

## Racemization-free and scalable amidation of L-proline in organic media using ammonia and biocatalyst only

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## Supporting Information

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## Solubility of L-proline

**Table S1** Solubility of L-proline in different solvents at various temperatures (data provided by Thermo Fisher Scientific, Linz). The effect of adding L-prolinamide or ammonia on the solubility of L-proline was investigated. 2M2B = 2-methyl-2-butanol.

Solvent	Temperature [°C]	Additive [%w]	L-proline [%w]	L-proline [mM] <sup>a</sup>
H <sub>2</sub> O	25 °C	-	162 <sup>1</sup>	13029
MeOH	21 °C	-	17.5	1202
EtOH	19 °C	-	1.5	103
<i>n</i> -BuOH	21 °C	-	3.1	218
<i>iso</i> -BuOH	21 °C	-	0.4	28.1
1,4-dioxane	70 °C	-	<0.01	<0.7
1,4-dioxane	70 °C	L-prolinamide 3.1	0.03	2.7
1,4-dioxane	70 °C	L-prolinamide 5.1	0.06	5.4
2M2B	21 °C	-	0.02	1.4
2M2B	21 °C	L-prolinamide 8.5	0.09	6.3
2M2B	70 °C	-	0.05	3.5
2M2B	70 °C	L-prolinamide 8.3	0.30	21.0
2M2B	70 °C	L-prolinamide 14.8	0.59	41.3
2M2B	21 °C	NH <sub>3</sub> 2.4	0.46	32.2

<sup>a</sup>calculated using the density of the pure solvent at 21 °C.

Additional solubility data (provided by Thermo Fisher Scientific, Linz):

Solubility of NH<sub>3</sub> in 2M2B: 2.4 %w NH<sub>3</sub>  
Solubility of L-prolinamide in 2M2B: at 21 °C: 8.5 %w  
at 70 °C: 18.0 %w

Dissolved concentrations of L-proline and L-prolinamide were quantified by HPLC measurements. The NH<sub>3</sub> concentration was determined potentiometrically by acid-base titration.

## Enzyme engineering

### Rational engineering of CalB

Interesting amino acid exchanges were selected from literature focussing on diverse strategies to improve product formation.

### Solvent stability

Increased stability in organic solvents is a desired parameter. In literature enzyme variants with improved stability in MeOH have been reported. 1,4-dioxane is hydrophilic and miscible with water. In a paper by Park *et al.* the stabilization of CalB in hydrophilic organic solvents was performed by rational design of hydrogen bonds based on information of previously found stabilized variants by directed evolution.<sup>2</sup> The concept behind that study was to increase hydrogen bonds between the

enzyme surface and surrounding water molecules to reduce enzyme deactivation by stripping of water molecules from the enzyme surface. Variants were tested with up to 80% methanol and residual activities were higher than that of the wild type. However, no absolute activities were given, only the residual activity of each variant normalized to 0% methanol in aqueous systems. Purely organic solvent systems have not been used. Other publications also describe inhibiting effects by methanol rather than enzyme destabilization.<sup>3</sup> Recently, the obtained variants with increased stability in hydrophilic solvents were combined with amino acid exchanges for modulating the flexibility within the substrate binding region.<sup>4</sup> The aim of this study was to increase the stability as well as activity of CalB by multiple-site mutagenesis.

### **Thermostability**

Next to engineering for improved solvent stability, thermostability was another engineering goal in order to enable increased reaction temperatures and prolonged reaction times. Several variants, which have been reported in literature have been selected for investigation under the desired reaction conditions including V210I/V221D/A281E,<sup>5</sup> R249L,<sup>6</sup> A162C/K308C,<sup>7</sup> A251E,<sup>8</sup> P218N/L219K/F220T/V221S and T57A/A89T/G226R/R168K.<sup>9</sup> Interestingly the amino acid exchanges T57A and A89T are already naturally occurring in a CalB homologue from ATCC 32657 *Pseudozyma aphidis* used as parental enzyme for engineering by circular permutation.<sup>10</sup>

### **Docking study of L-proline into the active site of CalB**

A docking study was performed to visualize the location of the substrate in the active site. For the modeling the structure of CalB PDB-ID: 5A71, chain A in 'open' form was used. Energy minimization was performed with YASARA Structure utilizing AMBER03 Force Field. Based on the modeling of the substrate in the active site, surrounding amino acid residues, including Val190, Ile189, Thr138, Asp134 and Gln157, were selected to evaluate their influence on substrate binding. The docking experiment indicated that there was a lot of free space around the substrate in the active site. This was in agreement with the fact that bulkier substrates, such as Cbz-protected L-proline, were very well converted. One strategy consisted in increasing the size of the amino acid side chains to test whether it was possible to achieve tighter binding of the substrate when the available space was reduced. The opposite hypothesis, to enable alternative binding modi by insertion of smaller amino acid residues, was tested as well.

### **Water tunnel**

Several alternative approaches to increase amidation activity of CalB were pursued. One strategy consisted in closing the water tunnel towards the active site of the enzyme to avoid unwanted hydrolysis. Although this might also decrease the accessibility for NH<sub>3</sub>, due to the similar size of water and ammonia, it may allow the use of increased water concentrations in the reaction media to improve the substrate solubility without increasing the effect of hydrolysis. In a study by the group of K. Hult, the double variant Q46A/S47L was designed which resulted in strongly reduced hydrolysis as well as increased transacylation activity.<sup>11</sup>

**Table S2:** Results of rational enzyme engineering normalized to protein loading. This table summarizes the intention behind each amino acid exchange and describes whether the idea for the amino acid exchange comes from a literature reference or from the present work.

Amino acid exchange	Conv. norm. [%] <sup>a</sup>	Intention	Source/Reference
N264Q	57.9	Solvent stability	2
A251E	46.8	Thermostabilization	8,12
T245S	46.1	Solvent stability	4,12
N97Q	45.7	Solvent stability	2,4,12
A8T	45.1	Solvent stability	4,12
Q46A/S47L	42.6	Water tunnel	<sup>11</sup> , for S47 see also 13
K308C	38.2	Thermostabilization	7
<b>CalBopt-24</b>	<b>38.1</b>	<b>Thermostabilization (T57A/A89T/G226R/R168K)</b>	<b><sup>9</sup>, T57A and A89T occur in CalB homologue<sup>14</sup></b>
A92E/V139E	37.4	Combination solvent stability	4,15
R249L	37.0	Thermostabilization	6
A92E	33.8	Solvent stability	4,12
A282L	32.1	ester synthesis, near active site	16,17
<b>CalB WT</b>	<b>31.6</b>	<b>Wild type</b>	-
L278A	31.5	rigidity, alcohol binding pocket	L278A in <sup>16,18,19</sup> , Leu278 in <sup>17,20–22</sup>
Novozym 435	26.1	Novozym 435 as reference	Novozymes
I285Q	25.8	rigidity, alcohol binding pocket	16,17,23
V139E/T245S	24.9	Combination solvent stability	4,12,15
P218N/L219K/ F220T/V221S	23.7	Combination solvent stability	<sup>9</sup> , L219 in <sup>21</sup>
V139E	21.9	Solvent stability	4,15
L278P	21.3	rigidity, alcohol binding pocket	L278P in <sup>20,21</sup> , Leu278 in <sup>17–19,22,24</sup>
A162C	19.4	Thermostabilization	7
A281E	15.4	Improved resistance towards irreversible thermal inactivation, ester synthesis	17,25
V210I/V221D/ A281E	13.1	Improved resistance towards irreversible thermal inactivation, ester synthesis	17,25
V190L	12.8	Modifying substrate binding mode	Present work
T138S	12.2	Modifying substrate binding mode	Present work, also in <sup>26</sup>
I189L	10.6	Modifying substrate binding mode	Present work, also in <sup>26</sup>
I189F	6.6	Modifying substrate binding mode	Present work
T138F	5.9	Modifying substrate binding mode	Present work, also in <sup>26</sup>

T138H	4.1	Modifying substrate binding mode	Present work
T138L	2.6	Modifying substrate binding mode	Present work
V190A	0.8	Modifying substrate binding mode	Present work, also in <sup>26</sup>
I189P	0.1	Modifying substrate binding mode	Present work
Q157L	0.1	Modifying substrate binding mode	Present work, also in <sup>26</sup>
T40V	0.0	Modifying substrate binding mode	Present work, <sup>17</sup>
V190H	0.0	Modifying substrate binding mode	Present work
VI90P	0.0	Modifying substrate binding mode	Present work
V190F	0.0	Modifying substrate binding mode	Present work

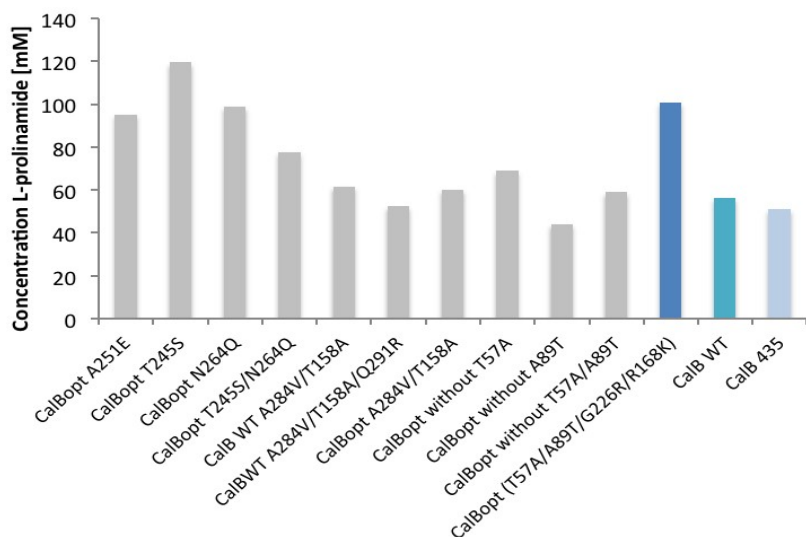
<sup>a</sup>Transformations of L-proline (33 mM) were performed in 2M2B with 0.5 M NH<sub>3</sub> (500 μL) for 16 h at 700 rpm and 70 °C. The reactions were catalyzed by different CalB variants (all resulting in similar enzyme titers as the wild type) immobilized by covalent binding on resin ECR8285 from Purolite and lyophilized (2 mg). Analysis was performed by HPLC-MS and results were normalized to the amount of protein bound on the beads.

All variants were immobilized and stored for roughly the same time (several months) before lyophilization and analysis of amidation of L-proline in 2M2B. In the initial screening of the variants, the beads were applied without lyophilization in 1,4-dioxane. In this screening, CalBopt-24 exceeded all other variants and the wild type significantly. Therefore, this variant was selected for subsequent random mutagenesis experiments.

### Combination of amino acid exchanges

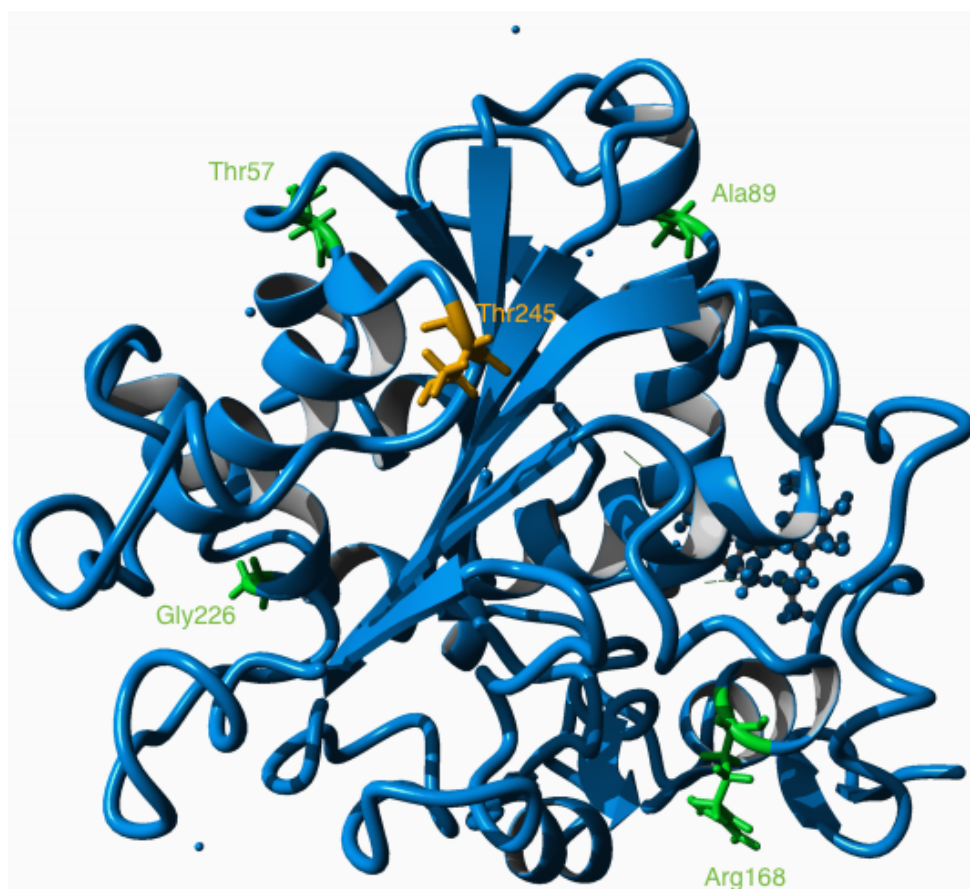
Based on the results of the first CalB variants (obtained by rational as well as random engineering), the amino acid exchanges of interesting variants were combined.

For industrial applications, high product concentrations are desired. The substrate loading was increased to 200 mM (only partially dissolved) and the enzyme loading to 4 mg immobilized beads. After 120 h, a product concentration of ~100 mM L-prolinamide was obtained with CalBopt-24 (shown in Figure S1). Under the same conditions, a 20% increase was observed with CalBopt-24 T245S (named CalB-S). The obtained results indicated, that the currently best variant for production of L-prolinamide is CalB-S (CalB T57A/A89T/G226R/R168K/T245S). The positions of the exchanged amino acid residues in the protein structure (shown in Figure S2) showed that the residues are spread all over the enzyme and not focused on the same region, e.g. not located in the active site, which suggests that involvement in substrate binding interactions seems to be less likely than a stabilizing effect on the structure.



**Figure S1:** Transformation of L-proline (200 mM) in 2M2B with 0.5 M  $\text{NH}_3$  (500  $\mu\text{L}$ ). The reactions were performed with different CalB variants and Novozym 435 (4 mg) for 120 h at 700 rpm and 70  $^\circ\text{C}$ ; addition of MeOH and HPLC-MS analysis of the reactions. The results were normalized to the protein loading on the beads.

#### Amino acid exchanges of CalB variant T57A/A89T/R168K/G226R/T245S (CalB-S)



**Figure S2:** Cartoon representation of the location of the five amino acid exchanges (T57A/A89T/R168K/G226R/T245S) in the protein structure of CalB-S.

## Testing alternative hydrolases

### Commercially available enzymes

Reports in literature suggest that alternative enzymes or enzyme formulations can be applied for amide formation beside immobilized CalB.<sup>27–29</sup> Different enzyme preparations were tested with L-proline and Cbz-L-proline as substrate with 2M2B and NH<sub>3</sub> at 70 °C. Low levels of L-prolinamide formation were obtained with CalB in a liquid preparation. Only trace amounts were obtained with CalB in lyophilized form (DSM) and lyophilized lipases from *Candida rugosa* and *Mucor miehei*, as well as immobilized Lipolase 100T and Lipozyme TL IM (shown in Table S3).

**Table S3: Alternative enzyme preparations for biocatalytic amidation of L-proline.** Transformation of L-proline (33 mM) in 2M2B with 0.5 M NH<sub>3</sub> (500 µL).

	L-proline [mM]	L-prolinamide [mM]	Conversion [%] <sup>a</sup>
Novozym 435 (immob.)	12.9	20.9	61.9
CalB lyoph, DSM (lyoph.)	33.8	0.02	0.06
Alcalase CLEA 201UF (CLEA)	31.0	n.d.	n.d.
Lipase Typ VII <i>Candida rugosa</i> (lyoph.)	32.8	0.01	0.03
Lipase RM IM Rh. <i>Mucor miehei</i> , NOVO (immob.)	34.5	0.02	0.05
Lipase <i>Candida lipolytica</i> (lyoph.)	35.8	n.d.	n.d.
Lipolase 100T (50 mg) (immob.)	27.5	0.02	0.08
Lipozyme TL IM (15 mg) (immob.)	24.9	0.02	0.07
Lipase <i>Antarctica</i> B liqu.(5 µL) <sup>b</sup> (liquid)	24.5	2.24	8.37

<sup>a</sup> The reactions were performed with different enzyme preparations (8.3 mg, unless otherwise stated) for 16 h at 700 rpm and 70 °C; addition of MeOH and HPLC-MS analysis of the reactions. The enzyme preparations were applied as obtained by the supplier. <sup>b</sup> additional peak after 3.1 min at m/z (pos mode) 115 and 158 (115 m/z represents L-prolinamide), peak was not further characterized. n.d. = not detected.

A 'Hydrolase Enzyme Screening Kit (HESK-4800)' from Almac Group (Craigavon, UK) was purchased, which contained 48 hydrolases. The enzymes were provided in lyophilized, liquid or immobilized form. The latter were directly used for the target amidation reaction. The liquid enzyme formulations were added to the beads (ECR8285) and the buffer for immobilization. The lyophilized enzyme preparations were incubated in the immobilization buffer over night under gentle mixing for solvation prior to the immobilization on the epoxy beads. Those enzymes, which were soluble in the immobilization buffer, were immobilized on resin ECR8285 and their capability to catalyze the amidation of Cbz-L-proline or L-proline was investigated. The experiments were run for 62 h in 1,4-dioxane at 40 °C with 8.3 mg enzyme (immobilized, without lyophilization). The results showed that substantial amidation of L-proline was only achieved with AH 39 'Lipase from *Candida antarctica*' (15% conversion) and traces with AH 2 'Lipase B from *Alcaligenes sp.*' (0.14% conversion).

To reduce the water content of the beads after immobilization of the enzymes from the Almac Hydrolase Kit, the beads were lyophilized for 24 h. No amidation activity was detected in 2M2B at 40 °C, 700 rpm, 16 h with NH<sub>3</sub> (0.5 M) and L-proline (33 mM) with the immobilized and lyophilized preparations (2 mg).

Furthermore, Lipase Amano PS-IM (immobilized on diatomaceous earth) was tested and the following commercial preparations were immobilized on ECR8806 and tested for amidation: Acylase I from porcine kidney and Acylase I from *Aspergillus melleus*, Acylase Amano 30000, Penicillinase from

*Bacillus cereus* and Amidase from *Rhodococcus erythropolis* (2 mg). In none of these cases formation of L-prolinamide was obtained.

## Enzyme preparation

The effect of different methods for the stabilization and immobilization of CalB WT in organic solvent were investigated. Highest amounts of product formation were obtained with Novozym 435 and CalB expressed in *P. pastoris* and immobilized on resin ECR8806 from Purolite (CalB 8806). Both preparations are based on immobilization via adsorption. According to respective product data sheets, Novozym 435 is adsorbed on macroporous Lewatit® VP OC 1600, consisting of poly(methacrylic acid) with divinylbenzene for cross-linking. Numerous applications of this preparation have been reported, often due to its exceptional activity and stability. Nevertheless, recycling of Novozym 435 can be problematic due to enzyme leaching and mechanical instability.<sup>30</sup> According to the supplier, the octadecyl activated methacrylate resin of ECR8806 allows reversible but at the same time very strong adsorption on the hydrophobic material.<sup>31</sup> In comparison to covalent binding adsorption causes less conformational change, which may explain the higher conversions of adsorbed preparations. When CalB is immobilized on resin ECR8285 (CalB 8285), covalent binding between the amine groups of the enzyme and the epoxy groups of the epoxy/butyl methacrylate carrier takes place.

Furthermore, cross-linked enzyme aggregates (CLEAs),<sup>32,33</sup> sol-gel entrapped CalB<sup>34</sup> and EziG™, immobilized on controlled porosity glass,<sup>35</sup> were tested. In Table S4 a comparison with the same amount of enzyme preparation, is shown. Novozym 435 and CalB 8806 led to highest amide formation. According to literature, the enzyme loading on the support of Novozym 435 ranges from 8.5 to 20 w/w%.<sup>30</sup> The varying enzyme loadings make a comparison difficult. When amide formation was normalized to the actual amount of enzyme, highest product concentrations were received with CalB Solgel, Novozym 435 and CalB 8806.

**Table S4** Effect of different CalB preparations on L-prolinamide formation in mM normalized to 1 mg of enzyme preparation and to 1 mg of enzyme.

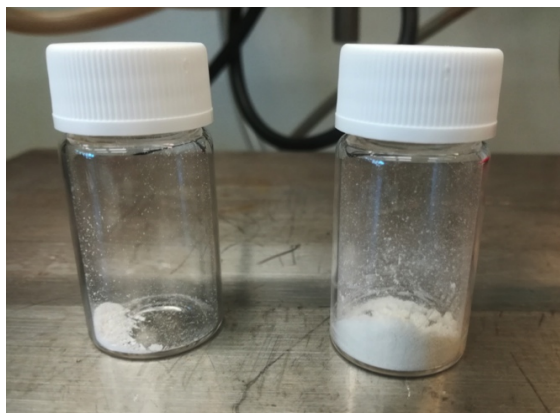
	mM product/mg enzyme preparation <sup>a</sup>	mM product/mg enzyme (max –min protein loading) <sup>a</sup>
Novozym 435	2.7	10.6 – 28.5
CalB WT 8285 (covalent)	2.0	9.7
CalB WT 8806 (adsorption)	2.7	20.6
CalB CLEA	1.6	n.d.
CalB Solgel	0.5	20.5 – 31.0
CalB EziG	0.4	2.3

<sup>a</sup>Transformation of L-proline (33 mM) in NH<sub>3</sub> in 2M2B (0.5 M, 500 µL) with various CalB preparations (8.3 mg) for 16 h at 700 rpm and 70 °C; conversions were analyzed by HPLC-MS. n.d. = no data about protein loading available.



## Preparative scale

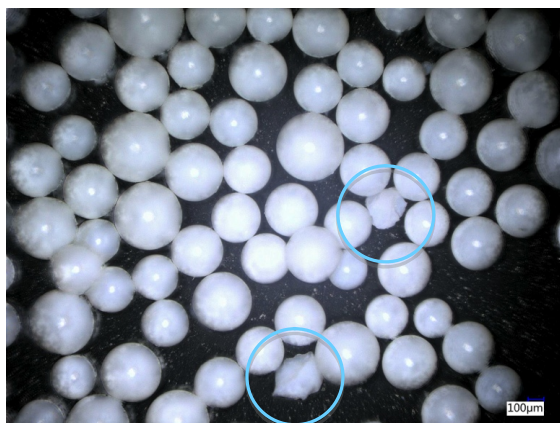
### Product isolation of 15 mL-scale reactions



**Figure S3:** Picture of isolated L-proline (~0.02 g, vial on the left hand side) and L-prolinamide (~0.10 g, vial on the right hand side) from a biotransformation with Novozym 435 (isolation by Thermo Fisher Scientific, Linz).

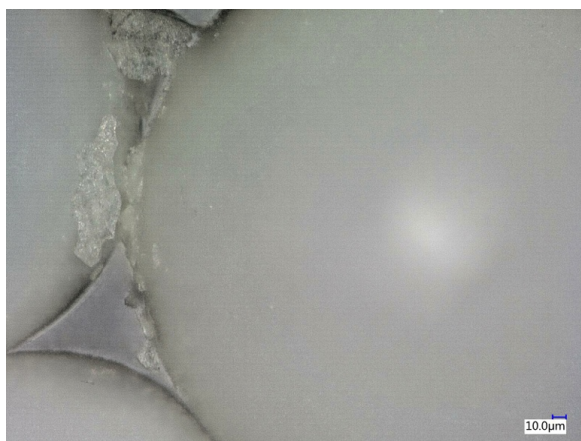
### Microscopy pictures after the reaction

The shape of the beads after the reaction was analyzed by microscopy (by Thermo Fisher Scientific, Linz, Austria) to assess whether mechanical damage has occurred. After shaking, the beads of resin ECR8806 were still largely intact (Figure S4).



**Figure S4:** Microscopy pictures of CalB immobilized on resin ECR8806 after biotransformation of L-proline in 2M2B with  $\text{NH}_3$ . Two uncharacterized fragments can be seen in the middle and the lower part of the picture, which are marked with blue circles. The surface of the beads showed no cracks.

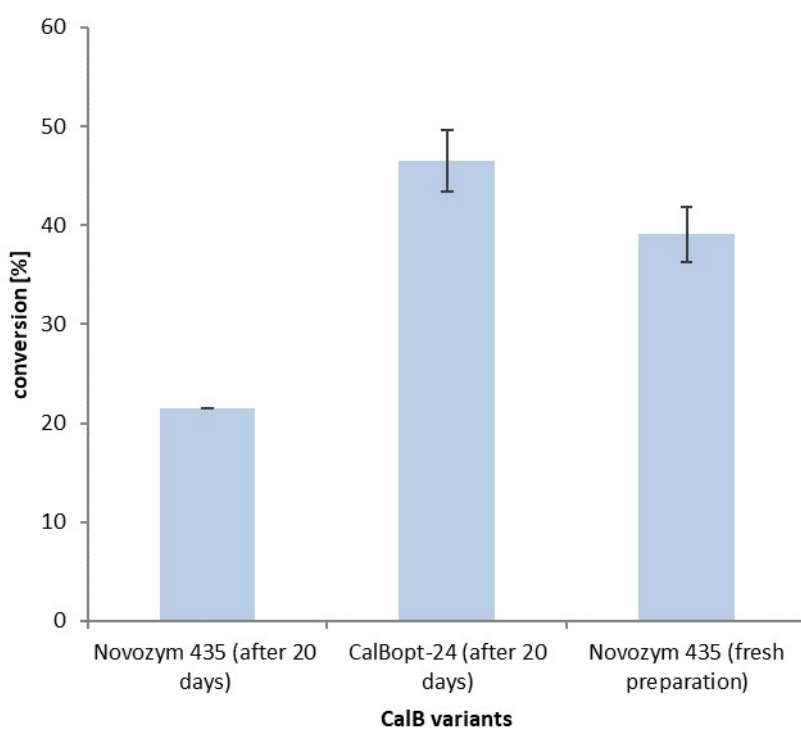
The next picture by transmitted-light microscopy revealed, that the surface was not damaged. Solid matter precipitated on the surface of the beads (Figure S5). The beads themselves remained intact.



**Figure S5:** Picture of CalB immobilized on ECR8806 after biotransformation of L-proline in 2M2B with  $\text{NH}_3$  by transmitted-light microscopy.

### Recyclability of the immobilized enzyme preparations

The enzyme preparations were reused for several amidation reactions of L-proline in 2M2B with  $\text{NH}_3$  for 20 days and washed with 2M2B in between. Comparing the conversion reached with the reused preparation with a fresh preparation showed, that reused Novozym 435 resulted in half of the conversion of fresh Novozym 435. In contrast, CalBopt-24 showed still higher conversion than fresh Novozym 435 (see Figure S6).



**Figure S6:** Transformation of L-proline (33 mM) in 2M2B with 0.5 M  $\text{NH}_3$  (500  $\mu\text{L}$ ). The reactions were performed with CalB435 or CalBopt-24 immobilized on ECR8806 (3 mg) for 16 h at 700 rpm and 70°C; addition of MeOH and HPLC-MS analysis of the reactions.

## Experimental part: cloning, mutagenesis and enzyme expression

### Rational engineering

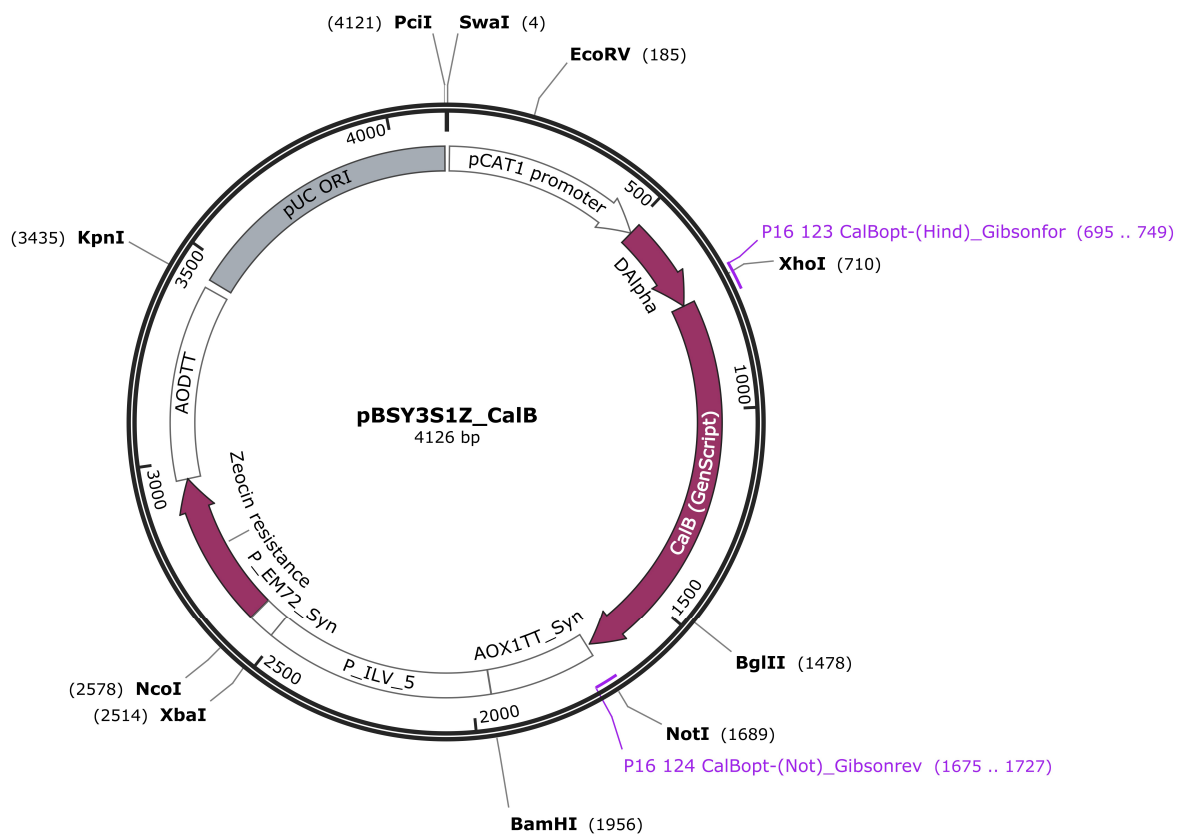
#### Chemicals, media, enzymes and kits for cloning

Oligonucleotides were purchased from IDT Integrated DNA Technologies (Leuven, Belgium). The GeneJET Plasmid Miniprep Kit from Thermo Fisher Scientific (Waltham, MA, USA) was used according to the protocol of the manufacturer for isolation of purified plasmid DNA. The Wizard SV Gel and PCR Clean-UP System from Promega GmbH (Mannheim, Germany) was used for DNA purification following agarose gel electrophoresis, PCR or restriction digestions. Chemicals were purchased from Fresenius Kabi Austria (Graz, Austria), Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), Becton, Dickinson and Company (Franklin Lakes, NJ, USA) and Biozym Biotech Trading (Vienna, Austria). PCRs were performed using Phusion High Fidelity Polymerase (Thermo Fisher Scientific) and GoTaq DNA polymerase (Promega, WI, USA) for error-prone PCR. Restriction enzymes were purchased from Thermo Fisher Scientific or New England BioLabs (Ipswich, MA, USA). The primer sequences are listed in the appendix. Gibson Assembly<sup>®</sup> cloning kits were purchased from SGI-DNA (La Jolla, CA, USA).

Media preparation: The following stock solutions were prepared: 500xB: 0.02% D-Biotin, filter sterilized; 10xYNB: 134 g/L Difco™ Yeast Nitrogen Base w/o Amino Acids, 10xD (20%): 220 g/L  $\alpha$ -D(+)-Glucose monohydrate, 10xG (20%): 200 g glycerol + 800 mL dH<sub>2</sub>O; 10x PPB (1 M PPB, pH 6.0): 30.13 g/L K<sub>2</sub>HPO<sub>4</sub>\*3 H<sub>2</sub>O, 118.13 g/L KH<sub>2</sub>PO<sub>4</sub>. For preparation of BMD1%/BMG1%/BMM5%: 10xYNB (100 mL/L), 10xPPB (200 mL/L), 10xD/10xG/MeOH (50 mL/L) and 500xB (2 mL/L) were added to dH<sub>2</sub>O after autoclaving.

#### Concept

The rational variants of CalB were generated based on the two-step site-directed mutagenesis protocol<sup>36,37</sup> using Q5<sup>®</sup> High-Fidelity DNA Polymerase (New England Biolabs GmbH). For expression of CalB a 500 bp fragment of the *CAT1* promoter was used.<sup>38</sup> The first step of the workflow was the insertion of the desired mutation(s) into the CalB wild type gene by PCR. After transformation of *E. coli* Top10 F' cells and sequencing, the DNA was used to transform *P. pastoris* by integration into the genome.



**Figure S7:** Vector map of pBSY3S1Z\_CalB. The vector contains a pUC origin for replication in *E. coli*, an *EM72\_syn* and *ILV5* promoter for expression of a Zeocin resistance marker in *E. coli* and *P. pastoris*, respectively, and an *AOD* terminator. The *CAT1* promoter (500 bp) and *AOX1\_syn* terminator were used for expression of CalBopt-24 and the  $D\alpha$  signal sequence for secretion into the supernatant. Restriction sites (*XhoI* and *NotI*) and primers used for amplification and cloning of CalB are shown.

### Cloning of CalB mutants in the pBSY3S1Z\_CalB expression vector

The vector backbone of pBSY3S1Z\_CalB (shown in Figure S7) was cut with *XhoI* and *NotI*. This vector included a deletion variant of the *S. cerevisiae* mating factor alpha signal sequence ( $D\alpha$ ). Two PCRs per mutation were started (PCR1: P16123 CalBopt-(Hind)\_Gibsonfor + rev primer for the desired mutation and PCR2: P16124 CalBopt-(Not)\_Gibsonrev + fw primer of the desired mutation). The plasmids were assembled via Gibson assembly or oePCR and used to transform *E. coli* Top10 F' cells. Cells were plated on LB-Zeocin (Life Technologies, Carlsbad, CA, USA; 25  $\mu\text{g}/\text{mL}$ ) plates, plasmid DNA was isolated and sent to Microsynth for sequencing.

When the correct sequence has been confirmed, plasmids were linearized with *SwaI* and used to transform *P. pastoris* BSY BG11 cells. After addition of DNA (1  $\mu\text{g}$ ) to the cells (30  $\mu\text{L}$ ), incubation on ice >5 min and electroporation with 1.5 kV, sorbitol (500  $\mu\text{L}$ ) and YPD (500  $\mu\text{L}$ ) were added to the cells. They were regenerated for 2 h at 28  $^{\circ}\text{C}$  and plated on YPD-Zeocin (100  $\mu\text{g}/\text{mL}$ ) plates.

## Directed evolution of CalB

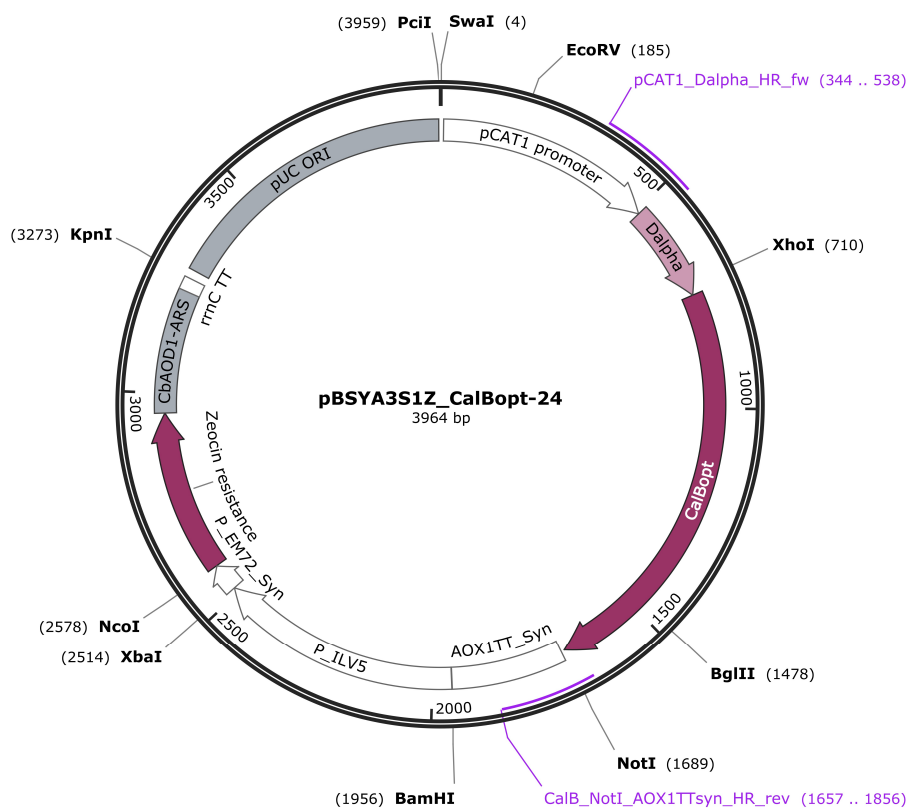
### Concept

Next to the rational design of CalB variants, a random engineering approach based on directed evolution was performed. CalBopt-24 (T57A/A89T/G225R/R168K), the best variant identified in the initial screening of the rational variants with 1,4-dioxane as solvent (data not shown), was chosen as starting point for mutagenesis by error-prone PCR (epPCR).

An ARS (autonomous replicating sequence) plasmid system was used, which consisted of inserting the gene in a plasmid containing an ARS by homologous recombination in *P. pastoris*. The advantages of this system compared to integration into the genome are that significantly lower amounts of DNA are required for transformation and that cloning in *E. coli* and linearization can be omitted. On the other hand, in some cases integration of the plasmid in the genome may occur and distort the results and addition of antibiotics to the growth media is required to avoid plasmid loss. When the landscapes of the different cloning procedures were compared, highest activity and uniformity of the clones was obtained with the pBSYA3Z ARS plasmid assembled by homologous recombination in *P. pastoris* BSY11dKU70 (obtained from bisy, Austria).

### Cloning

The ARS plasmid pBSYA3S1Z (shown in Figure S8) was used. The original stuffer fragment between the *CAT1* promoter and *AOX1TT\_Syn* terminator was removed by *SapI* digestion. For homologous recombination by *P. pastoris* primers with long overhangs to *P<sub>CAT1</sub>* and *TT<sub>AOX1\_Syn</sub>* were designed and the plasmid pBSY3S1Z\_CalBopt-24 including a D $\alpha$  signal sequence was used as template for epPCR.



**Figure S8:** Vector map of pBSYA3S1Z\_CalBopt-24. The vector contains a pUC origin for replication in *E. coli*, an *EM72\_syn* and *ILV5* promoter for expression of a Zeocin resistance marker in *E. coli* and *P. pastoris*, respectively, an *rrnC* terminator and a CbAOD1 ARS sequence as terminator and autonomous replicating sequence in *P. pastoris*. The *CAT1* promoter (500 bp) and *AOX1\_syn* terminator were used for expression of CalBopt-24 and the *D $\alpha$*  signal sequence for secretion into the supernatant. Restriction sites (*Xho*I and *Not*I) and primers used for homologous recombination of the CalB insert with the plasmid are shown.

### Random mutagenesis

Different epPCR conditions (0.1-0.5 mM MnCl<sub>2</sub>, varied dNTP ratios) were tested to determine a suitable error rate for mutagenesis of CalBopt-24 (T57A/A89T/R168K/G226R) and the secretion signal *D $\alpha$* . It turned out, that addition of MnCl<sub>2</sub> caused too many inactive variants. Therefore, the standard GoTaq® Flexi polymerase protocol (with MgCl<sub>2</sub>) was used once with equimolar dNTP ratios and once with an imbalanced ratio of dTTP+dCTP to dATP+dGTP of 3 to 1. The epPCR inserts (150 ng) were assembled with the cut backbone of pBSYA3Z (100 ng) by homologous recombination in yeast. BSY11dKU70 cells (obtained from bisy, Austria) were transformed with the DNA fragments (2kV; 500  $\mu$ L sorbitol and 500  $\mu$ L YPD-medium were added for 2 h regeneration at 28 °C, cells were plated on YPD Zeocin plates).

### Cultivation and screening

After transformation of *P. pastoris*, individual clones were picked and cultivated in 96-well DWPs in 250  $\mu$ L YPD-Zeocin (50  $\mu$ g/mL Zeocin, 1% glucose) for transformants containing ARS plasmids. Positive controls (CalBopt-24) and negative controls (blank, only buffer) were added on each plate. After cultivation for 60 h the cells were stamped on YPD-Zeocin plates to conserve the transformants and 250  $\mu$ L YPM (10% MeOH) was added for induction. Another induction step was performed after 72 h with 50  $\mu$ L YPM (10% MeOH). After 84 h total cultivation time, the cells were harvested by centrifugation for 10 min at 3000 xg.

The protein activity in the supernatant was determined with the *p*NPB assay before and after incubation with 1,4-dioxane as cosolvent. For the screening without co-solvent, substrate solution in Tris/HCl buffer (160  $\mu$ L) was added to the supernatant (40  $\mu$ L) and absorbance was followed over 10 min at 405 nm. Furthermore, supernatant (100  $\mu$ L) was incubated in 1,4-dioxane in Tris/HCl buffer (40  $\mu$ L and 60  $\mu$ L, respectively, in total 20% 1,4-dioxane) for 2 h at 60 °C in a polypropylene microtiter plate (MTP) sealed with aluminium foil. After centrifugation of the MTP at 4000 xg for 5 min, 40  $\mu$ L were added to *p*NPB substrate solution in Tris/HCl buffer (160  $\mu$ L) for activity measurement.

### Analysis of best variants

For isolation of plasmid DNA from *P. pastoris*, yeast lysis buffer (1 M sorbitol, 100 mM EDTA, 14 mM DTT in ddH<sub>2</sub>O) was prepared and the clones with highest activity were cultivated in ONCs (5 mL YPD-Zeocin). The ONCs were harvested by centrifugation (5 min, full speed) and the pellet was washed with ddH<sub>2</sub>O (1 mL). After another centrifugation step, the pellet was resuspended in yeast lysis buffer (1 mL) and zymolyase stock solution (4000 U/mL, 20  $\mu$ L) was added. The reaction was incubated for 2 h at 30 °C and the obtained spheroblasts were harvested by centrifugation (5 min, full speed). After removal of the supernatant the pellet was used for standard plasmid DNA isolation with the

Miniprep Kit and the DNA was used for transformation of *E. coli* cells, for preservation and sequencing of the genes. Most genes contained one mutation and sometimes one or two additional silent mutations.

After sequencing of clones with beneficial mutations and retransformation of *P. pastoris*, a rescreening of 20 clones per construct was performed to verify that the variants are indeed more active and contain only one plasmid per cell. The hydrolytic activity before and after incubation in organic solvent was measured with the *p*NPB assay and normalized to the protein concentration (determined with the Bradford assay). Consequently, the best variants were inserted in the integration vector pBSY3S1Z (*Xho*I and *Not*I) and the plasmid was used for transformation of *E. coli* and *P. pastoris* BSY BG11 cells (see description above). After cultivation in shaking flasks (450 mL BMG1%) and induction with MeOH, the cell cultures were harvested and the protein supernatant was concentrated and immobilized. The immobilized CalB variants were applied for amidation of the target substrate in organic solvent.

### Protein concentration

The total protein concentration in the samples was determined using the Bradford Assay (Bio-Rad; Art: 5000006). The Bradford solution was diluted 1:5 with dH<sub>2</sub>O. The samples (5 µL) were pipetted in the wells of a transparent 96-well MTP and Bradford solution (200 µL) was added. BSA standards with concentrations of 0.0625 to 2 mg/mL BSA and a blank sample were measured as well. All determinations were made in duplicates. After incubation of at least 5 min the absorbance at 595 nm was measured with a plate reader (with a 5 sec shaking period before measurements).

### Buffer exchange

PD10 desalting columns from GE Healthcare Life Sciences (Little Chalfont, UK) were used for buffer exchange at small scale according to the recommendations of the supplier. After washing the columns with ddH<sub>2</sub>O and target buffer, the sample (2.5 mL) was loaded onto the column. Target buffer (3.5 mL) was then applied to elute the protein and the fraction was collected. Depending on the total sample volume, the last two steps were repeated when required. At the end, the column was washed with ddH<sub>2</sub>O and stored in 20% EtOH. The obtained protein solutions were concentrated by centrifugation using Vivaspin 20, 10,000 MW CO centrifugation tubes from Sartorius at full speed and 8 °C.

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## Appendix

### List of primers

#### Fw and rev primer to create two fragments per construct:

Primer Nr.	Primer name	sequence 5'-3'
P16123	CalBopt-(Hind)_Gibsonfor	GAAGAGGGTGTCTCTCTCGAGAAGAGAGAGGCCGAAGCTTTGCCTTCAG GTTTCAG
P16124	CalBopt-(Not)_Gibsonrev	CTCAGGCAAATGGCATTCTGACATCCTCTTGAGCGGCCGCTTAAGGGGTA ACG
P16459	CalBopt-(XhoI)_Gibsonfor	CTAAGGAAGAGGGTGTCTCTCTCGAGAAGAGAGAGGCCGAAGCTTTGCC TTCAGGTTTCAG
P16121	CalBopt-(Hind)_for	AATCAAAGCTTTGCCTTCAGGTTTCAG
P16122	CalBopt-(Not)_rev	AATCAGCGGCCGCTTAAGGGGTAACG

#### Primers for rationally designed CalB variants:

P16460	CalB_T40V_fw	CTATTTTGCTTGTCCCAGGTGTTGGAACTACAGGTCTCAATC
P16461	CalB_T40V_rev	GATTGAGGACCTGTAGTTCCAACACCTGGGACAAGCAAATAG
P16462	CalB_T40Y_fw	CTATTTTGCTTGTCCCAGGTTACGGAACTACAGGTCTCAATC
P16463	CalB_T40Y_rev	GATTGAGGACCTGTAGTTCCGTAACCTGGGACAAGCAAATAG
P16464	CalB_D134E_fw	GATGGCATTGCTCCTGAGTATAAAGGTAAGTGTCTTG
P16465	CalB_D134E_rev	CAAGACAGTACCTTTATACTCAGGAGCAAATGCCATC
P16466	CalB_D134N_fw	GATGGCATTGCTCCTAACTATAAAGGTAAGTGTCTTG
P16467	CalB_D134N_rev	GACAGTACCTTTATAGTTAGGAGCAAATGCCATC
P16468	CalB_Q157L_fw	CTTCCGTCTGGCAATTGACCACTGGAAGTGC
P16469	CalB_Q157L_rev	GCACTTCCAGTGGTCAATTGCCAGACGGAAG
P16470	CalB_Q157T_fw	CCTCCGTCTGGCAAACAACCACTGGAAGTGC
P16471	CalB_Q157T_rev	GGCACTTCCAGTGGTGTGTTGCCAGACGGAAG
P16472	CalB_Q46L_S47L_fw	CTGGAACTACAGGTCTTTGCTTTTGTATTCCAAGTGG
P16473	CalB_Q46L_S47L_rev	CCAGTTGGAATCAAAAAGCAAAGGACCTGTAGTTCCAG
P16474	CalB_I285F_fw	CTGCCGAGCTGCCTTCGTTGCAGGTCC
P16475	CalB_I285F_rev	GGACCTGCAACGAAGGCAGCTGCGGCAG
P16476	CalB_L278M_fw	GTTGCTGCCGAGCTTTGATGGCCCTGCCGAGCTGC
P16477	CalB_L278M_rev	GCAGCTGCGGCAGGGGCCATCAAAGCTGCGGCAGCAAC
P16478	CalB_L278Y_fw	GTTGCTGCCGAGCTTTGTACGCCCTGCCGAGCTGC
P16479	CalB_L278Y_rev	GCAGCTGCGGCAGGGGCGTACAAAGCTGCGGCAGCAAC
P16480	CalB_D145V_fw	GGCTGGACCATTGGTTGCCCTTGCAAGTTTC
P16481	CalB_D145V_rev	GAAACTGCAAGGGCAACCAATGGTCCAGCC
P16482	CalB_K290V_fw	CATCGTTGCAGGTCTGTTCAGAATTGCGAGCCAG
P16483	CalB_K290V_rev	CTGGCTCGCAATTCTGAACAGGACCTGCAACGATG
P16484	CalB_G39A_fw	CTATTTTGCTTGTCCCAGCCACTGGAAGTACAGGTCC
P16485	CalB_G39A_rev	GGACCTGTAGTTCCAGTGGCTGGGACAAGCAAATAG
P16486	CalB_Q46A_S47L_fw	GTAAGTGGAACTACAGGTCTGCACTTTTGTATTCCAAGTGGATTG
P16487	CalB_Q46A_S47L_rev	GAATCCAGTTGGAATCAAAAAGTGCAGGACCTGTAGTTCCAGTAC
P16488	CalB_I285Q_fw	CCCTGCCGAGCTGCCCAAGTTGCAGGTCTTAAAC
P16489	CalB_I285Q_rev	GTTTAGGACCTGCAACTTGGGCAGCTGCGGCAGGG
P16490	CalB_L278P_fw	GGTTGCTGCCGAGCTTTGCCAGCCCTGCCGAGCTGCCATC

P16491	CalB_L278P_rev	GATGGCAGCTGCGGCAGGGGCTGGCAAAGCTGCGGCAGCAACC
P16492	CalB_A281E_fw	GCTTTGCTTGCCCCTGAAGCAGCTGCCATCGTTGC
P16493	CalB_A281E_rev	GCAACGATGGCAGCTGCTTCAGGGGCAAGCAAAGC
P16494	CalB_A282L_fw	CTTTGCTTGCCCCTGCCTTGCTGGCTGCCATCGTTGCAGG
P16495	CalB_A282L_rev	CCTGCAACGATGGCAGCCAAGGCAGGGGCAAGCAAAG
P16083	CalBopt-T138F_for	GCTCCTGACTATAAAGGTTTTGTCTTGCTGGACCATTG
P16084	CalBopt-T138F_rev	CAATGGTCCAGCCAAGACAAAACCTTTATAGTCAGGAGC
P16085	CalBopt-T138H_for	GCTCCTGACTATAAAGGTCATGTCTTGCTGGACCATTG
P16086	CalBopt-T138H_rev	CAATGGTCCAGCCAAGACATGACCTTTATAGTCAGGAGC
P16087	CalBopt-T138P_for	CTGACTATAAAGGTCCTGTCTTGCTGGAC
P16088	CalBopt-T138P_rev	GTCCAGCCAAGACAGGACCTTTATAGTCAG
P16089	CalBopt-I189F_for	CTCAGCTACCGATGAGTTTGTTC AACCTCAGGTCTC
P16090	CalBopt-I189F_rev	GAGACCTGAGGTTGAACAAACTCATCGGTAGCTGAG
P16091	CalBopt-I189P_for	CTCAGCTACCGATGAGCCTGTTCAACCTCAGGTC
P16092	CalBopt-I189P_rev	GACCTGAGGTTGAACAGGCTCATCGGTAGCTGAG
P16093	CalBopt-V190F_for	GCTACCGATGAGATCTTTC AACCTCAGGTC
P16094	CalBopt-V190F_rev	GACCTGAGGTTGAAAGATCTCATCGGTAGC
P16095	CalBopt-V190H_for	CTACCGATGAGATCCATCAACCTCAGGTCTC
P16096	CalBopt-V190H_rev	GAGACCTGAGGTTGATGGATCTCATCGGTAG
P16097	CalBopt-V190P_for	CTACCGATGAGATCCCTCAACCTCAGGTCTC
P16098	CalBopt-V190P_rev	GAGACCTGAGGTTGAGGGATCTCATCGGTAG
P16099	CalBopt-N97Q_for	GTATGCCGGTCCGGAAACCAGAAATTGCCAGTCTTACTTG
P16100	CalBopt-N97Q_rev	CAAGTAAGAACTGGCAATTTCTGGTTCCGGAACCGGCATAC
P16101	CalBopt-N264Q_for	GTAACCTTTGCCAGCTCAGGATCTTACTCCAGAAC
P16102	CalBopt-N264Q_rev	GTTCTGGAGTAAGATCCTGAGCTGGCAAAGGGTTAC
P16103	CalBopt-D265E_for	CCTTTGCCAGCTAATGAACTTACTCCAGAACAAAAG
P16104	CalBopt-D265E_rev	CTTTTGTCTGGAGTAAGTTCATTAGCTGGCAAAGG
P16105	CalBopt-A8T_for	GGTTCAGACCCAACCTTCTCACAGCC
P16106	CalBopt-A8T_rev	GGCTGTGAGAAGGTTGGGTCTGAACC
P16107	CalBopt-A92E_for	GCAATTACTGCTTTGTATGAAGGTTCCGGAACAATAAATTG
P16108	CalBopt-A92E_rev	CAATTTATTGTTTCCGGAACCTTCATACAAAGCAGTAATTGC
P16109	CalBopt-T245S_for	GCTTTGAGATCAACCTCTGGTCAGGCAAGATC
P16110	CalBopt-T245S_rev	GATCTTGCCTGACCAGAGGTTGATCTCAAAGC
P16111	CalBopt-V139E_for	CTGACTATAAAGGTAAGTGAATTGGCTGGACCATTGGATG
P16112	CalBopt-V139E_rev	CATCCAATGGTCCAGCCAATTGAGTACCTTTATAGTCAG
P16113	CalBopt-R249L_for	GATCAACCACTGGTCAGGCACTTTCTGCTGATTATGGAATTAC
P16114	CalBopt-R249L_rev	GTAATTCATAATCAGCAGAAAGTGCCTGACCAGTGTTGATC
P16115	CalBopt-A162C_for	CAGACCACTGGAAGTTGCTTGACAACCGCAC
P16116	CalBopt-A162C_rev	GTGCGGTTGTCAAGCAACTTCCAGTGGTCTG
P16117	CalBopt-K308C_for	GACCATTTGCCGTTGGATGTAGAATTGCTCAGGAATC
P16118	CalBopt-K308C_rev	GATTCCTGAGCAAGTCTACATCCAACGGCAAATGGTC
P16119	CalBopt-A251E_for	CTGGTCAGGCAAGATCTGAAGATTATGGAATTACCGAC
P16120	CalBopt-A251E_rev	GTCGGTAATTCCATAATCTTCAGATCTTGCCTGACCAG

CalBopt-T138Ln_for	CTGACTATAAAGGTCCTTGCTTGCTGGCTGGAC
CalBopt-T138Ln_rev	GTCCAGCCAAGACAAGACCTTTATAGTCAG
CalBopt-I189Ln_for	CTCAGCTACCGATGAGCTTGTTC AACCTCAGGTC
CalBopt-I189Ln_rev	GACCTGAGGTTGAACAAGCTCATCGGTAGCTGAG

CalBopt-V190Ln\_for CTACCGATGAGATCCTTCAACCTCAGGTCTC  
CalBopt-V190Ln\_rev GAGACCTGAGGTTGAAGGATCTCATCGGTAG

**Primers for reverting mutations to the wildtype:**

CalB\_A57T\_fw CAACTGGATTCCATTGAGTACTCAGCTTGGATACAC  
CalB\_A57T\_rev GTGTATCCAAGCTGAGTACTCAATGGAATCCAGTTG  
CalB\_T89A\_fw GTCAATGCAATTACTGCTTTGTATGCCGGTTC  
CalB\_T89A\_rev GAACCGGCATACAAAGCAGTAATTGCATTGAC  
CalB\_E251A\_fw CTGGTCAGGCAAGATCTGCTGATTATGGAATTACCGAC  
CalB\_E251A\_rev GTCGGTAATTCCATAATCAGCAGATCTTGCCTGACCAG  
CalB\_S245T\_fw GCTTTGAGATCAACCACTGGTCAGGCAAGATC  
CalB\_S245T\_rev GATCTTGCCTGACCAGTGGTTGATCTCAAAGC  
CalB\_A46Q/L47S\_fw GTACTGGAACACTACAGGTCCTCAATCTTTTGATTCCAACCTGGATTC  
CalB\_A46Q/L47S\_rev GAATCCAGTTGGAATCAAAGATTGAGGACCTGTAGTTCCAGTAC

**Primers to insert mutations found by directed evolution:**

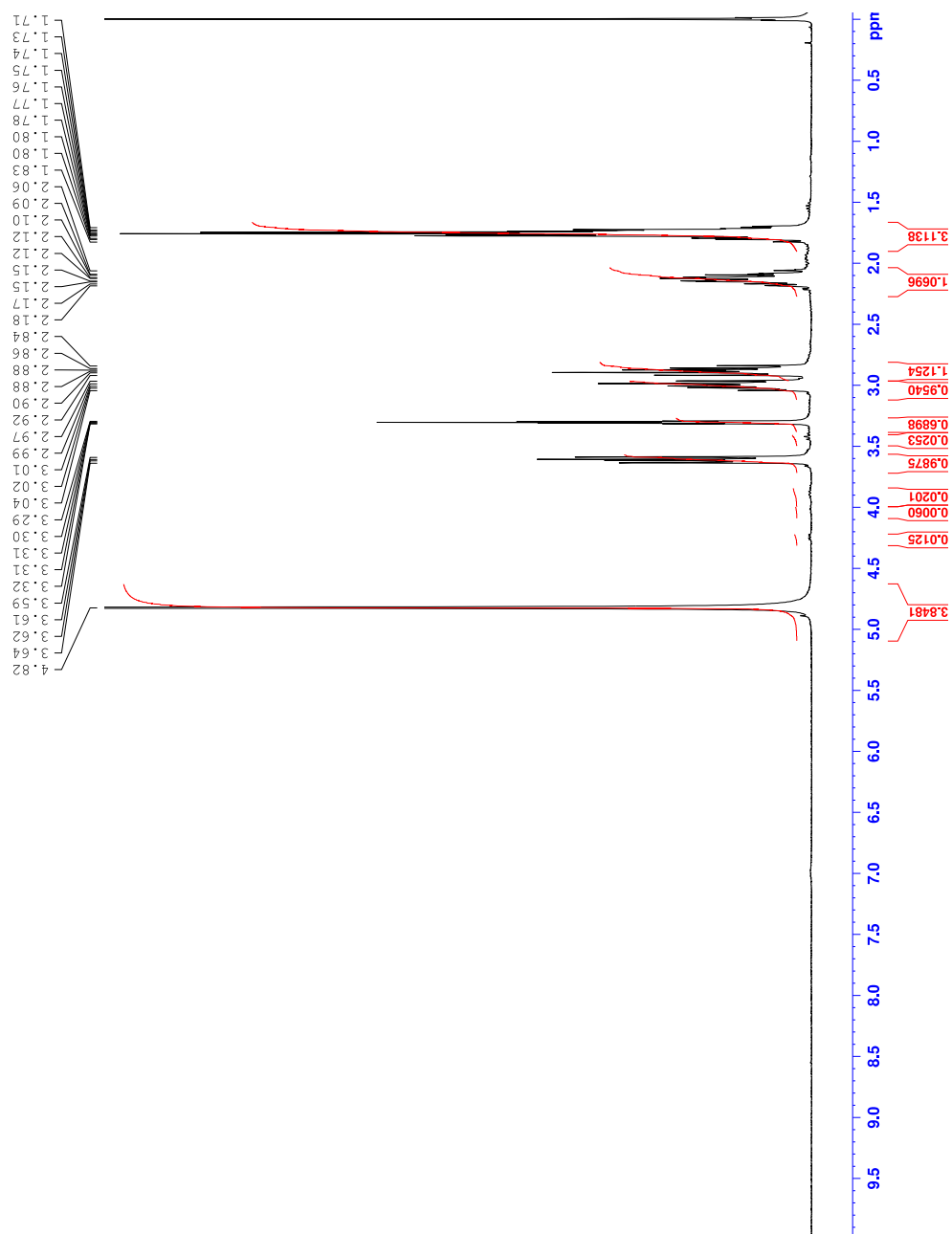
CalB\_A284V\_fw CTGCCGCAGCTGTCATCGTTGCAGGTCC  
CalB\_A284V\_rev GGACCTGCAACGATGACAGCTGCGGCAG  
CalB\_T158A\_fw CGTCTGGCAACAGGCCACTGGAAGTGC  
CalB\_T158A\_rev GCACTTCCAGTGGCCTGTTGCCAGACG

**Primers for sequencing:**

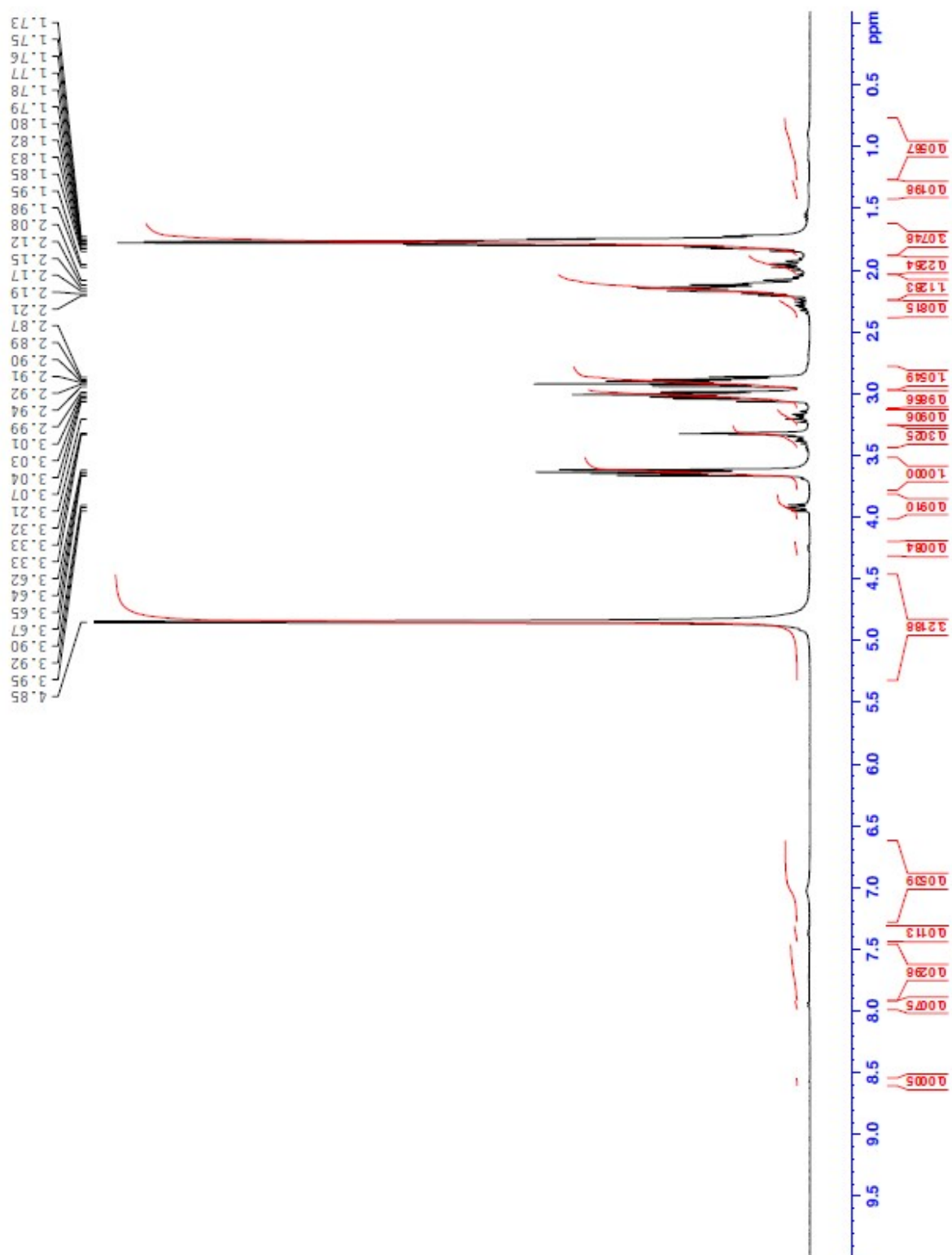
pCAT1-500\_seq\_fw CATATATAAGCTGTAGACCCAGCAC  
seqAOX1TT-120..143-rev CGAGATAGGCTGATCAGGAGCAAG

## NMRs after product isolation

### $^1\text{H}$ NMR after product isolation of biotransformation with CalBWT immobilized on ECR8806



<sup>1</sup>H NMR after product isolation of biotransformation with Novozym 435

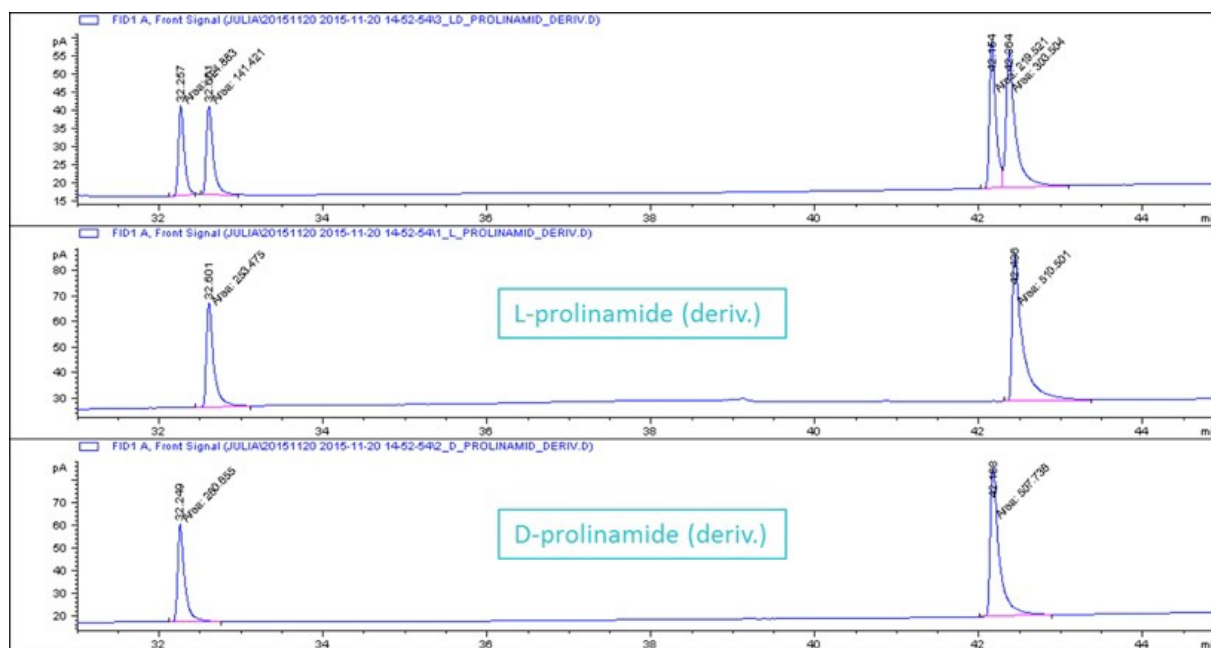


<sup>13</sup>C NMR after product isolation of biotransformation with Novozym 435

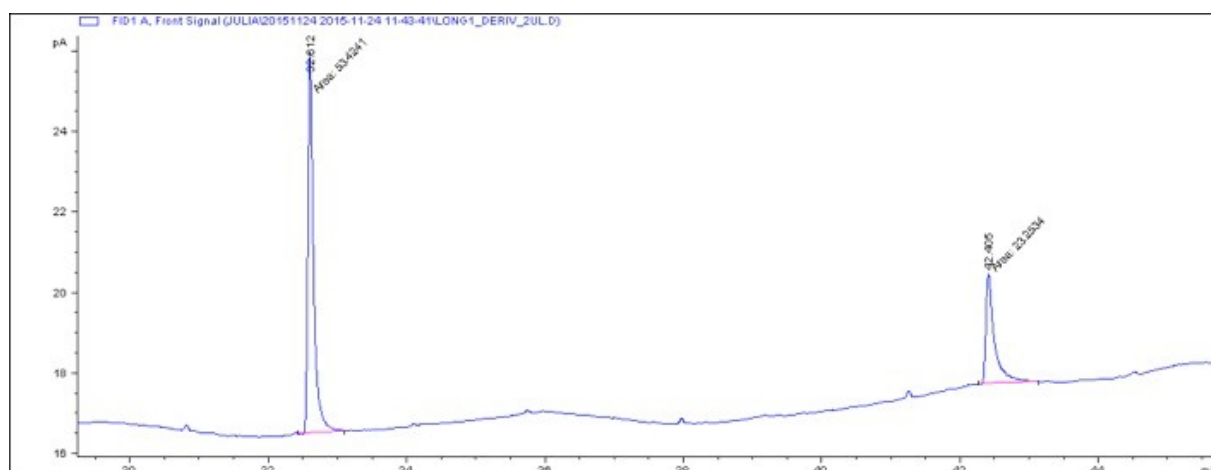


## Optical purity: D-/L-prolinamide racemisation

For analysis of D-/L-prolinamide racemization, derivatization with ethyl chloroformate and DMAP was performed. This method allowed the differentiation between the two enantiomers after derivatization and GC-measurements. In the biotransformations with L-proline as substrate, L-prolinamide was obtained.

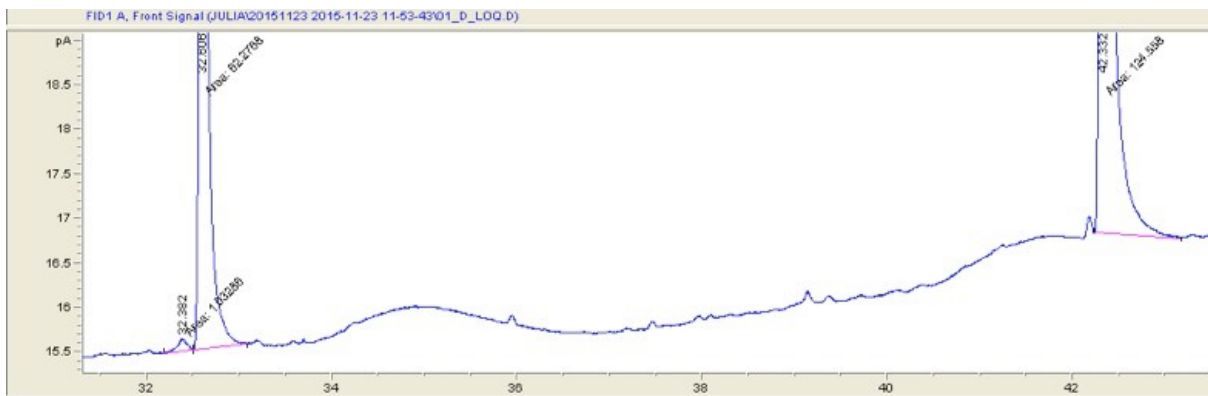


**Figure S9:** GC chromatograms of D,L-prolinamide as well as L-prolinamide and D-prolinamide after derivatization with ethyl chloroformate/DMAP.



**Figure S10:** GC chromatogram of biotransformation of L-proline to L-prolinamide with Novozym 435 and ammonia (derivatized).

In order to determine the limit of detection of the GC analysis, L-prolinamide solutions were spiked with 1% as well as 0.1% of D-prolinamide and analyzed by GC. In both cases it was possible to distinguish between the peaks of the two enantiomers and to detect the D-enantiomer.



**Figure S11:** GC chromatogram of L-prolinamide spiked with 0.1% D-prolinamide for determination of the limit of detection (afterderivatisation).