binding cleft. For horse liver dehydrogenase this residue was shown to be an aspartate residue [63]. In order to make the PDH-loop suitable for combination with NADP(H) dependent enzymes (for example as thermostable cofactor recycling enzyme), the principle of the cofactor specificity of PDH-11300 was investigated in detail.
Table 2: Melting points and $T_{50}^{60}$ of all established mutants and wild-type enzymes. The abbreviations in brackets refer to Table 3.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$T_m$ [°C]</th>
<th>$T_{50}^{60}$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH-158</td>
<td>43.2 ± 1.0</td>
<td>35.6 ± 0.4</td>
</tr>
<tr>
<td>PDH-11300</td>
<td>48.4 ± 0.5</td>
<td>48.3 ± 0.3</td>
</tr>
<tr>
<td>PDH-loop</td>
<td>53.6 ± 0.2</td>
<td>55.3 ± 0.2</td>
</tr>
<tr>
<td>PDH-loopD55N</td>
<td>65.5 ± 1.0</td>
<td>56.1 ± 0.2</td>
</tr>
<tr>
<td>PDH-loopN99L (N-L)</td>
<td>56.9 ± 0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>PDH-loopQ157A (Q-A)</td>
<td>52.9 ± 0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>PDH-loopV97A/N99L (2x)</td>
<td>53.8 ± 0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>PDH-loopV97A/N99L/Q157A (3x)</td>
<td>48.5 ± 0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>PDH-loopV97A/N99L/Q157A/N161M (4x)</td>
<td>48.8 ± 0.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Examining the homology model of the PDH-loop, the aspartate residue D55 could be identified pointing towards the ribose C2 hydroxyl group of the NAD$^+$ moiety. We assumed that this residue would repel the negatively charged phosphate group of the hypothetically bound NADP$^+$. After substitution of the C2 hydroxyl group with the phosphate group to build NADP$^+$ in silico, two subsequent energy minimizations were conducted. During this MD simulation the adenosine moiety of NADP$^+$ moved far out of the binding cleft of the PDH-loop. Based on this observation, the single mutant D55N was created in silico and subsequently a MD simulation was performed. As predicted, the phosphate group now was coordinated in a binding site flanked by amide-H-bond donor groups of the backbone of Q34, L56, N57 and amid groups of N57 and the newly introduced N55.

Compared to the PDH-loop variant, the $T_m$ of the cofactor mutant PDH-loopD55N was increased by 11.9°C to 65.5°C even though the enzyme differs in just one residue. For PDH-11300 and PDH-loop the $T_{50}^{60}$ values were nearly comparable to the melting points whereas for PDH-158 and the PDH-loopD55N variant the $T_{50}^{60}$ was about 8–9°C lower than the $T_m$ value. The $T_{50}^{60}$ of PDH-11300 was 48°C, which was 13°C higher than the value of PDH-158. The $T_{50}^{60}$ of PDH-loop (53.6°C) was increased by 7°C compared to the wild-type and the tendency roughly confirms findings of the stability measurements. The substrate scopes of the two PDH wild-type and seven mutant enzymes (Table 3) exhibit a bias towards vicinal diols. All enzymes preferably oxidize secondary compared to primary alcohols (Figure 12). 1,2-hexanediol, 1,2-butanediol and 1,2-propanediol (in the order of decreasing activity) were among the best substrates for each enzyme. 1,2-hexanediol was the best substrate for PDH-158. In contrast, 1,2-hexanediol was only second best for PDH-11300, here xylitol was the best substrate. Galactitol, the third best substrate for PDH-11300, was a bad substrate for PDH-158, even though galactitol is the naming substrate for this enzyme subclass. In mutant PDH-loop, the order of the best substrates resembled more the scope of PDH-158 than PDH-11300.
While glycerol was the sixth best substrate for PDH-158, the activity could not be recovered by rebuilding the hydrophobic binding pocket of the PDH-158 in the single or combined PDH-loop point mutants. The substrate scope of mutant PDH-loopV97A/N99L among the last five mutants of Table 3 had the highest correlation with the substrate scope of the PDH-loop variant \((r = 0.94)\). As expected, the correlation of substrate scopes was also high between the PDH-loop and PDH-loopD55N \((r = 0.93)\). The residues Q157 and N161 seem to have an essential role, especially for conversion of 1,2-diols, since enzyme variants containing mutations at these sites exhibited significantly decreased enzyme activities. The positions V97 and N99 were mutated without significant activity loss, and hence might be candidates for further saturation mutagenesis studies. In the case of 1,2-hexanediol the combination of the two substitutions V97A and N99L led to improved specific activity. The substrate scopes of PDH-158 and PDH-11300 correlate by \(r = 0.81\). In fact the correlations of PDH-loop with the PDH-158 \((r = 0.78)\) and PDH-11300 substrate scope \((r = 0.77)\) are slightly smaller. The explanation might be that the loop region is not the only determinant of substrate specificity.

PDH-11300 and PDH-loop both showed a \(K_m\) of 150 µM against NAD\(^+\). The substitution of the substrate recognition loop hence did not affect the co-substrate binding affinity, still the NAD\(^+\) turnover was increased by 68% to 2.7 \(s^{-1}\) (from 1.6 \(s^{-1}\) in PDH-11300). Activity towards NADP\(^+\) could be found neither for PDH-11300 nor for PDH-loop. The relaxed mutant PDH-loopD55N showed activity against both, NAD\(^+\) and NADP\(^+\), all the kinetic parameters where comparable resulting in a catalytic efficiency of \(-3.5 \text{ s}^{-1} \cdot \text{mM}^{-1}\) for either of the cofactors. The findings reinforce the prediction that the NADP(H) phosphate group is stabilized by polar contacts in an H-bond donor site. A minor disadvantage of PDH-loopD55N was the lower expression level for soluble enzyme compared to the PDH-loop variant, which was addressed in article 4.
The observation of an increased thermostability (10°C) of the chimeric enzyme compared to PDH-11300 was a rather unexpected finding. The mutant contained the flexible loop of the non-thermostable PDH-158 (merely stable for 6 h at 25°C). The increase in thermostability was proven by means of the $T_m$, long-term stability and $T_{50}$.

In literature the stabilization of enzymes is described as a gain of rigidity, or is reported as stabilization by substitution of flexible residues. An explanation might be that the corresponding wild-type loop is much more flexible than the PDH-158 loop, even though this seems unlikely since PDH-11300 turned out to be more thermostable than PDH-158. Multimeric enzyme stabilization can also

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**Table 3:** Substrate oxidation scope of PDH wild-types and mutants determined with 0.5 mM NAD$^+$ in bicin buffer (pH 9.0; 100 mM). For mutant PDH-loopD55N NADP$^+$ was used as cofactor. Specific activities are given in mU/mg, the level of the activity is indicated by gray shading. Concentrations are given in Article III.

<table>
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<th>Substrate</th>
<th>158 N-L $^a$</th>
<th>11300 N-L $^a$</th>
<th>loop D55N</th>
<th>N-L $^a$</th>
<th>Q-A $^a$</th>
<th>2x $^a$</th>
<th>3x $^a$</th>
<th>4x $^a$</th>
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$^a$ Abbreviations are explained in Table 2.
occur due to the enhancement of subunit interaction. The altered loop regions are not located at the multimerization interfaces of the tetramer but are located on the opposite sites. Due to the far distance no additional H-bonds or hydrophobic interactions were directly introduced. Nevertheless it might be possible that due to interactions or repulsions between residues inside the subunits longer ranging effects influence the subunit binding. To the best of our knowledge, the chimeric enzyme PDH-loop represents the first example of enzyme stabilization by introduction of a loop from a less thermostable to a moderately thermostable enzyme. The amino acid positions V97 and N99 were mutated without significant activity loss while the further introduced mutations led to severe activity loss. V97 and N99 hence might be candidates for further saturation mutagenesis studies. The activity of PDH-loop against CHL was improved remarkably. In order to make and PDH-loopD55N compatible with NADPH-dependent enzymes, the cofactor dependency to NAD(H) was successfully abolished. This point led us to the follow-up study in Article IV where we studied the utilization of PDH-loopD55N for the production of cyclohexanone and subsequently ε-caprolactone.

### 3.4 PRODUCTION OF ε-CAPROLACTONE WITH CLOSED LOOP RECYCLING

Having found a variant of a polyol dehydrogenase with a good activity towards cyclohexanol (CHL) a new self-sufficient recycling system for the conversion of CHL into ε-CL was established. The polyol dehydrogenase PDH-loopD55N (PDH-loopN) was initially engineered in order to enhance its thermostability, but as a secondary effect the activity against CHO was improved significantly (Article III). PDH-loopN was used to regenerate the NADPH for the subsequent CHMO-catalyzed conversion of CHO into ε-CL (Figure 13). In order to obtain an easy to use, self-sufficient catalyst, the CHMO and the PDH where co-immobilized on an amino-functionalized enzyme carrier material.

Poor expression of the PDH-loopN mutant made it necessary to optimize the heterologous expression. A commercial chaperone set (Takara, Cat. 3340) bearing five different chaperone combinations was applied to enhance the overexpression of PDH-loopN (Figure 15). It turned out that coexpression of the plasmid pGro7 bearing the chaperones GroES and GroEL yielded highest volumetric activity in *E. coli* BL21 (1350 U·l⁻¹). Three different *E. coli* strains, BL21, C41 and SHuffle were tested for the best expression as well. The best overall result was obtained with coexpression of PDH-loopN and GroES/GroEL in *E. coli* C41 (1592 U·l⁻¹). This represents a 4.9 fold increase compared to the initial conditions in *E. coli* BL21 without chaperones.
Successful biotransformations were carried out using solubilized enzymes. Application of high enzyme amounts during biocatalysis (0.5 U) led to a conversion of 84 and 80% based upon ε-CL formation with 5 and 10 mM CHL, respectively. This conversion was observed after 2 h and only slightly increased after incubation for 4 h.

The CHMO was identified as the bottleneck of the two-step reaction using a limited enzyme amount. Highest conversions (84%) were observed with a threefold excess of CHMO over PDH. Equal amounts of enzymes gave a conversion of 63% and even lower conversions were obtained with CHMO in shortfall (22%). The reaction turnover limitation of the CHMO could be explained by CHL inhibition. The initial activity of the CHMO strongly decreases to 30% already at 10 mM CHL. The PDH on the contrary showed good performance until a substrate concentration of 70 mM and had no serious activity loss at 90 mM CHL (Figure 14). Accordingly, the PDH is not as much affected by higher CHL concentrations as the CHMO and applies better for elevated substrate concentrations following this reaction scheme.

Subsequently to a small preparative scale biocatalysis, ε-CL was simply extracted with dichloromethane followed by the evaporation of solvent and was isolated in 55% yield with a purity >99%. After 2 h no residual CHL could be detected. The low yield might be attributed to the high volatility of CHL during biocatalysis and ε-CL during the evaporation step.

Reusable biocatalysts are beneficial due to higher total turnover numbers during synthesis resulting in higher economic efficiency. The reusability of the free enzymes was investigated firstly by simple filtration through a 10 kDa membrane leading to a low residual activity of only 10% in the second cycle.

Enzyme immobilization was carried out in order to facilitate the reuse of both enzymes and as well to enhance CHMO activity. The highest activity of co-immobilized biocatalyst was obtained using an enzyme ratio of 10:1 (CHMO:PDH) covalently bound to Relizyme™ HA403
Results

Using glutaraldehyde as linker molecule. Activity determination at increasing concentrations of CHL showed that CHMO was significantly stabilized against higher concentrations of CHL by immobilization. Up to 40 mM CHL could now be used without significant loss of activity, whereas the free CHMO retained only 15% activity at 25 mM CHL. The maximum conversion using the immobilized biocatalysts was only 34% at 10 mM (after 5.5 h). This low overall conversion again is due to the low stability of the immobilized CHMO.

Repeated 1 h biotransformations using the same co-immobilized biocatalyst for each batch resulted in a residual activity of 10% after the fifth cycle (Figure 16). The first reuse step already accounts for a loss of 60% of initial activity. By addition of fresh soluble CHMO to each cycle, the loss of activity in the first cycle was reduced by 35%. The activity of the biocatalyst just slightly decreased in this approach and a remaining activity of 53% was observed after the fifth cycle. By addition of fresh soluble PDH to each cycle no difference to the initial approach without additional enzyme could be observed.

In summary, the recent protein design to extend the PDH-loop for acceptance of the cofactor NADPH enabled the possibility to combine this dehydrogenase with a BVMO for the conversion of an alcohol directly to the corresponding pure lactone (>99%) without any intermittent purification steps. Covalent immobilization of dehydrogenase and BVMO could be successfully applied, but the immobilized CHMO showed a low stability with the support used resulting in lower conversion compared to the free enzymes.

With this promising proof-of-principle study we presented the first example for the combination of a PDH and a BVMO in soluble and immobilized form to enable the direct formation of ε-CL from CHL with no need for an additional cofactor recycling system.
4 CONCLUDING REMARKS

In this thesis new possibilities for the application of oxidative enzymes of different classes and origins were developed. Two new enzymes from *D. geothermalis*, polyol and aldehyde dehydrogenase and an electrolytic cofactor recycling with new electrode geometry are introduced. A rather unsuspected way to engineer a thermostable enzyme and a new route for the formation of ε-caprolactone are presented.

The glycerol oxidation using alditol oxidase yielded in enantiopure D-glyceric acid with a productivity of 2.0 g·l⁻¹ after 60 h. Protein engineering of the alditol oxidase by saturation mutagenesis at selected positions did not lead to improved enzyme variants. However, improved enzymes were found after random mutagenesis of alditol oxidase by epPCR. These improved enzyme variants were further augmented by combination of the revealed point mutations leading to a 2.4-fold improved variant.

Not many enzymes from the radiation resistant and mesothermophilic organism *Deinococcus geothermalis* have been cloned, functionally expressed and characterized. The BRENDA database lists merely four enzymes with *D. geothermalis* as origin. The results gained during this thesis indicate that heterologous expressed enzymes of this strain show moderate thermostability and temperature optima between 45-50°C. The thermostability of *D. geothermalis* enzymes demands a reaction temperature of not more than 40°C for longer terms. Still, the enzymes have the potential for further stabilization by means of protein engineering as proven in the case of PDH-loop and PDH-loopN with increased thermostability by 7-8°C.

The second investigated enzyme of *D. geothermalis* was the aldehyde dehydrogenase. The substrate scope of this enzyme resembles the scope of the known homologue ALDH of *E. coli*, yet the *Deinococcus* enzyme had a higher activity towards glyceraldehyde. Racemic glyceraldehyde was converted into D-GA by means of electrolytic cofactor recycling, however, the conversion of the reaction has the potential to be further improved.

Throughout mutational studies in the new polyol dehydrogenase from *D. geothermalis* the thermostability was increased. The substrate scopes of the resulting dehydrogenase variants were highly similar, yet the activity of PDH-loop against cyclohexanone was improved. Alteration of the cofactor specificity led to the design of a relaxed mutant PDH-loopN accepting both NAD and NADP. This enabled us to combine the PDH-loopN with CHMO for the production of ε-CL in a closed loop recycling system. Additionally, in order to obtain a stabilized biocatalyst, the two enzymes were co-immobilized on commercial amino-functionalized carrier. The CHMO turned out not to be applicable for immobilization since it might loose its cofactor during repeated biocatalysis steps. During this successful proof-of-concept study, pure ε-CL was obtained in a satisfactory yield.
5 Literature


6 ARTICLES AND AUTHOR CONTRIBUTIONS


S.G., H.W. and U.T.B. designed the experiments, S.G. and H.W. conducted the experiments, S.G. authored the manuscript, H.W. co-authored the manuscript. K.H.M. and U.T.B. initiated the project. All authors revised the manuscript.


H.W., M.P., G.S. and U.T.B. designed the experiments. H.W., M.P. and G.S. conducted the experiments, H.W. authored the manuscript, M.P. and G.S co-authored the manuscript, F.S. and U.T.B. revised the manuscript.


* H.W. and H.M. contributed equally to this work. H.W., H.M. and U.T.B. designed the experiments. H.W and H.M. conducted the experiments and wrote the manuscript, U.T.B. revised the manuscript.


* H.M. and H.W. contributed equally to this work. H.W. conceived the concept, H.M. and U.T.B. designed the experiments. H.M. conducted the experiments, H.M., H.W. and U.T.B. wrote and revised the manuscript.

Prof. Dr. Uwe T. Bornscheuer
Asymmetric synthesis of D-glyceric acid by an alditol oxidase and directed evolution for enhanced oxidative activity towards glycerol

Sandra Gerstenbruch · Hauke Wulf · Nina Mußmann · Timothy O’Connell · Karl-Heinz Maurer · Uwe T. Bornscheuer

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Abstract Glycerol as a by-product of biodiesel production is an attractive precursor for producing D-glyceric acid. Here, we demonstrate the successful production of D-glyceric acid based on glycerol via glyceraldehyde in a two-step enzyme reaction with the FAD-dependent alditol oxidase from Streptomyces coelicolor A3(2). The hydrogen peroxide generated in the reaction can be used in detergent, food, and paper industry. In order to apply the alditol oxidase in industry, the enzyme was subjected to protein engineering. Different strategies were used to enhance the substrate specificity towards glycerol. Initial attempts based on rational protein design in the active site region were found unsuccessful to increase activity. However, through directed evolution, an alditol oxidase double mutant (V125M/A244T) with 1.5-fold improved activity for glycerol was found by screening 8,000 clones. Further improvement of activity was achieved by combinatorial experiments, which led to a quadruple mutant (V125M/A244T/V133M/G399R) with 2.4-fold higher specific activity towards glycerol compared to the wild-type enzyme. Through studying the effects of mutations created, we were able to understand the importance of certain amino acids in the structure of alditol oxidase, not only for conferring enzymatic structural stability but also with respect to their influence on oxidative activity.

Keywords Oxidase · Protein engineering · Directed evolution · Rational protein design · Glycerol · Enzyme catalysis

Introduction

The global awareness of upcoming crude oil shortage, an increase of the crude oil price and the philosophy of sustainable utilization of resources resulted in an increase in the biodiesel production in the last decade. Glycerol is formed as a by-product of biodiesel production, specifically the transesterification process of lipids, and thus became cheap and easily available (Johnson and Taconi 2007). The development of valuable products on the basis of glycerol provides a commercial benefit to the involved chemical industry and thus increases the planning reliability for the biofuel industry and related areas. Many oxidases are known to produce hydrogen peroxide (H₂O₂) during the oxidation of their substrates, which can directly or indirectly serve as bleaching agent in washing and laundry applications. The development of a glycerol oxidase would combine both, the use of glycerol to build valuable compounds for the chemical industry and the production of H₂O₂ for the detergent industry.
Enzyme optimization is rapidly gaining relevance and is of great importance for the engineering of tailor-made biocatalysts (Lutz and Bornscheuer 2009). This protein engineering of industrially relevant enzymes is well described for instance for lipases, proteinases, and cellulases. Directed evolution is often used in combination with rational protein design to enhance enzymatic properties such as thermostability, pH stability, enantioselectivity, catalytic efficiency, or substrate specificity. Protein engineering of oxidative enzymes appears to be much more challenging and this is presumably related to toxicity problems. H₂O₂ can be critical for enzyme stability and especially functional expression of the protein in a microbial host such as Escherichia coli.

As “true” glycerol oxidases suitable for washing applications have not been described yet, a polyol oxidase was chosen as starting enzyme in this protein engineering approach. Alditol oxidase (AldO) from the actinomycete Streptomyces coelicolor A3(2), first described by Heuts et al. (2007a), is a soluble monomeric flavoenzyme containing one FAD molecule covalently attached to a histidine residue. The crystal structure of AldO (PDB code: 2vft) was solved by Forneris et al. (2008). This enzyme catalyzes the regioselective oxidation of several alditols at a terminal C-atom to the corresponding aldehyde. FAD is reduced and regenerated in a second reaction while electrons from molecular oxygen are transferred to generate H₂O₂ (Baron et al. 2009). It is known that the choline oxidase from Arthrobacter globiformis oxidizes the alcohol not only to the corresponding aldehyde but also converts the aldehyde into its acid (Gadda 2008). Van Hellemont et al. (2009) described that AldO wild-type already accepts glycerol as a substrate, but with poor catalytic efficiency compared to its preferred substrate sorbitol. Van Hellemont et al. furthermore proposed that substrates oxidized at the primary alcohol group are subsequently further oxidized to the corresponding carboxylic acid. Hence we assumed that AldO is able to oxidize glycerol up to glyceric acid (GA) in a two-step reaction via glyceraldehyde (GLA). Experiments were carried out towards elucidation of the products of this reaction, also regarding the enantiomeric composition (Fig. 1).

GA serves as a building block in glycerate-phospholipid-analogs, e.g., acylglycerate derivatives, bearing an “inverse ester” function compared to natural phospholipids at the carboxylic group of the GA, that circumvents the hydrolysis by phospholipase PLA₁ (Rosseto et al. 2008). Recently, GA has been converted into dilinoleoyl-D-glyceric acid for elucidation of its applicability in skin care products (Sato et al. 2011).

Here, we describe the enzymatic synthesis of D-GA from glycerol by oxidation with purified AldO following the proposed reaction pathway outlined in Fig. 1 as well as the directed evolution and rational design of this enzyme for enhanced specific activity towards glycerol to generate hydrogen peroxide.

Material and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Germany), Roth (Germany), VWR (Germany), New England Biolabs (USA), and Fermentas (Germany) unless stated otherwise.

Cloning of AldO and transformation in E. coli

The AldO gene (PDB code: 2vft) was synthesized by Entelechon GmbH, digested with BamHI and HindIII (Fermentas) and ligated into the pQE80L vector (Qiagen), including a N-terminal His₆-tag. The ligated DNA was transformed into E. coli BL21 (DE3), C41 (DE3) or C43 (DE3) (Lucigen).

Generation of a randomized library

The library based on the AldO-His gene was constructed by error-prone PCR (GeneMorph ™ II Random Mutagenesis Kit, Stratagene) using the primers Muta_pQE_Bamf CATCACGGATCCATGAGCGAC and Muta_pQE_Hindr CTAATTAAGCTTCCGCGAGT ACCCG. A reaction mixture of 50 µL was prepared as follows: vector DNA (30 ng gene DNA), reaction buffer, 0.8 mM dNTP mix, 2.5 U mutazyme II polymerase, 0.25 mM of each primer and run under following conditions: 96 °C for 5 min, 30 cycles of 96 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min, with a final step of 72 °C for 10 min. PCR products were digested, ligated, and the DNA library was transformed in E. coli using standard protocols.

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Fig. 1 Proposed reaction pathway of glycerol oxidation at the C1 position catalyzed by AldO. In the first step, glycerol is oxidized to GLA and subsequently to GA by oxygen.
Creation of site-directed mutants

This was performed using the QuikChange site-directed mutagenesis kit following the manufacturer’s instruction manual (Stratagene). Amino acid positions Q288, E320, and T345 of AldO were subjected to saturation mutagenesis. Positions P249, V250, F275, and T345 of the mutant ep274_E4 were chosen for single amino acid substitutions. The forward primers are listed in ESM Table S1 (supporting information) and the reverse primers are the complements of these primers. Original methylated plasmid DNA was digested with DpnI, and 3 μL of the newly synthesized plasmid DNA was directly transformed into XL10 competent cells (Stratagene). The plasmids were analyzed by sequencing to confirm the presence of the desired mutation. The transformants were incubated overnight in 5 mL LB medium containing 100 μg/mL ampicillin. The plasmids or the plasmid libraries were isolated by mini-prep, and 1 μL DNA was transformed into the expression host E. coli C43 (DE3).

Plate assay for AldO-His-expressing variants

Transformants were selected on LB agar plates containing ampicillin and 1% glucose, and were then transferred with a nitrocellulose membrane first onto an IPTG containing induction agar plate for two hours. Next, they were incubated on an agar plate containing 1 M IPTG, 1% glucose, and were then transferred to a corresponding microtiter plate. After sequencing of the DNA to locate mutations, interesting variants were expressed in 250 mL or 1 L shake flasks with baffles and gas-permeable adhesive seal films. Volumes of 50 or 200 mL auto-induction media were inoculated with a fresh single bacteria colony, and grown at 30 °C and 200 rpm for 24 h. The lysate was prepared as described above. Crude extract and inclusion body fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970). The crude enzyme solution was filtered through a 0.2 mm filter before directly applying onto a 1 mL HisTrap crude FF column (GE Healthcare) preloaded with Ni²⁺ ions and equilibrated with 5 volumes washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). The column was washed using 5 volumes of the washing buffer, and the enzyme of interest was then eluted with 5 volumes elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 150 mM imidazole, pH 7.4). The enzyme-containing fractions were subsequently pooled.

Protein expression and purification of mutants

Characterization of mutants

Protein concentrations were determined using the Bradford assay (Bradford 1976). The purified protein was observed as a single band in SDS-PAGE analysis. The activity of the purified enzyme was measured as described above using the

Activity screening in microtiter plates

This assay is based on a quantitative spectrophotometric determination of hydrogen peroxide formed by active oxidases using a system composed of 4-aminooantipyrine, chromotropic acid and horseradish peroxidase (Wong et al. 1981). Cell lysates (30 μL) were incubated with 50 μL substrate solution (160 mM glycerol, 0.16 mM hydroxylammonium sulfate as catalase inhibitor in 200 mM Tris-HCl pH 9.0) for 10 min at 37 °C and 300 rpm; after which, the oxidase reaction was stopped by addition of 100 μL 200 mM sodium acetate pH 5.5 containing 12.5 mM chromotropic acid and 0.6 mM 4-aminooantipyrine (stopping solution). The color formation was induced through addition of 15 μL of peroxidase (104 U mL⁻¹ in 200 mM sodium acetate pH 5.5). The absorbance (endpoint concentration of H₂O₂) was measured immediately in a spectrophotometer at 600 nm. One unit was defined as the generation of 1 μmol hydrogen peroxide per minute under the reaction conditions. To determine the blank value, controls without enzyme were carried out.

Protein expression for screening of AldO-His-expressing variants in deep-well plates

Single active colonies were picked and inoculated in 200 μL LB media containing ampicillin in 2 mL V-bottom deep-well plates (Greiner). Four wells were inoculated with wild-type AldO-His for reference purposes. The plates, covered with gas-permeable adhesive seal films, were incubated at 30 °C and 800 rpm. After 18 h incubation, 1 mL of Overnight Express auto-induction media (Novagen) containing ampicillin was added to the wells to induce protein expression. The bacteria were grown for 24 h at 30 °C and 1,000 rpm. Cells were separated by centrifugation (4 °C, 30 min, 6,400 × g) and cell lysis was performed in 150 μL 200 mM Tris-HCl pH 9.0 containing 1 × BugBuster reagent (Novagen) and DNase. The plate was then centrifuged (4 °C, 30 min, 6,400 × g), after which

an aliquot of the crude extract from each well (30 μL) was transferred to a corresponding microtiter plate.
The steady-state kinetic parameters characterization were performed in duplicate or triplicate. Incubation for 30 min. All measurements for the enzyme (Davies studies were performed in Davies buffer pH 3.0) were measured in a spectrophotometer at 600 nm. pH-dependent stability measurements were made after incubation for 30 min. All measurements for the enzyme characterization were performed in duplicate or triplicate.

Kinetic data analysis

The steady-state kinetic parameters $k_{cat}$, $K_M$, $k_{cat}/K_M$ for purified enzymes were determined using the described assay. Concentrations of glycerol were varied over the range of 0.05–2 M. Kinetic data were calculated using a Michaelis–Menten model (Eadie–Hofstee plot). The concentration of purified enzyme used for each single measurement was 0.02 mg mL$^{-1}$ for the wild-type and the mutants.

Biotransformation and product determination

Small-scale oxidation reactions were performed in 1.5 mL micro-test tubes, closed with a breathable film (AeraSeal film, Excel Scientific). Purified AldO-His was incubated with 30 mM glycerol and 67.5 U catalase (Sigma Aldrich) in 100 mM bicine buffer, pH 9.0 for 40 h.

Whole-cell biotransformation was carried out with resting E. coli BL21 (DE3) cells containing the AldO-His plasmid. Cells were grown and harvested as described and resuspended in 20 mL 100 mM bicine buffer pH 9.0, catalase was added for the decomposition of H$_2$O$_2$. The reaction was started by adding 300 mM glycerol and incubated at 37 °C while shaking at 200 rpm. The concentration of glycerol, GLA, and GA was monitored over 60 h by HPLC. The determination of reaction products and enantiomeric composition was conducted as described earlier (Wulf et al. 2012). Briefly, glycerol, GLA, and GA were separated with a Knauer Eurokat-H column and quantitated by refractive index detection. Chiral analysis of GA was conducted using a Daicel Chiralpak® QN-AX column and evaporative light scattering detection. Due to the evaporation of water, and the subsequent raise in substrate concentration during biocatalysis, the measured concentrations were corrected according to:

$$c_i^t = c_i^0 \sum \frac{c_m^0}{c_m^t}$$

The measured concentration at a given time point $c_i^t$ was multiplied with the quotient of the sum of all the measured substrate concentrations at the beginning of catalysis $c_m^0$ and the sum of the analyte concentrations at the sampling point $c_m^t$.

Molecular modeling

The crystal structure of AldO with sorbitol (PDB code: 2vfi) was used as a template for molecular dynamic (MD) simulations. A cubic simulation cell (15 Å side length) was located into the binding site of the protein structure surrounding the sorbitol moiety; afterwards, the sorbitol was deleted from the structure. Docking studies with glycerol based on the program YASARA (Krieger et al. 2002) were carried out using the build-in Autodock algorithm (Morris et al. 1998) automated in the “dock_run” macro with 100 runs and a minimum root-mean-square-deviation (RMSD) of 2.0 Å for clustering. To generate a model for the mutated enzyme variants, the mutations were introduced into the AldO crystal structure in silico using YASARA. The structures obtained were energy minimized using the macro “md_refine” making 20 snapshots in 500 ps at default parameters. Glycerol was then docked into the structure with the lowest energy using the “dock_run” macro as described above.

Results

Determination of the specific activity towards glycerol

The gene encoding AldO from S. coelicolor A3(2) was synthesized and cloned into the vector pQE80L, including a N-terminal His$_6$-tag. The recombinant protein AldO-His was expressed in three E. coli strains, BL21 (DE3), C41 (DE3), and C43 (DE3) after induction by lactose in an auto-induction medium and was then purified by affinity chromatography. SDS-PAGE analysis revealed a molecular weight around 46 kDa. Using E. coli C43 (DE3) resulted in the highest amount of active AldO-His (data not shown). The specific activity of AldO in E. coli C43 (DE3) was investigated for glycerol and GLA compared to the preferred substrate sorbitol (Table 1). The highest activity of 2,167 mU mg$^{-1}$ was shown for sorbitol. An activity of 260 mU mg$^{-1}$ (12% of sorbitol activity) was observed with glycerol. Towards GLA, the enzyme activity was 8.5% of

### Table 1 Specific activity of purified AldO-His towards the substrates sorbitol, glycerol and DL-GLA after lactose-induced expression in E. coli C43 (DE3) cultivated in 50 mL

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity [mU mg$^{-1}$]</th>
<th>Specific activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol</td>
<td>2,167 ($\pm$135)$^a$</td>
<td>100</td>
</tr>
<tr>
<td>Glycerol</td>
<td>260 ($\pm$25)</td>
<td>12</td>
</tr>
<tr>
<td>D,L-GLA</td>
<td>185 ($\pm$29)</td>
<td>8.5</td>
</tr>
</tbody>
</table>

All data are based on three individual experiments

$^a$ standard deviation
that with sorbitol. Activity towards dihydroxyacetone (DHA) could not be detected.

Biocatalysis: oxidation of glycerol

The reaction with glycerol using purified AldO-His confirmed that the final oxidation product of glycerol is glyceric acid (Fig. 1). The oxidation of 30 mM glycerol resulted in 15.7 mM GA and 3 mM GLA after 40 h (Fig. 2). Chiral HPLC showed that the final product of the two-step glycerol oxidation was $d$-($R$)-GA with $>$99%ee. Biotransformation with whole cells resulted in 19 mM $d$-GA (2.0 g/L) with 99.6% ee after 60 h at a conversion of 6.3%.

Directed evolution of AldO-His to improve its activity towards glycerol

Oxidase mutants were generated using two parallel approaches: site-directed mutagenesis based on structure-function considerations, and error-prone PCR to create random mutations.

The wild-type AldO-His gene was mutated using error-prone PCR and cloned into $E. coli$ C43 (DE3), which resulted in a library of 8,000 clones. A colony-staining method based on the qualitative detection of $H_2O_2$ was used for the pre-screening and minimization of screening effort. Indicated by a blue color formation, a total of 1,600 clones showed activity in this filter plate assay. Even the quite low mutation rate of one to four mutations per gene already led to an inactivation of 80%. Active clones were subsequently cultivated in glucose/lactose mediated auto-induction medium in deep-well plates for detailed investigation of their properties. Candidates were selected on the basis of both the activity towards glycerol of the crude extracts and the cell density compared to four wild-type samples per plate. The best variants were further evaluated by cultivation in shake flasks followed by affinity purification. As a result of screening these 1,600 independent clones under optimal conditions, five mutants were selected with significantly improved activities relative to AldO-His. Table 2 reports the specific activities determined in atmospheric oxygen at pH 9 for AldO-His and the mutants. These contained substitutions at positions 64, 125, 133, 236, 244, and 399, predominantly within the FAD-binding domain. As shown in Table 2, the best mutant (ep274_E4) showed up to 1.5-fold higher specific activity towards glycerol compared to wild-type AldO-His.

The mutant ep274_E4 was hence used as template for the second round of mutagenesis. The resulting library, comprising approximately 2,700 mutants, was screened but no variants with higher levels of activity than ep274_E4 were identified from the microtiter plate assay. Therefore, we used as an alternative a rational design approach for enzyme engineering, which focused on the active site of the oxidase.

Molecular modeling

The X-ray crystal structure of AldO revealed that the position of the substrates xylitol (PDB code: 2vfs) and sorbitol (PDB code: 2vft) at the bottom of the active-site funnel is mainly mediated by a hydrogen-bonding network in which S106 interacts with the substrate oxygen atoms O2 and O3, E320 with O2 and O4, and T345 with O5. For oxidation to proceed (deprotonation of O1 and hydride transfer), it is important that the O1 of the substrate is located very close to the flavin, which is normally mediated by hydrogen bonds formed with the two positively charged amino acid residues K375 and R322, as well as by H343 “sandwiching” the substrate between the imidazole of its side chain and the flavin ring which modulates the size of the active site (Forneris et al. 2008). All five oxygen atoms of both substrates are involved in the hydrogen-bonding network.

Results of docking in the two different force fields AMBER03 (Duan et al. 2003) and YASARA (Krieger et al. 2009) were compared with the 2vft structure by docking of sorbitol in the empty crystal structure with YASARA. The YASARA force field indicated a slightly better coverage with the crystal structure (RMSD of the sorbitol moieties of the crystal structure vs. modeled structure: 1.07 Å for AMBER03, 0.76 Å for YASARA). The carbon atoms 1–3 are located in the same position, and the hydroxyl groups share the same polar contacts like the sorbitol moiety of the crystal structure. The same holds true for position C5. The O6 of sorbitol is known from the crystal structure to have no interaction with the substrate (Forneris et al. 2008), this is also true for our model. In contrast, O4 interacts with H274, whereas in the crystal structure O4 showed polar contacts with E320.
Glycerol was docked into the structure as described above applying the YASARA force field. Prior to docking, the water of the crystal structure was removed. Docking of glycerol indicates that E320 has polar contacts with all of the three hydroxyls of glycerol (Fig. 3). Furthermore, R322 interacts with the glycerol O1 and H274 with O3. It was assumed that the small glycerol moiety is mainly fixed by E320 and does not reach far enough to the bottom of the cavity to transfer its hydride to the FAD isoalloxazine N5 atom effectively. Due to the distance of 5.0 Å, no polar contact is observed between O1 of glycerol and K375, which also could explain the low conversion of glycerol compared to sorbitol because the K375 is known to be important for effective oxidation (Forneris et al. 2008).

Site-directed (saturation) mutagenesis

Site-directed mutagenesis was used to modify the active site specificity from the preference towards long chain substrates to the tight binding of the small substrate glycerol. To circumvent the fixation of the glycerol moiety at position E320 while strengthening the interaction with Q288, these residues were subjected to simultaneous saturation mutagenesis. To avoid that T345 instead of E320 gets into contact with glycerol, which would increase the distance of the substrate to FAD, this residue was also selected for mutation. Three different libraries were constructed using the NNK codon for the selected positions. The first one included positions Q288 and E320, and the second library positions Q288 and T345. For creation of the third library, all three positions were mutated simultaneously with an NNK codon. Almost all active mutants were found to contain the original amino acid residue unchanged at these positions. Sequencing of several inactive clones showed that these variants were single and double mutants and contained changes at amino acid positions E320, T345, and/or Q288. Screening of 800, 270 and 500 clones of the three libraries failed to identify any mutations that gave improved activity to glycerol oxidation. Additional experiments revealed a lack of oxidase expression in all investigated single and double mutants. ESM Fig. S1 (supporting information) highlights the poor levels of expression observed for three mutants in comparison to well-expressed mutant ep274_E4,

![Fig. 3 Model of the active site of AldO (magenta) docked with glycerol (green) and sorbitol (blue) in comparison showing the hydrogen-bonding network within the enzyme and the proposed hydrogen-bond interactions between AldO and glycerol (yellow). Residues E320, T345 and Q288 were investigated in this study](image-url)

### Table 2

<table>
<thead>
<tr>
<th>Designation</th>
<th>Mutations</th>
<th>Cell density [OD$_{600}$]</th>
<th>Activity crude extract [mU]</th>
<th>Activity purified enzyme [mU]</th>
<th>Purified protein [mg]</th>
<th>Specific activity [mU mg$^{-1}$]</th>
<th>Improvement [x-fold]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldo-His</td>
<td>–</td>
<td>10</td>
<td>208 ($\pm$5.5$^a$)</td>
<td>226</td>
<td>0.87</td>
<td>260 ($\pm$8.6)</td>
<td>1</td>
</tr>
<tr>
<td>Mut 1 ep274_E4</td>
<td>V125M/A244T</td>
<td>9</td>
<td>467 ($\pm$27)</td>
<td>466</td>
<td>1.15</td>
<td>405 ($\pm$14)</td>
<td>1.55</td>
</tr>
<tr>
<td>Mut 2 ep274_G6</td>
<td>G399W</td>
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<td>281 ($\pm$9.4)</td>
<td>319</td>
<td>1.08</td>
<td>294 ($\pm$7.4)</td>
<td>1.13</td>
</tr>
<tr>
<td>Mut 3 ep279-1_E7</td>
<td>V133M</td>
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<td>304 ($\pm$4.6)</td>
<td>385</td>
<td>1.17</td>
<td>330 ($\pm$8.7)</td>
<td>1.27</td>
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<tr>
<td>Mut 4 ep279-1_D10</td>
<td>G236D</td>
<td>12</td>
<td>328 ($\pm$8.6)</td>
<td>405</td>
<td>1.35</td>
<td>300 ($\pm$10)</td>
<td>1.15</td>
</tr>
<tr>
<td>Mut 5 ep279-3_E8</td>
<td>A64V/G399R</td>
<td>11</td>
<td>316 ($\pm$10.5)</td>
<td>394</td>
<td>1.39</td>
<td>283 ($\pm$4.5)</td>
<td>1.09</td>
</tr>
</tbody>
</table>

All data are based on three individual experiments

$^a$ standard deviation

The table includes the cell density, activity crude extract, activity purified enzyme, purified protein, specific activity, and improvement for different mutant designs.


[1248] Springer
which yielded the improved activity. These results suggest that these three positions within the binding site are very critical to the structural integrity of the enzyme.

Combination of random mutations

Some mutations of the oxidase variants with higher activity towards glycerol from the error-prone PCR (Table 2) were combined with the best mutant ep274_E4. Site-directed mutagenesis was used to introduce the mutations into the ep274_E4 mutant and the specific activities towards glycerol were investigated. The mutations G236D and G399W integrated into ep274_E4 showed no effect or even a decrease in activity compared to the original ep274_E4 (data not shown). When introducing the mutation V133M, just little improvement of activity to 161% was observed. A 2-fold higher activity was found for mutant ep274_E4 G399R. V133M in combination with G399R led to a 2.4-fold increased specific activity for this quadruple mutant compared to wild-type enzyme (Table 3). This shows the importance of the combination of both mutations.

Biochemical characterization of the improved mutants in comparison to AldO-His wild-type

Mutants ep274_E4 and ep274_E4 V133M/G399R, and the Aldo-His wild-type were cultivated in 200 mL glucose/lactose mediated auto-induction medium, and His-tag purification was performed. Figure 4 shows an SDS-PAGE analysis of the mutant ep274_E4 V133M/G399R, which confirms the high quality of the His-tag purification. The temperature and pH optima and the stability of purified enzymes were investigated using the standard assay. Mutant ep274_E4, ep274_E4 V133M/G399R, and Aldo-His wild-type showed similar temperature and pH profiles, with no indication of altered properties for the mutants. For all three enzymes, the temperature optima were found to be 50 °C, and at 20 °C the residual activity was still 60%. Aldo-His wild-type and the mutant ep274_E4 retained 60% of their activity even at 60 °C, whereas mutant ep274_E4 V133M/G399R showed 100% activity at this temperature (data not shown). Nevertheless, there was no indication of higher temperature stability of ep274_E4 V133M/G399R. All three enzymes were stable after 30 min incubation at temperatures up to 50 °C (70% activity retained) and they lost their activity after 30 min at 60 °C. The pH optimum of both mutants and Aldo-His wild-type was between pH 8.0–9.0, while no activity could be detected between pH 3–5. All three enzymes retained 75% of their original activity across a pH range 7.0–11.0 in the presence of the substrate glycerol after 30 min. In general, fungal and bacterial oxidases differ in pH optimum, with many sugar oxidases exhibiting an acidic or neutral pH optimum (Hiraga et al. 1998; Oda and Hiraga 1998; Shin et al. 1993; Yamashita et al. 2000). Aldo-His and its mutants have a basic pH optimum and possess stability over a wide range and thus show great potential for use in washing detergents.

Enzyme kinetics were determined using the standard assay with variations in the substrate concentration over the range 0.05–2.0 M. No inhibitory effects were observed when using glycerol as substrate. Table 4 shows glycerol to be converted by the mutant ep274_E4 with a K_M value of 157 mM, whereas the Aldo-His showed a K_M value of 266 mM, which highlights an improved affinity of 1.7-fold of this mutant. This confirms the findings of van Hellemond et al. (2009) who described a K_M value of 350±50 mM for AldO using glycerol as substrate. The catalytic efficiency (k_cat/K_M) of the mutant ep274_E4 for glycerol was higher than that of the wild-type. Glycerol was converted by ep274_E4 V133M/G399R with a K_M value of 111 mM and a k_cat/K_M of 5.14·10^4 M^{-1}·s^{-1}, which shows an increase of activity of 2.4-fold compared to Aldo-His wild-type. Table 4 reports the steady-state kinetic parameters determined in atmospheric oxygen at pH 9 for Aldo-His and the mutant enzymes ep274_E4 and ep274_E4 V133M/G399R. Both mutants, ep274_E4 and

### Table 3

Cell density, activity, and yield of purified protein of oxidase variants towards glycerol after lactose-induced expression in _E. coli_ C43 (DE3) cultivated in 50 mL compared to the ep274_E4 mutant

<table>
<thead>
<tr>
<th>Designation</th>
<th>Mutations</th>
<th>Cell density [OD_600]</th>
<th>Activity crude extract [µU]</th>
<th>Activity purified enzyme [µU]</th>
<th>Purified protein [mg]</th>
<th>Specific activity [µU mg^{-1}]</th>
<th>Improvement [x-fold]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldo-His</td>
<td>–</td>
<td>10</td>
<td>208 (±5.5)</td>
<td>226</td>
<td>0.87</td>
<td>260 (±8.6)</td>
<td>1.00</td>
</tr>
<tr>
<td>ep274_E4</td>
<td>V125M/A244T</td>
<td>9</td>
<td>432 (±7.1)</td>
<td>331</td>
<td>0.87</td>
<td>381 (±5.7)</td>
<td>1.47</td>
</tr>
<tr>
<td>ep274_E4 V133M</td>
<td>V125M/A244T/V133M</td>
<td>10</td>
<td>335 (±11)</td>
<td>311</td>
<td>0.74</td>
<td>418 (±19)</td>
<td>1.61</td>
</tr>
<tr>
<td>ep274_E4 G399R</td>
<td>V125M/A244T/G399R</td>
<td>10</td>
<td>547 (±6.6)</td>
<td>471</td>
<td>0.89</td>
<td>528 (±13)</td>
<td>2.03</td>
</tr>
<tr>
<td>ep274_E4 V133M/ G399R</td>
<td>V125M/A244T/V133M/ G399R</td>
<td>9</td>
<td>776 (±1.4)</td>
<td>588</td>
<td>0.94</td>
<td>624 (±7.9)</td>
<td>2.40</td>
</tr>
</tbody>
</table>

All data are based on three individual experiments

*standard deviation
ep274_E4 V133M/G399R, showed the same enantioselectivity as the wild type, forming D-GA as product of glycerol oxidation.

It is surprising that the specific activity is improved with mutations that are not directly involved in substrate binding or oxygen access. To clarify if structural changes due to the mutations could have led to an alteration in substrate binding, thus explain the increasing activity, MD simulations were conducted. Since no mutation in the active site, and therefore no change of the direct binding of the substrate glycerol took place, the increased specific activity must be explained by long ranging structural effects. Both mutants, ep274_E4 with V125M and ep274_E4 V133M/G399R with V125M and V133M introduce sterically more demanding amino acids at the intersection of a loop region and an α-helix (V125) and two disordered loops (V133). This could have influence on the tertiary structure of the both enzymes. Especially for the region around V133, a considerable movement of the opposite strand of the mutation is detectable in MD simulation. The A244T mutation in both improved variants and the G399R mutation in the most reactive variant are located on the surface of the protein. Surface mutations would not be expected to influence the shape of the active site, but could have strong effects on solubility and folding. We believe that the improved activity resulted from both, an increase in catalytic rate and from a higher amount of correctly and more actively folded enzyme in *E. coli* C43 (DE3) compared to AldO-His wild-type. The modeling results suggest some structural changes even inside the active site of the oxidase mutants. The RMSD value of the AldO and the minimized ep274_E4 is 0.9427 Å considering the peptide chain. The deviation between the AldO and the best mutant ep274_E4 V133M/G399R is 1.0486, which reflects structural deviation and could be responsible for the increased activity of the mutants.

Our modeled structure of the active site of the mutant ep274_E4 suggests that each of the three hydroxyls of glycerol is contacted by residue E320 as shown for the AldO wild-type. In contrast, the amino acid residue K375 of ep274_E4 interacts with the O3 of glycerol (3.2 Å) and shares polar contacts with S106. In addition, this mutant has a higher predicted binding affinity for glycerol (4.73 kcal/mol vs. 4.01 kcal/mol). One important change in the binding pattern of glycerol represents the decreased distance between the hydride accepting N5 atom of FAD and glycerol from 3.84 Å in the AldO wild-type to 3.74 Å. Table 5 displays the distances of N5 of the FAD and the substrate hydroxyl groups 1 and 3 of the three best clusters and the calculated binding energies. Considering the hydride shift distance as major important impact on enantioselectivity, it could be predicted that all of the three enzymes favor the oxidation of the proR carbon, as was verified experimentally.

The predicted structure of the mutant ep274_E4 V133M/G399R suggests a similar binding pattern for glycerol as for the mutant ep274_E4, but with fewer contacts. E320 interacts with O1 and O2, but not with O3. S106 contacts O3 of glycerol like ep274_E4, but a polar contact with K375 is missing. The long distance to O1 of 5.8 Å results from a backward movement of K375 compared to the AldO wild-type. A possible acceptor for the O1 proton of glycerol might be R322 with a distance of 3.0 Å. In comparison to that, the sorbitol O1 of the crystal structure 2vft contacts both, the K375 and the R322 with a distance of 2.8 Å. The distance of sorbitol C1 and flavin N5 in the crystal structure 2vft ranges 3.3 Å. All of the resulting calculated structures of ep274_E4 V133M/G399R have worse binding energies

**Table 5** Calculated binding energies and distances between the N5 of the flavin and the C1 or C3 atom of the glycerol ligand. The three clusters with the smallest distance are shown

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Distance N5→ C1 (proR) [Å]</th>
<th>Distance N5→ C3 (proS) [Å]</th>
<th>Binding energy [kcal mol⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AldO</td>
<td>3.84</td>
<td>5.38</td>
<td>4.01</td>
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<tr>
<td></td>
<td>5.95</td>
<td>7.33</td>
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<td></td>
<td>6.39</td>
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<td>ep274_E4</td>
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<td></td>
<td>6.08</td>
<td>6.82</td>
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<td></td>
<td>6.48</td>
<td>7.36</td>
<td>3.72</td>
</tr>
<tr>
<td>ep274_E4 V133M/G399R</td>
<td>3.42</td>
<td>3.88</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>6.72</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>5.14</td>
<td>3.45</td>
<td>3.54</td>
</tr>
</tbody>
</table>
predicted than ep274_E4 and AldO wild-type, but the distance of C1 to the N5 (3.42 Å) was the best compared to both other enzymes due to the residue H343, which seems to push the glycerol a bit more in the direction of the flavin. We believe that the increased activities of the AldO-His mutants correlate with the decreased distance between C1 and N5 of the calculated structures. Altogether, it remains hard to predict what structural changes are responsible for the improvement of activity.

Discussion

The widespread use of enzymes in industrial applications requires the development of new biocatalysts that are both effective and economical. The generation of an oxidase would be a great benefit for a variety of biotechnological applications when using an easily available and cheap substrate like glycerol. The known glycerol oxidases are fungal enzymes from Penicillium sp. TS-622 (Lin et al. 1996) and Aspergillus japonicus (Uwajima et al. 1984), which contain copper. This makes them less suitable for industrial or washing applications and hence we investigated AldO from S. coelicolor A3(2) as alternative enzyme. It was already known that AldO has activity towards glycerol generating H₂O₂ as side-product, which can serve as a bleaching agent in detergent industry. A second potential commercial value of a glycerol oxidase is the production of d-glyceraldehyde (d-GA) as chiral compound.

In this paper, we confirm the proposal by van Hellemond et al. that the product of glycerol oxidation with AldO-His indeed gave the corresponding acid d-(R)-GA with excellent selectivity (>99.6%ee) although the conversion was low compared to reports from Habe et al. (2009) and references herein) for biotransformations of glyceric acid with whole cell catalysts.

In general, glycerol can be oxidized by alcohol dehydrogenases and subsequently aldehyde dehydrogenases. Using dehydrogenases, cofactor recycling systems like enzyme-coupled or substrate-coupled systems are hence necessary to reoxidize expensive cofactors like NAD⁺ or NADP⁺ (Kroutit et al. 2004). With AldO, this is not necessary, which highlights the value of this enzyme not only for the use of H₂O₂ in detergents but also for the industrial production of d-GA via a biocatalytic route.

A specific activity of 260 mU mg⁻¹ towards glycerol corresponds to 11% of the activity towards its preferred substrate sorbitol and hence the amount of hydrogen peroxide generated is too low for an application in bleaching. Consequently we used random mutagenesis and rational protein design of AldO to improve its activity. We were able to identify five mutants with improved activity towards glycerol within the first cycle of directed evolution after screening 1,600 active mutants. The best mutant, ep274_E4, showed a 1.5-fold higher specific activity and attempts for further improvement by the random approach failed. Saturation mutagenesis of three positions identified from substrate docking based on this variant were rather disappointing as 80% of the mutants were inactive and even single substitutions inside the substrate binding site prevented cytoplasmic expression and hence production of active oxidase. This clearly shows the difficulty in making reliable predictions by rational design with respect to the creation of active enzymes.

As an alternative approach to the rational design of the active site, we have combined certain mutations which were found to increase the activity towards glycerol. Introduction of mutations V133M and G399R into the ep274_E4 mutant led to a quadruple mutant with 2.4-fold higher activity compared to AldO-His wild-type. The modeling suggested that the improved activity of the AldO-His mutants originates from a higher affinity to glycerol compared to the larger substrate sorbitol. The decreased distance between the O1 of glycerol and the N5 of FAD from AldO wild-type to the best mutant seems to benefit the position of the glycerol in the cavity to favor catalysis.

In conclusion, we could show that directed evolution and combination of best mutants enabled the creation of variants with 2.4-fold higher specific activity although further efforts are needed to make this oxidase suitable enough for applications in detergent industries or biocatalysis.

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References

Kinetic resolution of glyceraldehyde using an aldehyde dehydrogenase from *Deinococcus geothermus* DSM 11300 combined with electrochemical cofactor recycling

H. Wulf, M. Perzborn, G. Sievers, F. Scholz, U.T. Bornscheuer

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**Abstract**

Glyceraldehyde and glycic acid are both valuable chiral starting materials. Aldehyde dehydrogenases (ALDHs) accept a broad scope of endo- and exogenous aldehydes, such as glyceraldehyde, and convert them into the corresponding carboxylic acid. Here we present cloning, overexpression and kinetic data on two ALDHs from *Escherichia coli* BL21 and *Deinococcus geothermus*. The two ALDHs have a similar substrate scope and favor short to medium chain aldehydes, both oxidize glyceraldehyde to glycic acid. The ALDH variant of *D. geothermus* shows the higher specific activity towards glyceraldehyde and has an elevated activity optimum compared with the BL21 enzyme. The ALDH of *G. geothermus* was also applied to conduct a kinetic resolution of glyceraldehyde with electrochemical cofactor recycling.

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1. Introduction

Aldehyde dehydrogenases belong to an enzyme class widely spread over every kingdom of life, catalyzing the irreversible oxidation of a wide variety of endo- and exogenous aldehydes to their corresponding carboxylic acids. The protein YdcW of *Escherichia coli* has been described belonging to the superfamily of aldehyde dehydrogenases (ALDHs), and was annotated as betaine aldehyde dehydrogenase (EC 1.2.1.8). Kinetic studies revealed the enzyme should be more specifically described as medium chain aldehyde dehydrogenase (EC 1.2.1.3) [1,2]. Like many other ALDHs the crystal structure of the protein reveals a homotetrameric quaternary structure with a catalytic site and NAD⁺ bound to each subunit. The subunits consist of a catalytic domain, a cofactor binding domain, and an arm-like oligomerisation domain including the C-terminus. The enzyme Dgeo,1120 represents an ALDH from the radiation resistant and thermophilic organism *Deinococcus geothermus* (type strain DSM 11300) [3,4] and has been to the best of our knowledge not yet characterized. The enzyme was annotated as succinic-semialdehyde dehydrogenase.

The reaction mechanism of human ALDHs has been identified as an ordered sequential mechanism [5]. First, the NAD⁺ cofactor binds to the enzyme. Secondly, an aldehyde is bound followed by a nucleophilic attack by the sulfur of a catalytically cysteine residue. The carbonyl hydride of the tetrahedral intermediate is then transferred to the C4 of the nicotinamide moiety of the NAD⁺. The conformational change of the NADH then favors the activation of a water molecule by a glutamate residue. The water molecule attacks the substrate–enzyme thioester bond, and the corresponding carboxylic acid is released [5–7]. Studies on YdcW regarding conserved residues and NADH positioning, derived from crystal structures, suggest that a similar mechanism in bacterial ALDH can be assumed [2].

Glyceraldehyde (GLA) and glycic acid (GA) are possible substrate and product of ALDH catalyzed reactions and both can serve as chiral building blocks. GLA for example can serve as structural motif in 4-(dihydroxyalkyl)-β-lactams [8,9]. GA is known as a liver stimulant in dogs and has effects on the acetaldehyde detoxification in rats [10,11]. GA is a building block in glycerate phospholipid analogues, e.g. acyl glycerate derivatives bearing an “inverse ester” function compared to natural phospholipids, at the carboxylic group of the GA that circumvents the hydrolysis by phospholipase PLA₁ [12].

The enzymes YdcW of *E. coli* BL21 (DE3) (ALDH-BL21) and dgeo,1120 of *D. geothermus* 11300 (ALDH-11300) were cloned and examined during this work. Since ALDHs depend on expensive cofactors like NAD⁺ or NADP⁺ as electron acceptors, preparative scale biotransformations using these dehydrogenases require
cofactor recycling systems like enzyme-coupled or substrate-coupled systems to reoxidize the cofactors [13]. In order to employ low concentrations of NAD\(^+\) in the biotransformation of glyceraldehyde to glycric acid, an electrochemical cofactor recycling system can be used. ALDHs in immobilized form have already been used in aldehyde oxidations with electrochemical cofactor regeneration [14]. Also glyceraldehyde was oxidized this way applying immobilized PQQ dependent oxidoreductases at modified glassy carbon electrodes [15]. NADH can be oxidized at carbon electrodes at neutral to alkaline pH but high overpotentials (above 1 V vs. Ag/AgCl) have to be applied [16,17]. The high positive potentials may lead to unspecific side products and enzyme deactivation. To circumvent the high potentials, NADH oxidation at glassy carbon could successfully be carried out using the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS) as mediator in a horse liver alcohol dehydrogenase based biotransformation approach [18].

In this paper we describe the cloning, overexpression and properties of two ALDHs such as kinetic data and oxidative kinetic resolution of glyceraldehyde (GGA) combined with electrochemical cofactor recycling using ALDH-11300 to yield glycric acid (GA) (Fig. 1).

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, USA) and Carl-Roth (Karlsruhe, Germany). Polyamides were obtained from Roboklon (Berlin, Germany), Restrictions enzymes and DNA ligases were obtained from Fermentas (Burlington, Canada).

2.2. Bacterial strains, plasmids and growth conditions

E. coli BL21 (DE3) was purchased from Novagen (Darmstadt, Germany) and used as expression strain and for the isolation of genomic DNA. D. geothermalis DSM 11300 was obtained from DSMZ (Braunschweig, Germany). E. coli One Shot\textsuperscript{®} TOP10 cells (F' marA Δ(mrr-bsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX74 recA1 araD139 Δ(araE::lacU7697 gwiU gwiI rpsL(Strept) endA1 nupG) and vector pCR\textsuperscript{®}-TOPO\textsuperscript{®} for subcloning were purchased from Invitrogen (Karlsruhe, Germany). E. coli strains were routinely cultured in lysogeny broth [19] without glucose at 37 °C, when necessary supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml). D. geothermalis was cultured in LB medium at 45 °C for 4 days. The expression plasmids pET-22b(+) and pET-28b(+) were obtained from Novagen (Darmstadt, Germany), the latter was used for enzyme expression with N-terminal His\textsubscript{6}-tag.

2.3. Cloning of ydcW and aldh-11300

Genomic DNA (gDNA) from E. coli BL21 (DE3) and D. geothermalis was isolated using the innuPREP Bacteria DNA Kit (Analytik Jena, Jena, Germany). Plasmid isolations (Fermentas, Burlington, Canada), PCR-purification and gel extraction (Analytik Jena, Jena, Germany) were performed according to the manufacturer protocols. Standard procedures such as DNA cloning and manipulations were performed as described by Sambrook and Russell [20]. DNA sequencing was carried out by GATC Biotech AG (Konstanz, Germany).

The gene ydcW (aldh-BL21, GenBank accession code: GI:313848522, locus tag B21_01412, protein: YP_604587 (NCBI)) was amplified from gDNA of E. coli BL21(DE3) by PCR using the primer pair forward: 5'–CAT ATG CAA CAT AAG TTA CTG ATT AAC GGA G–3' and reverse: 5'–GGA TTC TTA ATG TTT AAC CAT GAC GTG–3'. The gene aldh-11300 (GenBank accession code: GI:945545390, locus tag Dgeo_1120, protein: Q1Z1B6.DEIGD (UniProt)) was amplified from gDNA of D. geothermalis DSM 11300 using the primer pair forward: 5'–CAT ATG ACC CCT GAC CCC CAC CCT GAG AAC AC–3' and reverse: 5'–GGA TCC TCA GCC CAC CTG GGC AGC CTG GCC CCC GCT TCT GGA T–3'. PCR products were separated in a 1.2% agarose gel from which the desired fragments were purified, cloned into the vector pCR\textsuperscript{®}-II-TOPO\textsuperscript{®} and E. coli One Shot\textsuperscript{®} TOP10 cells were transformed with the plasmids bearing the respective insert. YdcW (ALDH-BL21) and ALDH-11300 in pCR\textsuperscript{®}-II-TOPO\textsuperscript{®} were sequenced and cloned into pET-22b(+) and pET-28b(+) via Ndel and BamHI restrictions sites (underlined). Expression strain E. coli BL21 (DE3) was transformed with the four different originated vectors.

2.4. Expression of ALDH-BL21 and ALDH-11300 and enzyme purification

ALDH-BL21 and ALDH-11300, both with and without N-terminal His\textsubscript{6}-tag, were expressed in E. coli BL21 (DE3) using 5 ml LB medium supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin for expression control and activity measurements. Purified enzyme was obtained by the expression of His\textsubscript{6}-tagged variants of the two enzymes in 500 ml LB medium containing 50 μg/ml kanamycin. Cells were grown at 37 °C to an optical density of 0.6 at 600 nm. Enzyme expression was induced with 0.4 mM IPTG and proceeded for 5–24 h at 30 °C. Cells were centrifuged (4000 × g, 4 °C, 15 min), the cell pellet was washed twice with 20 mM Tris–HCl buffer (pH 7.5) and disrupted in the same buffer by sonication (2–4 min, 30% power, 50% pulse). For biotransformation experiments cells were washed and resuspended with 50 mM sodium phosphate buffer (pH 7.5) (PB) and subsequently lysed in 50 mM sodium phosphate, 0.5 M NaCl (buffer A) using a French\textsuperscript{®} Press (Thermo Fisher, Waltham, MA). The crude cell extract was centrifuged for 15 min at 16,000 × g and 4 °C and the supernatant was diluted 1:10 with buffer B (50 mM sodium phosphate, 0.5 M NaCl, 0.3 M imidazole, pH 7.5). For the purification of ALDH-BL21 and ALDH-11300 with the hexahistidine affinty tag (His\textsubscript{6}-tag) the diluted crude extract was injected into a 5 ml HisTrap\textsuperscript{TM} FF crude column for immobilized metal ion affinity chromatography (IMAC) using the ÄKTApurifier\textsuperscript{TM} (GE-Healthcare, Munich, Germany). The column with bound enzyme was washed with 20% buffer B and fractions were collected while bound enzyme was eluted by applying 100% buffer B. For the storage of purified enzyme in solution or lyophilization, the purified enzyme was subjected to gel filtration for the removal of imidazole and high salt concentration using ÄKTApurifier\textsuperscript{TM} equipped with Sephadex G-25 Superfine column (26 × 110 mm).

2.5. Determination of protein concentration and SDS-PAGE

Polycrylamide gel electrophoresis was carried out using 4% stacking gel and 12% resolving gel according to the method described by Laemmli [21]. Roti-Mark\textsuperscript{®} STANDARD (Roth, Karlsruhe, MW: 14–200 kDa) was used as protein standard. The
protein concentration was determined either with the BCA-assay (Uptima, Montluçon, France) or with Coomassie Brilliant-Blue using Roti®-Nanoquant (Carl-Roth, Karlsruhe, Germany) according to manufacturer protocols using BSA as standard.

2.6. Enzyme assays

The aldehyde dehydrogenase activity was determined spectrophotometrically at 30 °C by monitoring the rate of reduction of NAD+ at 340 nm. The standard reaction mixture (1 ml) contained 80 mM Tris–HCl buffer (pH 9.0), 0.35 mM NAD+, 2.5 mM d,L-glyceraldehyde and an appropriate amount of the enzyme. The reaction was started by adding d,L-glyceraldehyde to the mixture. One unit of aldehyde dehydrogenase activity was defined as the amount of enzyme that catalyses the formation of 1 μmol NADH per minute.

In order to determine the apparent KM and vmax values for different aldehydes the described activity test was carried out with varying substrate concentrations at a fixed concentration of 0.35 mM NAD+. Initial velocities were recorded during the first 11 s of catalytic turnover. Apparent KM and vmax-values were obtained by linear regression fitting of a Hanes plot. These values were used for non-linear regression, which effectively fits the data directly to the hyperbola according to the method of Wilkinson [22] and Duggleby [23] using the program HYPER (J.S. Eastern, http://www.liv.ac.uk/~jse/software.html).

The effect of temperature on the enzyme activity was determined by incubating the standard reaction mixture at different temperatures ranging from 12 to 59 °C for 10 min at pH 9.0. For determining the effect of the pH towards enzyme activity, purified enzyme was incubated at 30 °C for 5 min in the following buffers at 100 mM: sodium acetate (pH 4.0–5.5), Bis–Tris–HCl (pH 5.5–7.0), sodium phosphate (pH 6.0–8.0) and Tris–HCl (pH 6.5–9.7) Activity was measured as described above.

2.7. Electrochemical measurements and biotransformations

Electrochemical measurements were carried out with an Autolab PSTAT 10 (Eco Chemie, Utrecht, Netherlands). All potentials refer to the Ag/AgCl electrode (3 mol l−1 KCl, E = 0.208 V vs. SHE). In cyclic voltammetry the second scan was used. The sweep rate was 5 mVs−1 and the potential range between 0 and 1 V vs. Ag/AgCl. The buffer system consisted of 100 mM sodium phosphate pH 8.0 (buffer PB).

Biotransformation experiments were carried out using the electrochemical cofactor recycling system. A flow glassy-carbon electrode (30 PPI RVC Duocell®, ERG Materials and Aerospace, Oakland, USA) was used as working electrode. A Pt-net surrounded the working electrode and served as auxiliary electrode. The above-mentioned Ag/AgCl reference electrode contacted the reactor via a KCl saturated agar tube (Fig. 2).

The buffer reaction solution consisted of 0.5 mM ABTS and 2 mM NADH in 20 ml of 100 mM sodium phosphate (pH 8.0). Experiments were performed at room temperature and the potential of the working electrode was hold at 0.7 V vs. Ag/AgCl. As biocatalyst a preparation of 5–10 U of purified and lyophilized ALDH−11300 was used. Racemic d,L-glyceraldehyde was added up to 40–50 mM.

2.8. Product determination by HPLC

Concentrations of glyceraldehyde (GLA) and glyceraldehyde (GA) were determined using a Shimadzu LC 10 HPLC equipped with a Eurokot-H precolumn (30 x 8 mm) and column (300 x 8 mm, both Knauer, Berlin, Germany) at 80 °C with 0.01 N sulfuric acid at 0.8 ml/min flow rate and refractive index detection. Chiral analysis of glyceric acid was conducted using a Merck Hitachi LaChrom HPLC equipped with a Chiralpak® QN-AX precolomn (4 x 10 mm) and column (4.6 x 150 mm, both Chiral Technologies Europe, Illkirch, France) at 23 °C with methanol/acet acid/ammonia acetate 98/2/0.5 (v/v/w) as eluent at 1 ml/min flow rate and evaporative light scattering detection. The retention time of L-GA was 5.9 min and the d-GA retention time was 7.1 min.

3. Results and discussion

3.1. Amplification, cloning and protein expression of ALDH−BL21 and ALDH−11300

The genes encoding proteins ALDH−BL21 and ALDH−11300 could be successfully amplified and cloned into pET−22b (+) without His6−tag and pET−28b (+) bearing the genes with N-terminal His6−tag in order to enhance and simplify the protein purification procedure. The cloned DNA sequence of the ydcW differed in one nucleotide (A994G) to the database entry B21_01412. This difference leads to the mutation S332G at protein level in ALDH−BL21. Both genes could be overexpressed in the host strain E. coli B21 (DE3). The His6−tag variants of ALDH−BL21 and ALDH−11300 were purified to apparent homogeneity by IMAC (as determined by SDS–PAGE, data not shown) at yields of 39% (ALDH−BL21) and 66% (ALDH−11300) of the total activity.

3.2. Temperature− and pH−optimum

Since both enzymes could be expressed in active form with and without His6−tag, subsequent experiments were performed with the His6−tagged enzyme variants using glyceraldehyde as substrate. The temperature optimum of ALDH−BL21 from E. coli was between 30 and 35 °C (Fig. 3). The maximum activity for the ALDH−11300 from the mesothermophilic organism D. geothermalis was determined to be more than 10 °C higher, between 43 and 47 °C (Fig. 3), making this enzyme more useful for applications at elevated temperatures around 45 °C. The temperature range corresponds to the optimum growth temperature of D. geothermalis of 45−50 °C [3] since chaperones and compatible solutes may additionally stabilize the enzyme at temperatures of over 45 °C in its physiological environment.
Both enzymes showed the highest activity at pH 8.5 in Tris–HCl. At pH 5.5 the enzymes showed residual activities of 10% and a complete loss of activity took place at pH 4.0 (Fig. 4).

3.3. Activity and steady-state kinetics

For ALDH–11300 from D. geothermalis, the oxidation of glyceraldehyde and other substrates was measured for the first time in this work. For the homolog ALDH enzyme from E. coli K12 activity against glyceraldehyde was previously described by Gruetz et al. [2], Activity could also be confirmed here for ALDH–BL21 from E. coli BL21, which differs in one amino acid (V197I) from the K12 variant. For this enzyme a specific activity of 0.6 U/mg was determined after IMAC purification. The newly characterized ALDH–11300 with a specific activity of 3.5 U/mg is more active against glyceraldehyde compared to ALDH–BL21. The purified ALDH–BL21 had only 2% activity against GLA with NADP⁺ compared to NAD⁺ as cofactor. ALDH–11300 showed 15% activity against GLA using NADP⁺ instead of NAD⁺. Gruetz et al. found 6% activity using NADP⁺ in GLA oxidation, whereas the $v_{\text{max}}/K_M$ ratio for NADP⁺ is still 25% of the NAD⁺ value [2]. The biocatalytic reduction of GLA and GA with NADH was investigated too. However no activity was observed with both enzymes towards GLA or GA. Eriksson et al. [10] showed that treatment of rats with GA enhances ethanol and acetaldehyde clearance. The authors suggest that GA enhances acetaldehyde detoxification by enhanced cofactor regeneration through ALDH catalyzed substrate reductions. Since the BRENDA–Database [24] solely lists one example of a ALDH converting an acid to an aldehyde, namely 3-hydroxypropionic acid to 3-hydroxypropionaldehyde with a poor activity of 4% of the oxidation capacity [25] and data of this work show no action of ALDH against glyceraldehyde this hypothesis seems to be unlikely. Because of the high difference in the redox potential, acids need to be phosphorylated prior to be metabolized and reduced by aldehyde dehydrogenases at reasonable reaction velocities.

Both aldehyde dehydrogenases showed no activity towards 1-propanol, 1,2-propanediol and glycerol as substrates. These findings indicate the chemoselectivity of these enzymes towards the oxidation of a terminal carbonyl group.

The activity of ALDH–11300 against several substrates was determined, and the kinetic parameters of the Michaelis–Menten equation were determined for six substrates at various concentrations (Table 1). The highest turnover rates were found for the oxidation of isobutyraldehyde. The slowest turnover was observed with the aromatic substrate phenylacetaldehyde. However with 3.76 $\text{s}^{-1}$ $\text{mM}^{-1}$ the $k_{\text{cat}}/K_M$ value was still better than that of glyceraldehyde, which had the second best turnover but also the second highest $K_M$. The best $k_{\text{cat}}/K_M$ was determined for butyraldehyde. Since no constants could be determined for valeraldehyde (C5) and caproaldehyde (C6), butyraldehyde represents the best kinetic data for the longest unbranched carbon chain. Valeraldehyde showed a significant inactivation above 150 $\mu\text{M}$ (Fig. 5). The plots for caproaldehyde and valeraldehyde showed a non Michaelis–Menten like, sigmoidal slope.

Kinetic data were also determined for the ALDH–BL21. Similar to the findings of Gruetz et al. our data show an increase in the turnover as the chain length of the substrate increases. In contrast to the findings for the K12–ALDH [2], the best substrate according to $v_{\text{max}}$ values is the C4 substrate butyraldehyde. The best $k_{\text{cat}}/K_M$ value has isobutyraldehyde before caproaldehyde (Table 2).

The enzyme activity after storing at 0 °C and at room temperature (RT) was determined during more than 40 days. ALDH–BL21 was more stable than ALD–11300 during both storing conditions. Activity of ALDH–BL21 after storing at 0 °C for 20 days showed 95%
of the initial value, whereas for ALD-11300 activity was reduced by 40% (0°C) to 80% (room temperature) (Fig. 6). Storage of purified enzymes at −20°C resulted in complete loss of activity, but freeze dried ALD-11300 retained 80% of its activity towards GLA.

3.4. Biotransformation and determination of reaction products

Glyceraldehyde should be oxidized to GA in an ALDH catalyzed kinetic resolution. For these biotransformation experiments 100 mM sodium phosphate pH 8.0 was used instead of Tris-buffer, because tris (tris(hydroxymethyl)-aminomethane) is not applicable in reactions with acyl-enzyme intermediates since it may act as nucleophile itself [26]. Cyclic voltammetry measurements show the two-electron transfer system of ABTS. The first redox reaction is reversible and important for the NADH reoxidation. The second (more positive) redox system is irreversible and must be avoided as otherwise ABTS is eliminated from the equilibrium. The cyclic voltammogram of NADH shows an irreversible system with a current peak at 580 mV vs. Ag/AgCl (Fig. 7, plot d). A solution of 4 mM NADH and 1 mM ABTS shows an irreversible oxidation peak at 500 mV since ABTS•+ is immediately

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Table 1
Kinetic parameters of ALDH-11300 for six substrates, ordered according to increasing \( k_{cat} \)-values. The error estimations represent a confidence interval of 95%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( v_{max} ) [U mg⁻¹]</th>
<th>( K_M ) [mM]</th>
<th>( k_{cat} ) [s⁻¹]</th>
<th>( k_{cat}/K_M ) [s⁻¹ mM⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetaldehyde</td>
<td>1.74 ± 0.24</td>
<td>0.13 ± 0.07</td>
<td>1.56 ± 0.22</td>
<td>12.4 ± 3.35</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1.97 ± 0.14</td>
<td>0.21 ± 0.06</td>
<td>1.78 ± 0.13</td>
<td>8.59 ± 2.06</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>2.76 ± 0.16</td>
<td>0.14 ± 0.03</td>
<td>2.49 ± 0.14</td>
<td>17.39 ± 4.65</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>2.9 ± 0.4</td>
<td>0.07 ± 0.04</td>
<td>2.61 ± 0.36</td>
<td>37.7 ± 9.9</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>3.05 ± 0.35</td>
<td>2.52 ± 0.95</td>
<td>2.75 ± 0.32</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>Isobutyraldehyde</td>
<td>3.22 ± 0.15</td>
<td>2.67 ± 0.4</td>
<td>2.9 ± 0.13</td>
<td>1.09 ± 0.33</td>
</tr>
</tbody>
</table>

Fig. 5. The activity of ALDH-11300 towards several substrates at different concentrations.

Table 2
Kinetic parameters of ALDH-BL21 for eight substrates, ordered according to increasing \( k_{cat} \)-values. The error estimations represent a confidence interval of 95%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( v_{max} ) [U mg⁻¹]</th>
<th>( K_M ) [mM]</th>
<th>( k_{cat} ) [s⁻¹]</th>
<th>( k_{cat}/K_M ) [s⁻¹ mM⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetaldehyde</td>
<td>0.08 ± 0.03</td>
<td>4.87 ± 3.98</td>
<td>0.07 ± 0.03</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.42 ± 0.01</td>
<td>0.56 ± 0.06</td>
<td>0.38 ± 0.01</td>
<td>0.67 ± 0.14</td>
</tr>
<tr>
<td>Isobutyraldehyde</td>
<td>0.55 ± 0.03</td>
<td>0.02 ± 0.004</td>
<td>0.49 ± 0.03</td>
<td>26.47 ± 5.94</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>0.95 ± 0.1</td>
<td>8.44 ± 2.66</td>
<td>0.86 ± 0.09</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>1.08 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>0.97 ± 0.04</td>
<td>21.05 ± 4.21</td>
</tr>
<tr>
<td>Valeraldehyde</td>
<td>1.24 ± 0.11</td>
<td>0.07 ± 0.02</td>
<td>1.12 ± 0.1</td>
<td>16.28 ± 4.73</td>
</tr>
<tr>
<td>Caproaldehyde</td>
<td>1.41 ± 0.11</td>
<td>0.05 ± 0.02</td>
<td>1.27 ± 0.1</td>
<td>25.21 ± 6.04</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>1.64 ± 0.25</td>
<td>0.08 ± 0.04</td>
<td>1.48 ± 0.23</td>
<td>18.98 ± 5.8</td>
</tr>
</tbody>
</table>

Fig. 6. Activity of the enzymes ALDH-BL21 und ALD-11300 after storing at room temperature and at 0°C for 43 days.
oxidized by NADH (Fig. 7 and [18]). The addition of enzyme and GA shifts the current peak of the recycling system to higher overpotentials, which may be due to blocking of catalytic active sites at the electrode. The increase of the overpotential of the recycling system requires a higher potential as it would be needed for normal ABTS mediated NADH reoxidation. The potential for the biotransformation should be at least 650 mV vs. Ag/AgCl (Fig. 7) thus for biotransformation experiments 700 mV where applied.

During the biotransformation experiments, the cofactor NADH was indirectly recycled at constant potential of 700 mV at a glassy carbon foam electrode. The foam electrode was used to generate a greater contact surface, where ABTS and subsequently NADH could be reoxidized (Fig. 1). The reaction vessel contained 20 ml buffer solution with dissolved enzyme and substrates. Since NADH was applied initially, the reaction started only after the current was switched on. Samples were taken during the biocatalysis and analyzed via ion-exclusion and chiral HPLC, and the transferred electric charge was monitored (Fig. 8). In biotransformation A the concentration of GA increased by 16.4 mM while the concentration of GLA decreased by 14.5 mM (Fig. 8). Biotransformation B does not show such a high accumulation of D-GA, it increases by 7.9 mM while GLA drops in 12.7 mM (Fig. 8). The small experimental discrepancies between drop of educts and gain of products are mostly probable experimental errors. The measured transferred charge \( q_m \) apparently fits the theoretically transferred charge \( q_t \) best in the beginning of the biocatalysis (Fig. 8). The difference between \( q_m \) and \( q_t \) gains significance with progression of the reaction. A subsequent oxidation of glyceric acid at the anode is not possible, as proven in separate CV experiments (data not shown), hence further oxidation of GA is not an explanation for the differing \( q_t \) and \( q_m \). A possible explanation could be the big surface and the large number of pores and cavities of the carbon foam electrode. GLA and GA may diffuse into the pores, and hence evade the chromatographic quantitation. Washing steps of the working electrode after the biotransformation revealed that GA and GLA where retained by the electrode and perhaps remained in the cavities. This can lead to a systematic error in the determination of the extent of the biotransformation. The structure of

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**Fig. 7.** The plot a displays the CV of ABTS and NAD, b displays ABTS, NAD and ALDH-11300, c displays ABTS, NAD, ALDH-11300 and GLA, d displays NAD (4 mM) and e displays ABTS (1 mM). All CVs were measured in PB (100 mM, pH 8.0).

**Fig. 8.** Plots of biotransformation experiments A and B. The reactions differ solely in the prolonged reaction time and greater number of sample points in biotransformation B. \( q_m \) and \( q_t \) refer to the left axis, concentrations of GLA, GA and \% ee to the two left axes.
the porous working electrode will certainly lead to lower potentials inside the pores, which will decrease the electrolysis efficiency [27]. This feature must receive further attention when an upscaling of the technique is intended. It might also be possible that with reaction time the enzyme becomes oxidized at the electrode as described by Manjón et al. for glucose dehydrogenase [28], which can be an explanation for the uncompleted conversion of the GLA.

The enantiomeric excess of the resulting D-GA decreased during both biotransformations although no direct oxidation of GLA at the electrode could be observed at the applied potential. At the first measurement, the enantiomeric excess of both reactions was still 98.5%ee and 100%ee but ended up in 91.8 and 87.9%ee, respectively (Fig. 8).

4. Conclusion

In this work we showed the cloning, purification, characterization and biocatalysis with two ALDH from E. coli BL21 and D. geothermalis. Both enzymes could be overexpressed and purified with IMAC yielding 39–66% active enzyme. The ALDH-11300 with 3.5 U/mg had a better specific activity against glyoxaldehyde then ALDH-11300 with 0.6 U/mg. The pH profile was similar for both enzymes and showed the highest activity at a pH of 8.5. The temperature optimum of activity at 45–50 °C was 10 °C higher with ALDH-11300 then with ALDH-11300. The best substrate according to \( k_{\text{cat}}/K_M \) for ALDH-11300 and ALDH-11300 were butyraldehyde and isobutyraldehyde, respectively. Biotransformations of GLA to GA with ALDH-11300 and electrochemical cofactor recycling led to concentrations up to 1.8 g/l d-GA with 88%ee.

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References

Protein engineering of a thermostable polyol dehydrogenase

H. Wulf1, H. Mallin1, U.T. Bornscheuer*

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

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ABSTRACT

The polyol dehydrogenase PDH-11300 from Deinococcus geothermatis was cloned, functionally expressed in Escherichia coli and biochemically characterized. The enzyme showed the highest activity in the oxidation of xylitol and 1,2-hexanediol and had an optimum temperature of 45 °C. The enzyme exhibited a T50-value of 48.3 °C. The T50 is the temperature where 50% of the initial activity remains after incubation for 1 h. In order to elucidate the structural reasons contributing to thermostability, the substrate-binding loop of PDH-11300 was substituted by the loop–region of a homolog enzyme, the galactitol dehydrogenase from Rhodobacter sphaeroides (PDH-158), resulting in a chimera enzyme (PDH-loop). The substrate scope of this chimera basically represented the average of both wild-type enzymes, but surprisingly the T50 was noticeably increased by 7 °C up to 55.3 °C. Further mutations in the active site led to identification of residues crucial for enzyme activity. The cofactor specificity was successfully altered from NADH to NADPH by an Asp55Asn mutation, which is located at the NAD+ binding cleft, without influencing the catalytic properties of the dehydrogenase.

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1. Introduction

Biocatalytic processes are frequently applied in organic synthesis and due to constant innovation in the protein engineering area, the application of biocatalysts to replace conventional chemistry is an emerging field [1,2]. Nevertheless, newly introduced biocatalysts always have to compete with established processes and need to match both chemical and economical demands. Especially thermostable enzymes are highly useful as they exhibit stability over a broad range of temperatures. This feature is essential if the advantages of elevated temperatures like better substrate solubility, reduced medium viscosity and a lowered risk of microbial contaminations [3] should be exploited.

The known structural reasons for thermostability in enzymes are due to the elevation of structural rigidity by stronger interactions like salt bridges, H-bondings and π–π effects [4]. A contribution to the engineering of the thermostability was the development of the ‘B-factor iterative test’ (B-FIT [5]), which is based on the observation that certain residues in protein crystal structures cannot be fully resolved due to a high flexibility of these amino acids. The B-value is a measure for this flexibility and can be easily retrieved from pdb-files of protein structure data by means of the program PyMol or the B-FIT software and consequently can help to guide the creation of more thermostable variants of enzymes by protein engineering as recently shown by Reetz et al. [5] for Bacillus subtilis lipase A, and by us for an esterase from Pseudomonas fluorescens [6].

Eijssink et al. [3] suggested three ways to access enzymes with improved thermostability: (1) isolating enzyme variants from organisms living in extreme environments, (2) rational-based mutagenesis considering all accessible structural information towards enzyme structure stabilization, and (3) directed evolution with random mutagenesis followed by screening or selection rounds [3]. Rational protein engineering strategies towards increasing thermostability of especially multimeric enzymes involve enhancement of covalent or electronic interactions of the subunits for example by introduction of disulfide bonds and reinforcement of H-bond networks or hydrophobic interactions [7].

Polyol dehydrogenases comprise a large family of oxidoreductases active towards di- or polyhydroxylated species. Their relevance in industry is due to their regio- and enantioselective catalysis leading to valuable chiral products. Here we combined approaches (1) and (2) given above for the engineering of a polyol dehydrogenase towards higher thermostability. The enzyme galactitol dehydrogenase from Rhodobacter sphaeroides D, which is a polyol dehydrogenase (PDH), was evolved from a chemostat culture grown at selective pressure and classified as a tetrameric short-chain dehydrogenase/reductase (SDR [8]). The enzyme is strictly dependent on NAD+, active against a broad range of polyols, with prevalence for aliphatic 1,2-diols and capable for the production of l-taragote. Tagatose is a ketohexose C-4 fructose epimer present in

Abbreviations: PDH, polyol dehydrogenase; SDR, short chain dehydrogenase; MD, molecular dynamics.
* Corresponding author. Tel.: +49 3834 86 4367; fax: +49 3834 86 794367.
E-mail address: uwe.bornscheuer@uni-greifswald.de (U.T. Bornscheuer).
1 Both authors equally contributed to this work.
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http://dx.doi.org/10.1016/j.enzmictec.2012.06.006
nature and a potential low calorie sweetener that can be obtained by oxidation of galactitol [9,10]. The polyol dehydrogenase used within this work [PDH-158], origins from *Rhodobacter sphaeroides* DSM 158, and differs in five amino acid residues from the galactitol dehydrogenase from *R. sphaeroides* D (Fig. S1). The gene product of *Deinococcus geothermalis* (Dgeo2_2865, abbreviated as PDH-1300) was annotated as SDR as well and shares 51% sequence identity with PDH-158. The radiation resistant and mesothermophilic organism *D. geothermalis* (DSM 11300) was isolated from hot springs near Naples, Italy [11]. To date, only a few enzymes from *D. geothermalis* species have been cloned, expressed and functionally characterized. The BRENDA database lists only three enzymes with *D. geothermalis* as origin (1,4-alpha-glucan branching enzyme, amylosucrase and S-ribosylhomocysteine lyase). Recently published results indicate that heterologous expressed enzymes of this strain show moderate thermostability and temperature optima from 45 to 60 °C [12–14].

This paper describes the first study towards a SDR and likewise a polyol dehydrogenase of *D. geothermalis* regarding kinetic and thermic properties and substrate scope. In order to alter and understand the substrate binding, a chimeric enzyme was constructed using PDH-11300 as scaffold. The chimera was created by substitution of a loop sequence of the PDH-11300 with the homolog loop of PDH-158, that was determined as substrate binding domain [15]. Point mutations in this newly created substrate binding site were introduced in order to evaluate the influence of these residues on the binding site hydrophobicity and the substrate scope.

In biocatalytic redox reactions the recycling of the cofactor is the crucial step in order to develop new cost effective synthesis routes. Some enzymes are strictly dependent to one cofactor of either NAD(H) or NADP(H). To broaden the cofactor specificity of an enzyme can thus contribute to a higher versatility in the application of this enzyme. In this paper a further point mutation was introduced in order to change the cofactor specificity of the enzyme. The bases of the NAD(H) dependence of SDRs and hence the incapability to use NADP(H) as cofactor is mostly due to ionic repulsion of certain residues in the cofactor binding cleft. For horse liver dehydrogenase this residue was shown to be an aspartate residue [16]. By means of homology modeling, we identified and mutated the inhibiting residue in the newly built thermostable chimeric PDH mutant, and were able to generate an enzyme with extended cofactor dependency.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and Carl Roth (Karlsruhe, Germany). Polymerases were obtained from Roboklon (Berlin, Germany), restriction enzymes and DNA ligases were obtained from Fermentas (Burlington, Canada).

2.2. Bacterial strains, plasmids and growth conditions

*Escherichia coli* BL21 (DE3) (8 dcm ompT hsdS (r m +) gal) was purchased from Novagen (Darmstadt, Germany) and used as expression strain. *D. geothermalis* DSM 11300 was obtained from DSMZ (Braunschweig, Germany). *E. coli One Shot® TOP10 cells (F- mcrA Δ(mr-hsdS-McrBC) Δ(lacIQZΔM15) lacY1Δ719 recA1 araD139 Δ(strr) proA+ rpsL100 endA1 nupG50) and vector pCR®II-TOP® for subcloning were purchased from Invitrogen (Karlsruhe, Germany). *E. coli* strains were routinely cultured in lysogeny broth (LB) [17] at 37 °C, when necessary supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml). *D. geothermalis* was cultivated in LB medium at 35 °C for four days. The expression plasmid pET-22b (+) was obtained from Novagen (Darmstadt, Germany) and used for heterologous enzyme expression.

2.3. Cloning of PDH-158 and PDH-11300

Genomic DNA (gDNA) from *E. coli* BL21 (DE3) and *D. geothermalis* was isolated using the innuPREP Bacteria DNA Kit (Analytik Jena, Jena, Germany). Plasmid isolations (Fermentas, Burlington, Canada), PCR-purification and gel extraction (Analytik Jena, Jena, Germany) were performed according to the manufacturers protocols. Standard procedures such as DNA cloning and manipulations were performed as described by Sambrook and Russell [18]. DNA sequencing was carried out by GATC Biotech AG (Konstanz, Germany).

The gene pdh-158 encoding for the short chain dehydrogenase (GenBank accession code: GI:77386383, locus tag RSP2363, (NCBI)) was amplified from genomic DNA of *R. sphaeroides* DSM 158 by PCR using the PDH-158 forward and reverse primer pair (Table S1). The SDR gene of *D. geothermalis* (GenBank accession code: GI:4074094, locus tag Dgeo2_2865 (NCBI)) was amplified from genomic DNA of *D. geothermalis* DSM 11300 using the PDH-11300 forward and reverse primer pairs (Table S1). PCR products were separated in a 1.2% agarose gel from which the desired fragments were purified and cloned into the vector pCR®II-TOP®. *E. coli* One Shot® TOP10 cells were transformed with the plasmids bearing the respective insert. The genes were sequenced and cloned into pET-22b (+) via Ndel and BamHI restriction sites (underlined). A N-terminus His6-tag was introduced simultaneously. The expression strain *E. coli* BL21(DE3) was transformed with these constructs.

2.4. Construction of the chimeric enzyme

The chimeric enzyme was constructed using the protein scaffold of PDH-11300 and the loop region E195-R203 of the substrate-binding domain of PDH-158. Mutagenesis was carried out by overlap extension PCR. By using a combination of primers containing the loop mutation and gene flanking primers, two fragments with overlapping ends were amplified in a first PCR. During a second PCR round with these fragments and gene flanking primers the chimeric gene was amplified (Table S1). After restriction with Ndel and BamHI the corresponding gene was ligated into pET22b (+) and transformed into chemical competent *E. coli* BL21 (DE3) cells (CaCl2/RBCl) [19]. The chimeric enzyme is abbreviated as “PDH-loop”.

2.5. Homology model and site-directed mutagenesis

For model prediction, design and docking studies of PDH-11300 and PDH-loop, the software YASARA structure (version 9.10.14 [20]) was used. As force field YAMBER2 [21] was taken and the best found template was the crystal structure of the GatDH of *R. sphaeroides* D (pdb code: 2W02). The resulting model of PDH-11300 had a z-score of -0.363, a structural coverage of 97% and an amino acid coverage of 53%. The model was refined and energy minimized. For PDH-loop the same template with comparable results was used. Energy minimizations of the PDH-loopD55N were performed after in silico mutation of D55N and substitution of the C2 hydroxyl group of NADP+. A first minimization simulation using the YASARA software every residue of the objects were set as fixed, just the Asn residue and the phosphate residue were free. In the subsequent energy minimization both, the NADP+ and enzyme were free.

Mutations of the active site and NAD+ binding site were introduced by site-directed mutagenesis (QuickChange) using complementary primers to introduce the desired mutations. PDH-loop was taken as template for the two single (PDH-loopN99L and PDH-loopQ157A) and the double (PDH-loopN99L/Q157A) mutations. The double mutant PDH-loopN99L/Q157A was used as template for PDH-loopN99L/Q157A and PDH-loopN99L/Q157A/Q157A (NCBI) as template for PDH-loopN99L/Q157A/Q157A/N161M (NCBI). For expression, all mutants were transformed into E. coli BL21 (DE3) chemocompetent cells.

2.6. Expression of PDH variants and enzyme purification

All enzyme variants were expressed in *E. coli* BL21 (DE3) cells grown at 37 °C in shaking flasks. Protein expression was induced at an OD600 of 0.8–1.0 with 0.1 mM IPTG. After 4–6 h of protein expression at 37 °C cells were harvested by centrifugation (15 min, 3939 × g, 4 °C), washed with 100 mM sodium phosphate buffer (pH 7.5, 500 mM NaCl) and disrupted in the same buffer by sonication on ice (3 times for 5 min, 0.5 s pulse, 50% power). The supernatant was collected by centrifugation (30 min, 10 000 × g, 4 °C) and was passed through a 0.2 µm filter prior to chromatography. Affinity chromatography was performed on an AKTA purifier™ (GE Healthcare, Munich, Germany) using a 5 ml Ni-Sepharose 6 Fast Flow crude column (GE Healthcare). The crude extract containing 60 mM imidazole was loaded on the column at a flow rate of 5 ml/min. The bound protein was washed with 100 mM sodium phosphate buffer (pH 7.5, 500 mM NaCl, 60 mM imidazole) and eluted with the same buffer containing 300 mM imidazole. The active fractions were pooled. For storage, the purified enzyme was subjected to gel filtration for removal of imidazole and dialysis against high salt concentration using an AKTA purifier™ equipped with a S5/20 Superfine column (25 × 110 mm). Storage of pure enzyme was performed at 4 °C or lyophilized at −20 °C. Enzyme purity and expression level were verified with SDS-PAGE (data not shown).

2.7. Determination of protein concentration and SDS-PAGE analysis

Polyacrylamide gel electrophoresis was carried out using 4% stacking gel 12% resolving gel according to the method described by Laemmli [22]. RotiMark® STANDARD (Carl Roth, Karlsruhe, Germany, Mw: 14–200 kDa) was used as protein standard. The protein concentration was determined either with the BCA-assay (Uptima, Montluçon, France) or with Coomassie Brilliant-Blue using Roti®-Nanoquant (Carl-Roth, Karlsruhe, Germany) according to manufacturers protocols using BSA as standard.
2.8. CD-spectroscopy for the determination of $\tau_m$

The melting points ($\tau_m$) of PDH variants were determined by circular dichroism (CD) spectroscopy. The purified and desalted enzyme was subjected to CD-spectroscopy in 5 mM sodium phosphate buffer (pH 7.5) using a jasco V-650 at heat rates of 1 °C/min or 2 °C/min.

2.9. Enzyme assays

The dehydrogenase activity was determined spectrophotometrically at 25 or 30 °C by monitoring the rate of NADH ($\varepsilon = 6.22 \text{mM}^{-1} \text{cm}^{-1}$) or NADPH ($\varepsilon = 6.12 \text{mM}^{-1} \text{cm}^{-1}$) formation at 340 nm. The standard reaction mixture (1 ml) contained 100 mM bicine–HCl buffer (pH 9.0), 0.25 mM NAD+, 1–500 mM of different substrates and an appropriate amount of the enzyme. The reaction was started by adding NAD+ to the mixture. One unit of dehydrogenase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol NADH or NADPH per minute.

In order to determine the $K_m$ and $V_{max}$ values for different alcohols the spectrophotometric activity test was carried out with varying substrate concentrations at a fixed concentration of 0.25 mM NAD+ or NADP+ for PDH-loopD55N. Initial velocities were recorded during the first 60 s of catalytic turnover. Apparent $K_m$ and $V_{max}$ values were obtained by linear regression fitting according to the method of Weisiger [23] using the program HYPER (J.S. Easterby, http://www.liv.ac.uk/~jesoftware.html).

The effect of temperature towards enzyme activity was determined by spectrophotometric measurements at different temperatures ranging from 20 °C to 65 °C for 3 min at pH 9.0. Stability studies were performed by incubation of purified enzyme solution for 6 h at the desired temperatures followed by activity tests after different time periods.

For determination of the $K_m$ (that is the temperature where 50% of the initial activity remained after 1 h incubation) enzyme solutions were incubated in a thermostated Analytik Jena, Jena, Germany) at different temperatures. Depending on the studied enzyme, a temperature gradient of 30 °C was applied to 12 microreaction vessels containing enzyme solution. After 1 h incubation, residual enzyme activities were determined spectrophotometrically using a microtiter plate reader. The exact value was calculated by determination of the inflection point of a fit of the residual activities at certain temperatures to a sigmoidal plot (sigmoidal Boltzmann fit, using OriginPro 7.5).

For the determination of the effect of pH on enzyme activity, purified enzyme was measured at 25 °C for 3 min in the following buffers: 100 mM sodium phosphate (pH 5.5–8.0), bicine (pH 8–9) and glycine–NaOH (pH 9–12). For the extinction coefficient of NADH only negligible differences could be detected for the different pH and buffers.

Substrate scopes were determined in microtiter plates with 250 μl total volume in bicine buffer at pH 9.0. The NAD+ concentration was 0.5 mM and the substrate concentration for most of the substrates was 40 mM (Table 2). Different concentrations were employed for the substrates 1-phenylethanol (5 mM, 0.4 mM DMSO), 2-ethyl-1,3-hexanediol, 3-methyl-1-butanol (10 mM, 0.2% DMSO), cyclohexanol, cyclopentanol (10 mM, 0.4% DMSO), (10 mM, 0.2% DMSO), glyceraldehyde (20 mM), galactitol (26.6 mM).

3. Results

3.1. Cloning, mutagenesis and protein expression of polyol dehydrogenases

The enzyme PDH-11300 from *D. geothermalis* was expressed recombinantly in *E. coli* BL21 (DE3) resulting in good yields of active enzyme up to 3.16 kU (activity against xylitol) per liter culture broth. By analyzing the crystal structure of the GdDH from *R. sphæroides* D (pdb code: 2WDZ) a loop region (195–203 aa, EMTLMERER) containing amino acids with high B-values could be identified, and this particular stretch was also found in the sequence of PDH-158 (Fig. 1 and Fig. S1). This loop is located next to the active site and was described as a flexible substrate binding loop, which might fit to several different substrates in the homolog enzyme from *R. sphæroides* D [15]. Based on this knowledge a chimeric enzyme was constructed introducing this loop of PDH-158 into the PDH-11300 protein scaffold. In the alignment of the amino acid sequence of PDH-158 with the PDH-11300 sequence, this loop matched the amino acid positions 196–204 of PDH-11300 (PLTRRGLET). These amino acid residues of PDH-11300 were substituted by the loop of PDH-158 for the construction of the chimera PDH-loop. The resulting mutant PDH-loop could be expressed and purified with yields up to 1.27 kU (activity against xylitol) per liter culture broth.

The substrate binding site of PDH-158 consists of a small and a large cavity [15]. The smaller part is responsible for binding specificity and the larger part may accommodate a wide variety of different substrates. The assumed 4Å radius around the substrates of PDH-158 in the structure (PDB: 2WDZ) and the homology model of PDH-11300 covered the region surrounding the large cavity. 15 amino acid residues were identified, of which ten form the large binding pocket, and only four of the residues of PDH-11300 differed from the PDH-158 structure (Fig. 2). The large cavity of PDH-158 displays a highly apolar character whereas the large pocket of PDH-11300 shows a more polar character, which is due to the four different amino acids. These four residues therefore could represent determinants of substrate specificity and activity. To verify this assumption, four mutations (V97A, N99L, Q157A and N161M) were introduced to create mutants of PDH-11300 bearing the apolar large cavity of PDH-158. Based on the structural analysis two single mutants (PDH-loopN99L, PDH-loopQ157A), one double mutant PDH-loopV97A/N99L, one triple mutant PDH-loopV97A/N99L/Q157A and one quadruple mutant PDH-loopV97A/N99L/Q157A/N161M were constructed with PDH-loop as template, expressed and purified.

The cofactor dependency of PDH-11300 and mutants is strictly linked to NAD+*. To make the biocatalysts described herein suitable for combination with NADP(H) dependent enzymes (for example as thermostable cofactor recycling enzyme), a study on the cofactor specificity was performed. Performing the homology model of the PDH-loop, aspartate residue D55 could be identified pointing towards the ribose C2 hydroxyl group of the NAD+ moiety. It was assumed that this residue would repel the negatively charged phosphate group of the hypothetically bound NADP+. After substitution of the C2 hydroxyl group with the phosphate group in silico two subsequent energy minimizations were conducted. It could be observed that the adenine moiety of NADP+ moved far out of the binding cleft (Fig. 3) in the PDH-loop. Based on this structural analysis the single mutant D55N was created in silico. After introducing the given mutation and performing a MD simulation the phosphate group could be found coordinated in a binding site flanked by amide–H-bond donor groups of the backbone of Q34, L56, N57 such as the amide groups of N57 and the newly introduced N55.
3.2. Biochemical characterization: temperature- and pH-optima, thermostability

The temperature profiles of PDH-11300 and PDH-loop were examined using xylitol as model substrate. The activity was determined between 20 and 65 °C and both enzymes had the highest activity at 45 °C (Fig. 4). These results correspond well with the optimal growth conditions of *D. geothermalis*. At 55 °C a significant loss of activity could be found for PDH-11300 due to inactivation of the enzyme. For the loop mutant PDH-loop a broader temperature range could be determined. This mutant had 64% activity at 60 °C whereas the wild-type was not active any more. This implied a higher stability of the PDH-loop variant towards elevated temperatures. PDH-11300 showed best activity in bicine buffer at pH 9.0 and more than 80% activity remained between pH 8.5–10 during oxidations (Fig. 5). Interestingly, the PDH-loop variant was still active in the basic pH range above 9.0 and showed highest activity at pH 11 in glycine–NaOH buffer (140% compared to bicine buffer at pH 9.0).

Studies on the thermostability of PDH-11300 revealed no loss in activity during 6 h of incubation at 40 °C (Fig. 6b). During incubation at 45 °C a linear decrease in activity could be observed (12.5% residual activity after 6 h). The mutant PDH-loop was stable for 6 h of incubation even at a temperature of 50 °C (Fig. 6c). At 55 °C the activity dropped to 6% after 2 h. These results confirm the broader temperature range and an improved thermostability of +10 °C for the loop mutant. PDH-158, in contrast, exhibits a low stability as 25 °C is the highest temperature where the enzyme remained active for 6 h (Fig. 6a). During incubation at 20 °C a “maturation” of the enzymes could be observed, resulting in an increased enzyme activity after 6 h (for PDH-11300 less characteristic visible at 35 °C).

By comparison of the melting points (Tm) a similar ranking of the thermostability could be observed (Table 1 and Fig. S2). The PDH-158 possessed the lowest Tm whereas the PDH-11300 showed a 5.2 °C (∼48 °C) higher and the PDH-loop a 10.4 °C (∼54 °C) higher melting point. Compared to the PDH-loop variant, the Tm of the cofactor mutant PDH-loopD55N was increased by 11.9 °C to 65.5 °C. For PDH-11300 and PDH-loop the Tm values were nearly comparable to the melting points whereas for PDH-158 and the PDH-loopD55N variant the Tm was about 8–9 °C lower than the Tm value. In order to evaluate the thermostability, the Tm is more accurate than the Tm, because it is directly linked to the enzyme activity after 1 h of incubation at certain temperatures. The Tm of PDH-11300 was 48 °C which was 13 °C higher than the value of

![Fig. 2. Large binding pockets of PDH-158 (1) and PDH-11300 (2). Only the variable residues around the pockets of both enzymes and the nicotinamide ring of the NAD+ are displayed.](image1)

![Fig. 3. Positioning of NADP+ in the PDH-loop cofactor binding cleft (left) and NADP+ bound to PDH-loopD55N (right).](image2)
Fig. 4. Temperature dependency of initial velocities of PDH-11300 (■) and PDH-loop (▲). 100% activity refers to 3.2 U/mg (PDH-11300) and 4.1 U/mg (PDH-loop) purified protein.

Fig. 5. Initial activities of PDH-11300 (filled symbols, ◼, ◼, ▲, ▲) and PDH-loop (empty symbols, ◼, ◼, ▲, ▲) at different pH-values and buffer types. 100% activity refers to 5.5 U/mg (PDH-11300) and 2.4 U/mg (PDH-loop) purified protein.

Fig. 6. Activity plots of A: PDH-158, B: PDH-11300 and C: PDH-loop after incubation up to 6 h at the given temperatures for elucidation of the thermostability of the enzymes. The graph with filled circles in each plot represents the highest temperature where the enzyme is stable for at least 6 h.

Table 1
Melting points $T_m$ and $T_{50}$ values of wild type and mutant polyol dehydrogenases.

<table>
<thead>
<tr>
<th>PDH</th>
<th>$T_m$ (°C)$^a$</th>
<th>$T_{50}$ (°C)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>158</td>
<td>43.2 ± 1.0</td>
<td>35.6 ± 0.4</td>
</tr>
<tr>
<td>11300</td>
<td>48.4 ± 0.5</td>
<td>48.3 ± 0.3</td>
</tr>
<tr>
<td>Loop</td>
<td>53.6 ± 0.2</td>
<td>55.3 ± 0.2</td>
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<tr>
<td>LoopD55N</td>
<td>65.5 ± 1.0</td>
<td>56.1 ± 0.2</td>
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<tr>
<td>LoopD99L</td>
<td>56.9 ± 0.3</td>
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</tr>
<tr>
<td>LoopQ157A</td>
<td>52.9 ± 0.2</td>
<td>nd</td>
</tr>
<tr>
<td>LoopV97A/N99L</td>
<td>53.8 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>LoopV97A/N99L/Q157A</td>
<td>48.5 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>LoopV97A/N99L/Q157A/N161M</td>
<td>48.8 ± 0.4</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^a$ Melting point determined by circular dichroism spectroscopy.

$^b$ Temperature where 50% of enzyme activity remains after 60 min of incubation.

PDH-158. Again the thermophilic nature of PDH-11300 could be testified. The $T_{50}$ of PDH-loop was increased by 7 °C compared to the wild type and roughly confirms findings of the stability measurements. For the active site mutants of PDH-loop only the $T_m$ was investigated for a fast check of dramatically increased or decreased values. This was considered sufficient because the main focus of the mutant properties lay in the activity and alteration of substrate scope, and not on the stability. The $T_m$ showed that no mutant had a melting point lower than PDH-11300. The PDH-loopQ157A and PDH-loopV97A/N99L variants showed no decreased melting point, but the mutant PDH-loopN99L had an increase of the $T_m$ by 3 °C.
A combination of the double mutant and mutation Q157A gave a $T_m$ reduction by 5 °C for the PDH-loop/V97A/N99L/Q157A triple mutant. Heat treatment of PDH-11300 for 10–30 min at 50 °C for purification purposes gave a 1.7-fold higher purity without loss of activity.

3.3. Substrate scope and steady-state kinetics for NADH/NADPH

The substrate scopes of nine PDH variants were determined to evaluate the influence of the introduced mutations. Especially the reconstruction of the hydroporphic substrate binding-cleft of PDH-158 in the PDH-loop mutant by combination of several point mutations mentioned above was investigated. Regarding the substrate spectra of all enzymes a bias towards vicinal diols was obvious. In each analysis 1,2-hexanediol, 1,2-butane-diol and 1,2-propanediol (in the order of decreasing activity) were among the best substrates. 1,2-hexanediol was the best substrate for the PDH-158. In contrast, 1,2-hexanediol was only second best for PDH-11300, here xylitol was the best substrate. Galactitol, the third best substrate for PDH-11300, was a worse substrate for PDH-158, even though galactitol is the naming substrate for this enzyme subclass. By substitution of the substrate recognition loop leading to mutant PDH-loop, the order for the highest activity substrates resembled more the scope of PDH-158 than PDH-11300. The correlation coefficient between PDH-158 and PDH-loop such as PDH-11300 and PDH-loop are $r = 0.78$ and $r = 0.77$ respectively. Comparing the activity for 1-propanol and 1-butanol with the secondary alcohols 2-propanol and 2-butanol in PDH-158 and PDH-11300, it could be derived that these enzymes (and the mutants thereof) preferably oxidize secondary than primary alcohols. The activity of PDH-loopD55N against cyclohexanol was improved remarkably. While glycerol was the sixth best substrate for PDH-158, the activity could not be recovered by rebuilding the hydroporphic binding pocket of the PDH-158 in the single or combined PDH-loop point mutants. The substrate scope of mutant PDH-loop/V97A/N99L among the mutants PDH-loop/N99L, PDH-loop/Q157A, PDH-loop/V97A/N99L, PDH-loop/V97A/N99L/Q157A and PDH-loop/V97A/N99L/Q157A/N161M with $r = 0.94$ had the highest correlation with the substrate scope of the PDH-loop variant (Table 2). As expected, the correlation of substrate scopes was also high between the PDH-loop and PDH-loopD55N ($r = 0.93$). The amino acid positions Q157 and N161 in the PDH-loop variant seem to have an essential role, since enzymes containing mutations at these sites exhibited decreased enzyme activities (i.e. PDH-loop/Q157A, PDH-loop/V97A/N99L/Q157A and PDH-loop/V97A/N99L/Q157A/N161M, Table 2). The positions V97 and N99 seem to be susceptible to mutation since the variants V97A and N99L did not lead to a significant activity loss. In the case of 1,2-hexanediol the combination of these two substitutions (mutant PDH-loop/V97A/N99L) improved the activity compared to the

### Table 2

<table>
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<th>Substrate</th>
<th>158</th>
<th>11300</th>
<th>loop</th>
<th>loopD55N</th>
<th>N-L</th>
<th>Q-A</th>
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<td>714</td>
<td>337</td>
<td>232</td>
</tr>
<tr>
<td>Cyclopentanol</td>
<td>500</td>
<td>383</td>
<td>466</td>
<td>207</td>
<td>464</td>
<td>427</td>
<td>576</td>
<td>343</td>
<td>234</td>
</tr>
<tr>
<td>Ethanol</td>
<td>37</td>
<td>55</td>
<td>23</td>
<td>53</td>
<td>89</td>
<td>131</td>
<td>66</td>
<td>17</td>
<td>56</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>30</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>86</td>
<td>91</td>
<td>61</td>
<td>7</td>
<td>54</td>
</tr>
<tr>
<td>Galactitol</td>
<td>263</td>
<td>1427</td>
<td>508</td>
<td>213</td>
<td>206</td>
<td>95</td>
<td>172</td>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>Glycerinaldehyde</td>
<td>75</td>
<td>72</td>
<td>86</td>
<td>44</td>
<td>116</td>
<td>104</td>
<td>137</td>
<td>28</td>
<td>128</td>
</tr>
<tr>
<td>Glycerol</td>
<td>698</td>
<td>145</td>
<td>193</td>
<td>98</td>
<td>156</td>
<td>109</td>
<td>111</td>
<td>23</td>
<td>54</td>
</tr>
<tr>
<td>Hydroxyacetone</td>
<td>56</td>
<td>100</td>
<td>43</td>
<td>50</td>
<td>120</td>
<td>145</td>
<td>89</td>
<td>26</td>
<td>57</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>72</td>
<td>936</td>
<td>763</td>
<td>400</td>
<td>230</td>
<td>102</td>
<td>166</td>
<td>19</td>
<td>73</td>
</tr>
<tr>
<td>Thioglycerol</td>
<td>221</td>
<td>782</td>
<td>1036</td>
<td>1459</td>
<td>276</td>
<td>147</td>
<td>239</td>
<td>40</td>
<td>71</td>
</tr>
<tr>
<td>Xyitol</td>
<td>6651</td>
<td>4659</td>
<td>1521</td>
<td>1435</td>
<td>1096</td>
<td>128</td>
<td>1722</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

*a Activities are given in μM/mg and were determined with 0.5 mM NAD$^+$ in bicine buffer (pH 9.0; 100 mM). For mutant PDH-loopD55N NAD$^+$ was used as cofactor. Substrate concentrations are given in Section 2.*

b The abbreviation refer to following PDH mutants: N-L: loopN99L, Q-A: loopQ157A, 2x: loopV97A/N99L, 3x: loopV97A/N99L/Q157A and 4x: loopV97A/N99L/Q157A/N161M.
Table 3 Kinetic constants of PDH variants against the cofactor NAD$^+$ and NADP$^+$.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH-11300</td>
<td>NAD$^+$</td>
<td>0.15 ± 0.06</td>
<td>1.61</td>
<td>10,740</td>
</tr>
<tr>
<td>PDH-loop</td>
<td>NAD$^+$</td>
<td>0.15 ± 0.03</td>
<td>2.70</td>
<td>17,800</td>
</tr>
<tr>
<td>PDH-loopD55N</td>
<td>NAD$^+$</td>
<td>0.44 ± 0.26</td>
<td>1.53</td>
<td>3480</td>
</tr>
<tr>
<td>PDH-loopD55N</td>
<td>NADP$^+$</td>
<td>0.41 ± 0.09</td>
<td>1.42</td>
<td>3450</td>
</tr>
</tbody>
</table>

* Activities were determined against 1,2-hexanediol.

single mutants. Despite these two mutations, introduction of further modifications for the construction of the catalytic site of PDH-158 did not lead to improved enzyme activity.

The $K_m$ of PDH-11300 and PDH-loop against NAD$^+$ is 150 μM for both enzymes. The substitution of the substrate recognition loop hence did not effect the co-substrate binding affinity, still the NAD$^+$ turnover increased by 68% to 2.7 s$^{-1}$ (Table 3). Following mutagenesis and expression, activity tests of the purified mutant enzyme PDH-loopD55N showed activity towards both cofactors NAD$^+$ and NADP$^+$. For PDH-11300, PDH-loop and PDH-loopD55N the kinetic parameters were determined for NAD$^+$ and NADP$^+$. No activity could be found for PDH-loop and NADP$^+$. The relaxed mutant PDH-loopD55N showed activity against both, NAD$^+$ and NADP$^+$, all the kinetic parameters where comparable resulting in a catalytic efficiency of ~3.5 s$^{-1}$ μM$^{-1}$. However, the catalytic efficiency of PDH-loopD55N was decreased compared to PDH-11300. Still the findings reinforce the prediction that the NADP(H) phosphate group is stabilized by polar contacts in an H-bond donor site (Fig. 3). A minor disadvantage of PDH-loopD55N was the lower expression level for soluble enzyme compared to the PDH-loop variant.

4. Discussion

In this study a new polyol dehydrogenase from the mesothermophilic organism D. geothermalis 11300 (PDH-11300) was cloned, characterized and served as scaffold for a newly designed biocatalyst. The temperature optimum was examined and corresponds to the optimal growth conditions of the strain of 45 °C. Reported temperature optima of enzymes with D. geothermalis origin vary in a range from 45 °C to 60 °C. For instance amylolysurease of D. geothermalis was reported to have an optimal temperature of 50 °C and a half-life of 26 h at 50 °C [25]. The maximum half-life of the PDH-11300 characterized here was 3.5 h at 45 °C. Nevertheless, the chimeric mutant PDH-loop retained 100% of its activity for more than 6 h at 50 °C. A β–galactosidase from D. geothermalis was described having a half-life of 3 h at 60 °C [14]. The attempt to evaluate and compare these enzyme properties is difficult since different temperatures and timespans are used throughout different studies. It becomes obvious, that the $T_{50}^{\text{so}}$ values describe the thermostability of an enzyme in the most precise and unbiased manner since a fixed incubation time is applied and the $T_{50}^{\text{so}}$ value is determined by fitting the residual activities over several different temperatures.

The PDH-11300 showed the highest activity at pH 9.0. The properties of the enzyme were compared with the homolog PDH enzyme of R. sphaeroides 158 which shares high sequential and structural homology with the D. geothermalis enzyme. The new enzyme PDH-11300 was much more thermostable compared to PDH-158. For determination of the reasons of the different substrate scope and activity, an extensive protein engineering study on the binding site of PDH-11300 was carried out. At first a loop containing amino acids with high B-factors was identified in the crystal structure of PDH-158. It was assumed that this flexible loop highly influences the substrate conversion, especially for larger, bulkier substrates. The newly designed chimeric enzyme PDH-loop showed a similar substrate conversion for 16 compounds compared to PDH-158, which could be explained by the new PDH-158 like active site region. The overall substrate scope was not altered seriously as seen by the correlation coefficients. The substrate scopes of PDH-158 and PDH-11300 correlate by $r=0.81$. In fact the correlations of PDH-loop with the PDH-158 ($r=0.78$) and PDH-11300 substrate scope ($r=0.77$) are slightly smaller. An explanation might be, that the loop region is not the only determinant of substrate specificity.

Interestingly, a broader pH, and temperature profile, such as a 10 °C increased thermostability of the chimera was observed compared to PDH-11300. This was a rather unexpected finding, as the mutant contained the flexible loop of the non-thermostable PDH-158 (merely stable for 6 h at 25 °C). The increase in thermostability was proven by means of the $T_{m}$ long-term stability and $T_{D}^{\text{so}}$. In literature the stabilization of enzymes is described as a gain of rigidity, or is reported as stabilization by substitution of flexible residues. An explanation hence might be, that the corresponding wild-type loop from PDH-11300 is much more flexible than that from PDH-158. For multimeric enzymes stabilization can also occur due to the enhancement of subunit interaction. The altered loop regions are not located at the multimerization interfaces (Fig. 1) but are located on the opposite sites. Due to the far distance no additional H-bonds or hydrophobic interactions were directly introduced. Nevertheless it might be possible, that due to interactions or repulsions between residues inside the subunits longer ranging effects influence the subunit binding. Since no crystal structure of PDH-11300 and hence the PDH-loop variant is available yet, the explanation for the elevated thermostability remains elusive. Nevertheless the chimeric enzyme PDH-loop, represents the first example of enzyme stabilization by introduction of a loop from a less thermostable to a moderately thermostable enzyme. Even if single mutations showed no effect, a combination of the mutations was shown to have a big influence on the thermostolerance.

Following the chimera design and enzyme characterization, the structure–function relationship of the enzyme regarding substrate scope and activity was elucidated by a multiple point mutational study on the active site of PDH-loop. Therefore a map of the large cavity of the substrate binding site from PDH-loop and PDH-11300 was build based upon homology modeling. The goal was to identify key residues for substrate conversion and recognition by alignment of the crystal structure of PDH-158 and the homology model of PDH-11300. A reconstruction of the apolar large cavity of PDH-158 was conducted expecting the substrate scope to approach the template substrate scope. The amino acid positions Q157 and N161 were shown to be important for conversion of 1,2-diols in PDH-loop. The positions V97 and N99 were mutated without significant activity loss, and hence might be candidates for further saturation mutagenesis studies.

We performed a protein engineering of the cofactor specificity of PDH-loop and identified mutation D55N as crucial key residue for NADP$^+$ dependency. The resulting mutant PDH-loopD55N accepted both cofactors with similar activity and affinity (Table 3), neither the chimera nor the wild-type enzymes were active with NADP$^+$. A possible explanation for the change of cofactor activity was found by docking and energy minimization studies. Herein the asparagine residue repels the phosphate group, whereas the asparagine residue provides for additional stabilization of the phosphate.

Since 1,2-hexanediol or xylitol were among the best substrates for the newly designed enzymes, we propose that the enzyme might better be designated as polyol dehydrogenases rather than galactitol dehydrogenases.
5. Conclusion

We have successfully cloned and characterized a new thermostable polyol dehydrogenase PDH-11300 from D. geothermalis. Introduction of a loop of the less thermostable homolog enzyme PDH-158 led to a thermostabilized enzyme. The substrate scope and active site of the PDHs were studied extensively. The strict NAD\(^+\) dependency was expanded successfully to both cofactors (NAD\(^+\) and NADP\(^+\)) by a single point mutation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmitec.2012.06.006.

References

ARTICLE IV
A self-sufficient Baeyer–Villiger biocatalysis system for the synthesis of ε-caprolactone from cyclohexanol

H. Mallin¹, H. Wulf¹, U.T. Bornscheuer*

Department of Biotechnology & Enzyme Catalysis, Institute of Biochemistry, Greifswald University, Felix-Hausdorff-Str. 4, D-17487, Greifswald, Germany

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ABSTRACT

In order to establish a new route for ε-caprolactone production from the corresponding cyclohexanol with an internal cofactor recycling for NADPH, a recently redesigned thermostable polyol dehydrogenase (PDH) and the cyclohexanone monoxygenase (CHMO) from Acinetobacter calcoaceticus were combined. First, the expression of PDH could be improved 4.9-fold using E. coli C41 with co-expression of chaperones. Both enzymes were also successfully co-immobilized on glutaraldehyde-activated support (Relizyme™ HA-403). Cyclohexanol could be converted to ε-caprolactone (ε-CL) with 83% conversion using the free enzymes and with 34% conversion using the co-immobilized catalysts. Additionally, a preparative scale biotransformation of ε-caprolactone starting from cyclohexanol was performed using the soluble enzymes. The ε-CL could be isolated by simple extraction and evaporation with a yield of 55% and a purity of >99%.

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1. Introduction

Researchers in biocatalysis strive to find solutions for the replacement of conventional chemical routes by application of enzymes. The advantages of biosynthetic routes are lower energy cost due to lower reaction temperatures, less or no usage of organic solvents, and fewer efforts required for workup due to the high chemoselectivity of enzymes [1,2]. Especially useful are routes where several enzymatic steps are combined in order to achieve multistep synthesis and avoid isolation of intermediates. A further challenge is the cofactor dependency of especially NAD(P)H-dependent enzymes. We have focused here on the design of a system combining a Baeyer–Villiger monoxygenase (BVMO) with a polyol dehydrogenase in order to produce ε-caprolactone (ε-CL) directly from cyclohexanol (CHL, Scheme 1). On industrial scale, ε-CL is synthesized by Baeyer–Villiger oxidation [3] using peracetic acid and cyclohexanone (CHO) as substrates. ε-CL is used for the formation of biodegradable thermoplastic polyesters by ring opening polymerization [4] and was formerly used as precursor for ε-caprolactam and hence polyamide production [5,6].

Baeyer–Villiger monoxygenases are valuable enzymes for the regio- and stereoselective formation of ester functions by introduction of molecular oxygen via Baeyer–Villiger oxidation [3,7]. BVMOs accept a broad range of substrates; particularly the NADPH-dependent cyclohexanone monoxygenases (CHMO) from Acinetobacter calcoaceticus [8–10] was shown to convert a vast variety of ketones of different substance classes [11,12]. In order to perform cost effective scale-up, efficient cofactor recycling must be ensured for these biotransformations. This might be performed using resting cells as described by Geitner et al. [13] or by co-expression of NADPH-regenerating enzymes like glucose-6-phosphate dehydrogenase and CHMO in whole cell systems [14]. The application of isolated enzymes as biocatalysts can be advantageous for several reasons compared to whole cells especially if the reaction comprises only one (or few) reaction steps [15]. Consequently, several recycling systems using isolated enzymes, e.g. using a phosphate dehydrogenase fused to a BVMO [16], have been developed (for a review see Torres Pazmiño et al. [17]).

To achieve high total turnover numbers and thus to guarantee for the stability of biocatalysts, immobilization of enzymes has been applied since decades. An additional benefit of enzyme immobilization is the easier separation of the catalyst from the reaction product and eventually from the cofactor. Immobilized enzymes, mainly isomerases and hydrolyses are widely used in industrial applications, and many different classes of carriers have been employed [18–20]. The CHMO of A. calcoaceticus could be immobilized for example on polyethyleneimine-coated agarose support via adhesion or covalently bound to Eupergit® C [21].

Abbreviations: PDH, polyol dehydrogenase (PDH,loopN); CHMO, cyclohexanone monoxygenase from Acinetobacter calcoaceticus; CHL, cyclohexanol; ε-CL, ε-caprolactone; CHO, cyclohexanone; BVMO, Baeyer–Villiger monoxygenase; FID, flame-ionization detection; GA, glutaraldehyde.

* Corresponding author. Tel.: +49 3834 86 4367, fax: +49 3834 86 794367.
E-mail address: uwe.bornscheuer@uni-greifswald.de (U.T. Bornscheuer).

1 Both authors equally contributed to this work.

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NADP
pellet
manufacturer
mixture
(Steinheim,
PAGE.
2.1.
Thermoanaerobium
Willets
with
EMT-8459;
scaffold
from
g enase
gene
of
overnight
cell
OD
30
bear
inoculation
30
°
FID).
NADPH.
reduced
extracted
with
dichloromethane.
Dichloromethane
medium
was
determined
by
spectrophotometric
monitoring
do
NADPH
at
340
nm
using
the
same
buffer
with
0.6 mM
CHO.
The
reaction
was
started
by
addition
of
0.3
mM
NADPH.
One
unit
of
dehydrogenase
gene
and
BDV
activity
was
defined
as
the
amount
of
enzyme
that
catalyzes
the
formation
of
1
µmol
NADPH
or
NADP* per
minute
respectively.
For
biocatalysis,
different
amounts
of
pure
enzyme
were
used
in
1–3
ml
batches
(glass
vials
or
flasks)
in
100
mM
bicarbonate
buffer
(pH
9.0)
containing
5–10
mM
NADH
and
a
mixture
of
the
NADP* and
NADPH
biotransformations
were
carried
out
in
a
shaker
flask
containing
30
mM
bicarbonate
buffer
(100
mM,
PH
9.0),
10
mM
NADH
and
0.3
mM
NADP* (unit
corresponds
to
the
conversion
of
1
µmol
min−1
CHO)
and
0.6
mM
of
each,
reduced
and
oxidized
cofactor.
The
reaction
batch
was
taken
in
two
times
with
30
mM
dichlobromathene.
After
evaporation
of
the
organic
solvent,
the
extract
was
analyzed
by
GC–MS
and
also
used
with
the
same
column
used
above.
The
same
heating
conditions
were
used
except
that
the
maximum
temperature
was
increased
to
180°C.
2.6.
Co-immobilization
of
CHMO
and
PDH
For
co-immobilization
of
CHMO
and
PDH,
0.5–1
g
(dry
weight)
of
Relizyme
HA403
were
treated
with
4–8
ml
of
125
mM
glutaraldehyde
(GA)
in
50
mM
phosphate
buffer
(pH
7.5, 2 h).
Different
ratios
of
CHMO/PDH
(units)
dissolved
in
8
ml
were
added
to
the
glutaraldehyde
treated
carrier
and
incubated
for
16
h
at
4°C
and
20
rpm.
After
washing
with
two
times
20
ml
of
phosphate
buffer,
the
immobilized
enzymes
were
used
for
biocatalysis.
For
determination
of
activity,
5
mg
(dry
weight)
of
biocatalyst
were
used
in
a
volume
of
250
µl
reaction
buffer
(pH
9.0)
containing
different
amounts
of
CHL
and
the
cofactor
mixture.
Each
concentration
was
measured
in
duplicate.
After
1.5
h
the
whole
mixture
was
extracted
using
the
protocol
described
above.
Biotransformations
with
75
mg
(dry
weight)
immobilized
enzymes
were
carried
out
in
2
ml
bicarbonate
buffer
(100
mM,
PH
9.0,
18
ml
buffer)
containing
0.3
mM
NADP*,
0.3
mM
NADPH
and
10
mM
CHL
at
30°C.
Samples
were
taken
periodically
and
analyzed
as
described
above.
For
recycling
studies,
100
mg
(dry
weight)
biocatalyst
were
used
in
1
ml
reaction
buffer
(pH
9.0)
containing
5
mM
CHL
and
the
cofactor
mix.
After
1
h,
250
µl
sample
was
taken
and
analyzed
as
mentioned
above.
The
biocatalyst
was
washed
two
times
with
50
mM
cold
sodium
phosphate
buffer
(pH
7.5)
and
was
then
subjected
to
the
next
cycle.
For
the
approach
with
adding
of
two
units
of
the
enzyme
0.1
U
1−1
of
pure
PDH
and
1
U
1−1
of
pure
CHMO
were
added
to
each
cycle.
The
recovered
activity
was
calculated
by
comparison
of
the
missing
units
from
the
supernatant
after
immobilization
and
the
units
found
on
500
mg
(dry)
support
after
immobilization.
3.
Results
3.1.
Expression
optimization
of
PDH
Due
to
the
poor
expression
of
the
PDH
mutant
[22]
we
first
investigated
the
optimization
of
the
overexpression
of
the enzyme.
Because
of
the
large
insoluble
fraction
we
tried
an
expression
at
20°C
which
did
not
result
in
a
higher
activity
of
PDH.
Next,
we
used
different
chaperones
to
support
folding
during
overexpression.
Plasmids
bearing
eight
different
chaperones
(pGro7,
pGJKE8,
pJKE7,
pGTF2,
pF16
respectively)
were
tested
and
turned
out
that
pGro7
showed
an
increased
volumetric
activity
of
4.3-fold
and
pGKE8
an
increase
of
2-fold
compared
to
the
wild-type
without
chaperones
(Fig.
1).
Further
expression
optimization
was
achieved
by
using
different
E.
coli
strains
(BL21,
C41
and
Shuffle).
Regarding
the
protein
specific
activity
of
the
crude
extract,
the
E.
coli
strain
Shuffle
turned
out
to
be
the
most
effective
producer
of
the
overexpressed
enzyme
(Fig.
2).
Regarding
the
protein
specific
activity
of
the
crude
extract,
the
E.
coli
strain
C41
was
the
worst
compared
to
the
other
two
strains.
With
E.
coli
C41
and
expression
of
pGro7
chaperones
(GroES/ GroEL)
about
1592
U L−1
of
culture
broth
could
be
obtained.

corresponds to a 4.9-fold increase compared to initial conditions (Table S1) and represents the best expression system according to the obtained volumetric activity. The low specific activity found for this expression system is due to the high total protein amount of the E. coli C41. Furthermore the better expression was visualized by SDS-PAGE analysis where the same trend could be observed (Fig. S1).

3.2. Biocatalysis with dissolved enzymes and e-CL synthesis

With 0.5 U (per batch) of both enzymes during biocatalysis a conversion of 84 and 80% based upon e-CL formation with 5 and 10 mM CHL, respectively, could be obtained. This conversion was observed after 2 h and only slightly increased after incubation for 4 h. For both approaches after 2 h around 95% of the substrate CHL was consumed.

To determine the rate-limiting step of the two-step reaction, three different ratios of enzyme activity were studied with 5-fold lower amounts of enzymes (Fig. 3). The highest conversion of 84% was obtained after 4.5 h with a threefold excess of CHMO over PDH (94% of CHL was consumed at this time). Equal amounts of enzymes gave a conversion of 63% and even lower conversions were obtained with CHMO in shortfall. In these cases 78% (equal amounts of enzymes) and 52% (CHMO in shortfall) of the CHL was consumed after 4 h. The bottleneck of e-CL formation thus is due to the BVMO activity.

In order to identify the reason for the reaction turnover limitation by the CHMO, the activities of the single enzymes were determined spectrophotometrically at different concentrations of CHL. The initial activity of the CHMO already drops to 30% at 10 mM CHL. The PDH on the contrary showed good performance until 70 mM and no serious activity loss at 90 mM CHL (Fig. 4). Hence, the PDH compared to the CHMO works better with elevated substrate concentrations and indicates that the PDH has a higher stability in the system. As a consequence, the CHMO is the bottleneck for e-CL formation because the activity strongly decreased already at 10 mM CHL.

A preparative scale biocatalysis revealed that after 2 h no residual CHL could be detected. The e-CL product was simply extracted with dichloromethane followed by the evaporation of solvent and was isolated in 55% yield with a purity > 99%. The low yield can be attributed to the high volatility of CHL, which can easily be addressed in larger scale biocatalysis.

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3.3. Co-immobilization of CHMO and PDH on Relizyme™ HA403

To verify the reusability of the free enzymes first a simple filtration through a 10 kDa membrane was performed for both enzymes. This led to a low residual activity of only 10% in the second cycle based on e-CL formation. Thus, immobilization experiments were carried out in order to increase the stability of the CHMO against CHL and to facilitate the reuse of both enzymes. Therefore both enzymes were covalently bound to Relizyme™ HA403 using glutaraldehyde as linker. Investigation of different ratios of the enzymes revealed that a 10:1 (CHMO:PDH) ratio led to the highest activity of the co-immobilized biocatalysts. For the combined approach an activity of 500 mU/g dry carrier with a recovered activity of 28% could be detected. Activity determination at increasing concentrations of CHL showed that CHMO was significantly stabilized against higher concentrations of CHL by immobilization as up to 40 mM CHL could now be used without significant loss of activity (Fig. 5), whereas the free CHMO retained only 15% activity at 25 mM CHL (Fig. 4).

Comparing the initial activity of immobilized and dissolved enzymes, the immobilized enzymes showed a doubled conversion after 1 h, although the free enzymes had a 2.5-fold higher starting activity. Unfortunately, the maximum conversion using the immobilized biocatalysts was only 34% at 10 mM (after 5.5 h). It was supposed that this low overall conversion is due to the low stability of the immobilized CHMO. Repeated 1 h biotransformations using the same co-immobilized biocatalyst for each batch resulted in a residual activity of 10% after the fifth cycle (Fig. 6). The first reuse step already accounts for a loss of 60% of initial activity. By addition of fresh soluble CHMO to each cycle, the loss of activity in the first cycle could be reduced to 35%. The activity of the biocatalyst just slightly decreased in this approach and a remaining activity of 53% was observed after the fifth cycle. By addition of fresh soluble PDH to each cycle no difference to the initial approach without additional enzyme could be observed.

4. Discussion

This paper presents a novel two-step route for the formation of e-CL from CHL and CHO. The starting point was a newly designed chimeric PDH, for which analysis of the substrate scope exhibited an increased activity of PDH against CHL. Additionally the cofactor dependency was broadened allowing for the utilization of NADP+ as co-factor. The reaction product of CHL oxidation, CHO, can be used for a subsequent reaction with a CHO-convert ing Baeyer–Villiger monoxygenase, such as the CHMO of A. calcoaceticus used within this work. Here, the PDH forms the reduced cofactor NADPH during CHL oxidation. The PDH furthermore provides the precursor for e-CL formation, CHO. The irreversible CHMO catalyzed formation of e-CL withdraws CHO from the irreversible redox interconversion of CHL and CHO. This approach thus represents a closed-loop cofactor recycling system, which might be interesting for industrial application because of its self-sufficiency.

At first an optimization of the overexpression was performed. Therefore different chaperones and strains were examined, because variation of the expression temperatures did not result in a higher amount of soluble protein. By using the pGro7 plasmid the volumetric activity could be increased 4.3-fold compared to cultivations without expression of a chaperone. When we investigated different strains it turned out that with E. coli C41 and pGro7 the highest volumetric productivity of active protein could be obtained (4.9-fold higher) although with E. coli SHuffle higher specific activities (0.6 U mg−1 crude protein) were possible. The high differences in volumetric and protein specific were due to the additional expression of the chaperones and the different unspecific protein background of the used E. coli strains.

Using the internal cofactor recycling system by application of dissolved enzymes, conversions up to 84% at 5 mM CHL could be obtained. Here, it could be shown that the conversion is reduced by the evaporation of the substrate. This is due to the fact that no optimized reactor system was used (only flasks with breathable membranes).

The system allows for the production of pure e-CL by simple extraction and removal of the solvent, as could be shown in a small preparative scale biotransformation. 600 mg L−1 of pure e-CL (>99%) could be isolated with no residual substrate present.

The soluble PDH indicated a high stability against the organic solvent CHL whereas the soluble CHMO showed high susceptibility to CHL. The CHMO activity strongly decreased by 70% of relative activity at 10 mM CHL and above and seemed to be the bottleneck for e-CL formation. For this reason a co-immobilization in order to stabilize the CHMO was performed. The activity of CHMO against higher concentrations of CHL was strongly increased due to immobilization. Concentrations up to 3 g L−1 (30 mM) CHL could now be applied with no loss of activity. Concerning the ratio of CHMO:PDH units a 10-fold excess of CHMO in covalent fixation with GA led to highest activity compared to immobilization of equal units.

Recycling the immobilized biocatalyst, we could demonstrate that the main drawback of the approach is the instability of the
CHMO. The results show that the low overall activity and low recycling stability is due to the CHMO stability but not the PDH stability. After 5 reuse cycles the PDH still had an activity of 53%, and thus seems to be a promising candidate for covalent immobilization with GA. The CHMO is sensitive to higher CHL concentration as well as it loses activity during the batch recycling steps. The activity loss of CHMO is probably due to loss of the FAD cofactor as described for immobilized oxidases other than CHMO [27,28]. The functional immobilization of A. calcoaceticus CHMO could be demonstrated for Relizyme™ HA403, but further stabilization of the CHMO activity on the carrier has to be carried out. Such stabilization could involve covalent bonding of the FAD either artificially or by designing or finding of a CHMO with bound cofactor like present in e.g. alditol oxidase [29]. The screening for other carriers or a protein engineering of the CHMO would be further options for optimization. This protein engineering could include the introduction of amino acid residues (for example cysteins or lysines) for a targeted, orientated immobilization of the enzyme. Further optimization of the biotransformation could involve a continuous flow system with substrate feeding of CHL in moderate concentrations.

5. Conclusion

In summary, our recent protein design to extend the PDH for acceptance of the cofactor NADPH enabled the possibility to combine this dehydrogenase with a BVMO for the conversion of an alcohol directly to the corresponding pure lactone (>99%) without any intermittent purification steps. Covalent immobilization of dehydrogenase and BVMO could be successfully applied, but the immobilized CHMO showed a low stability with the support used resulting in lower conversion compared to the free enzymes. With this promising proof-of-principle study we present the first example for the combination of a PDH and a BVMO in soluble and immobilized form to enable the direct formation of e-CL from CHL with no need for an additional cofactor recycling system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2013.01.007.

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