Enhancement of organic solvent stability of an `ene´- reductase

Diploma thesis

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Kurzfassung

Das Potenzial das sich durch den Einsatz von Enzymen in der Biokatalyse ergibt ist einzigartig. Mittlerweile sind Biotransformationen nahe zu unverzichtbar in industriellen Prozessen. Dennoch gibt es Limitierungen, die beseitigt werden müssen. Ein Beispiel dafür ist die Anwesenheit von organischen Lösungsmitteln, die dazu dienen die Löslichkeit von diversen Substraten zu verbessern, die Produktabtrennung zu erleichtern und die Anzahl unerwünschter Seitenreaktionen zu verringern.

Das Ziel dieser Arbeit war es, die Stabilität einer heterolog exprimierten ‘Ene’-Reduktase in der Anwesenheit eines organischen Co-Solvens zu verbessern. Dafür musste zuerst ein Ausleseverfahren im Mikrotiterplatten- Maßstab entwickelt werden, das die Detektion von stabilitätsverbesserten Mutanten ermöglichte.

Die für das Screening eingesetzten Mutanten Bibliotheken wurden mittels error-prone PCR hergestellt und in DMF/Toluol (2%/10%) beziehungsweise NMP/Toluol (2%/10%) Gemischen in Puffer vermessen. Für das Screening wurde das durch Zellaufschluss entstehende Rohlysat in Anwesenheit des organischen Lösungsmittelgemisches geschüttelt und an verschiedenen Zeitpunkten (zwischen 0-240min) vermessen.

In Anschluss wurden die Mutationen jener Mutanten, die eine erhöhte Stabilität aufwiesen kombiniert. Dadurch gelangte man zu einer Mutante, deren Stabilität nach Inkubation mit organischen Lösungsmitteln gegenüber der Wildtypvariante signifikant erhöht war.

Abstract

There is a great potential for the use of enzymes in biocatalysis. Recently, biotransformations have become almost indispensable in industrial processes. However, there are still challenges which have to be complied. Unfortunately, natural enzymes are often not optimally suited for applications in the industry. An example therefore is the presence of organic solvents, which were used to increase the solubility of substrates, to simplify the product recovery and to reduce the number of side reactions.

The aim of the work was the stability enhancement of a heterologously expressed 'ene' - reductase in the presence of organic co-solvents. The study included the establishment of a screening system for the detection of stability improved mutants in microtiter plate format. Mutant libraries created by error-prone PCR were screened for more stable variants of the enzyme. The screening was performed in DMF/Toluene mixtures (2%/10%) or respectively in NMP/Toluene mixture (2%/10%). For the screening the crude cell lysates were shaken in the presence of the organic co-solvent mixtures for a duration of 0-240 min. Beneficial mutations from mutants with increased stability were combined. The combined variant showed a significantly improved stability after the incubation with organic solvents compared to the wild type.

Contents

Abbreviations

Table 1: Abbreviations used during this study

Abbreviation	Description
α-MCA	α-methyl-trans-cinnamaldehyd
AA	Amino acid
ADH	Alcohol dehydrogenase
AKR	Aldo-keto reductase
ALH	2-alkenal/one α,β-hydrogenase
Amp	Ampicillin
Amp_{100}	Final ampicillin concentration of 100 µg/mL
BSA	Bovine serum albumin
C=C	Carbon-carbon double bond
C=O	Carbon-oxygen double bond / carbonyl group
C_{50}	Threshold concentration
cDNA	Complementary DNA
CD-spectroscopy	Circular dichroism spectroscopy
ddH_2O	Double distilled water “Fresenius”
DMF	N,N-dimethylformide
DMSO	Dimethyl sulfoxide
DWP	Deep well plate
EC number	Enzyme commission number
EWG	Electron-withdrawing group
FMN	Flavin mononucleotide
$FMNH_2$	Flavin mononucleotide, reduced form
fw	Forward
GST	Glutathione-S-transferase
HNE	4-hydroxy-(2E)-nonenal
kDa	Kilo Dalton
LB	Low salt Luria Bertani
log P	Logarithm of the partition coefficient
Mb	Mega base pairs
MDR	Medium-chain dehydrogenases/reductases
MTP	Microtiter plate / microplate
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide, oxidised form
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NMP	N-Methyl-2-pyrrolidone
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
OYE	Old yellow enzyme
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PP	Polypropylene
PPB	Potassium phosphate buffer
PS	Polystyrene

rv	Reverse
rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TB	Terrific Broth
U	Unit
UV-spectroscopy	Ultraviolet spectroscopy
Wt	Wild type (pMS470syn)
ζ	Zeta

1 Introduction

1.1 Stability of enzymes in organic (co-)solvents

There is a great potential for the use of enzymes as biocatalysts, although some limitations regarding the environment required for catalysis are still met. Enzymes are adapted to the cellular environment and therefore they are usually not stable under process applications e.g. extremes of pH, increased temperature or the presence of organic solvents.
In the following sections aspects regarding the use of organic (co-)solvents in catalysis are discussed.

1.1.1 Reasons for the use of organic solvents

It is well known that there are many advantages for the use of organic solvents in enzyme catalysis. Organic (co-)solvents have been successfully applied in biocatalysis for many reasons:

- Increased solubility of substrates and/or products [1, 2, 3, 4]
- Altered substrate specificities [1, 4]
- Fewer side reactions [1]
- Easier product recovery [1, 2, 3, 4]
- Shift of thermodynamic equilibria [1, 2, 3, 4]
- Reduced microbial contamination [1, 2, 3, 4]
- Enhance thermostability [1, 2, 3, 4]

1.1.2 Limitations caused by the use of organic (co-)solvents

In general organic (co-)solvents can lead to enzyme denaturation e.g. by removing essential water molecules form the hydration shell [5, 6].

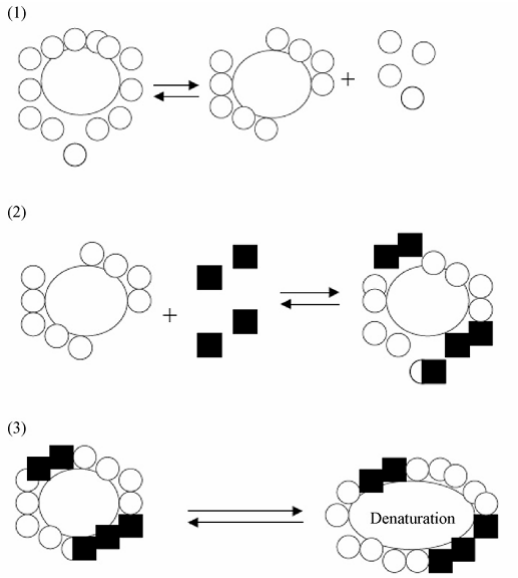

Figure 1: "Steps in enzyme deactivation in organic medium"[6]**:** (1) Protein with bound hydration shell; (2) water miscible organic solvent replaces water molecules from the hydration shell; (3) the consequent conformational change leads to denaturation of the enzyme.
○ water molecule
■ solvent molecule

Beside this, the increasing complexity of the reaction system [5], additional costs for the solvent [5] and the reduced activity of the enzyme [7] could be seen as reasons against the use of organic (co-)solvents.

1.1.2.1 Protein inactivation

For stability improvement, denaturation caused by unfolding of the enzyme's tertiary structure to a disordered polypeptide [6] must be overcome. The denaturation process can be described as follows [8].

Scheme of protein inactivation

$$N \xleftrightarrow{K} U \xrightarrow{k} I$$

N	Native form of the protein
U	Unfolded form of the protein
I	Irreversible inactivated form of the protein
K	Equilibrium constant for the reversible N↔U transition
k	Rate constant for the irreversible U→I reaction

Fagain [8] used the previous scheme to explain the terms of thermodynamic stability and long-term stability, respectively.

Thermodynamic or conformational stability affects the resistance of a protein against denaturation and is represented by the reversible transition N↔U.
Long-term or kinetic stability reflects the resistance of a protein against irreversible inactivation (e.g. dissociation of subunits, proteolysis, oxidation of Cys-residues etc.) and describes the transition from the native state to the irreversible inactivated form of the enzyme. This N→I inactivation is in most cases a first-order exponential decay.
Protein denaturation can be measured by UV- or fluorescence spectroscopy, CD (circular dichroism)-spectroscopy and urea gradient gel electrophoresis.

1.1.2.2 Solvent parameters

A lot of different parameters describe the effects of organic solvents on protein stability.
In this context the so called threshold behaviour [9, 10, 11] is often mentioned. The threshold behaviour is the association of activity and the amount of organic solvent and describes the protein performance in an organic solvent/water mixture. Furthermore, the term threshold concentration (C_{50}) [9, 10, 11] is used. This term determines the concentration of organic (co-) solvent, which is required to increase the enzyme activity to half of the initial activity.
C_{50} has been determined for enzymes like α-chymotrypsin [9, 11] and several other proteins (e.g. trypsin, laccases and cytochrome c) [9]
Based on the obtained results from α-chymotrypsin, Khmelnitsky and co-workers [9] established a system to predict the C_{50} of organic (co-)solvents. The so calculated parameter is called Denaturation Capacity (DC).
A further parameter that describes the effect of organic solvents on proteins is the log P [12, 13, 14]. It represents the logarithm of the partition coefficient of an organic solvent in standard octanol–water two-phase systems. Based on the log P values solvents can be divided into 3 classes:

Table 2: Influence of the log P on solubility and effects cased on the protein [12, 14]

Log P	Solubility in water (20°C) (Weight %)	Effects on the protein
log P ≤ 2	>0.4	Strongly distorts the water shelf, leading to inactivation or denaturation
2 < log P < 4	0.04 – 0.4	Weak water distorters, effect on the protein is unpredictable
log P ≥4	< 0.04	No interactions with the water shelf, leaving the protein in an active state

Laane and co-workers [14] used a lipase to determine Log P values of different solvents. The log P values of solvents used during this diploma thesis are summarized in Table 3.

Table 3: Log P values of the used solvents [14, safety data sheets]

Solvent	log P
DMSO	-1.3
DMF	-1.1
Toluene	2.5
NMP	-0.46

A very similar parameter to the log P value is the Hildebrand solubility parameter (δ) [12, 15], a parameter for the solvent polarity. In the case of the Hildebrand parameter the highest catalytic activity can be observed in organic solvents (δ<~8) with a low polarity and a molecular weight from under 150 g/mol.

Beside these parameters further correlations of enzyme activity or stability with the denaturation capacity of the solvent have been found. Examples are the Dimroth-Reichardt parameter [16] or the polarity index [17].

In summary, polar water miscible organic solvents have the highest potential to strip away or to replace parts of the hydration shell. Those solvents have the highest denaturation capacity.

Water immiscible organic solvents have a very low affinity to remove essential water from enzymes but degradation can occur at the interfaces [18].

Usually, enzyme stability strongly depends on the measuring conditions. Budde and Khmelnitsky [19] have shown that the obtained results depend on the measuring conditions. Their experiments with two different aldolases (form rabbit and trout muscle) showed that the results obtained after incubation depend either if the measurement is performed in the presence or after the removal of the organic solvent. Their results showed that DMF caused an increase of catalytic activity even without incubation. The removal of the solvent before the measurement partially restored most of the catalytic activity. This phenomenon was observed in DMF, all other tested solvents caused irreversible denaturation

In respect of enzyme stability in organic (co-)solvents, predictions are difficult. The stability of an enzyme depends on both the used solvent and the enzyme itself.

1.1.3 Methods for enzyme stabilisation in organic (co-)solvents

Many different methods for enzyme stabilisation in the presence of organic (co-)solvents are commonly used (Table 4).

Table 4: Methods for enzyme stabilisation

Method	Reference
Chemical modification	[8, 20, 21]
Immobilisation	[5, 6, 20, 21]
Reversed micelles	[5, 14, 21]
Lyophilisation and additives	[8, 20, 21]
Protein engineering	[1, 7, 8, 20, 22, 23, 24, 25]

1.1.3.1 Chemical modification

Chemical modification is used since the 1960s [20] to stabilize enzymes. As described by Fagain [8] free side chains of amino acids can be used to alter the properties of enzymes. Thiol groups of cysteines or the amino groups of lysine residues play an important role in enzyme modification. These residues can interact under quite mild conditions with specific reagents to yield chemically modified proteins. Nevertheless, modifications can lead to enzyme inactivation because some of the modified residues could be essential for catalysis.
Until now, three different strategies [8] to increase the stability of an enzyme by chemical modification exist:

- Cross-linking (intra- or intermolecular): For cross-linking bifunctional reagents are used. The reactive groups of the cross-linking reagent can be similar (homobifunctional) or different (heterobifunctional). Typical reagents for cross-linking are glutaraldehyde, diimidates, disulfonylchlorides, etc [6].
- Surface group modification: This method can be used to make the surface of a protein more hydrophilic. Thereby, the enzyme becomes better solvated and the denaturating effect of the solvent can be minimized. Mozhaev and co-workers used this technique to convert tyrosine residues of trypsin into aminotyrosines. This was done in two steps (introduction of a $-NO_2$ group into the aromatic ring of tyrosin by the use of tetranitromethane and reduction of the $-NO_2$ with dithionite) [8].
- Covalent coupling to polymers such as polyethylene glycol (PEG) or polysaccharides: The use of PEG to modify enzymes is described by Ogion and Ishikawa [21]. PEG interacts with the free amino groups of enzymes, the modification of an enzyme with PEG leads to a more soluble enzyme variant in organic solvents like benzene or

chlorinated hydrocarbons. Hydrated PEG chains can create an aqueous shell around the enzyme, which can prevent the enzyme from inactivation. A modification with PEG is useless when water-miscible organic solvents are used; because this sort of solvents destroy the aqueous shell built up by PEG and thereby inactivate the enzyme.

1.1.3.2 Immobilisation

Immobilisation is a common strategy to improve the operational stability of biocatalysts [6]. Thereby, an enzyme is immobilized on an insoluble support [20, 21]. Many different techniques for enzyme immobilisation are used, e.g. adsorption, covalent binding, entrapment and membrane confinement [6].
Alternatively, immobilisation techniques can also be used for heat stabilisation of enzymes [21].
The main advantages of immobilized enzymes are that they can be continuously used and easily recovered. On the contrary, the activity of the immobilized enzyme might be decreased, during coupling to the support. The coupling can result in partially blocked substrate binding pockets or furthermore the activity reduction can be a result of limited transport rate by diffusion through the supporting media.

1.1.3.3 Reversed micelles

Reversed micelles are formed when surfactants are dissolved in non-polar organic solvents. Examples for surfactants are detergents and phospholipids. These compounds can form closed aggregates. Thereby, the outer shell is formed by the hydrophobic parts, while inner parts are built up by the polar groups of the surfactants. The diameter of micelles is determined by the molar ratio of water to surfactant. In the inner of micelles, water and other polar molecules can be solubilized. The entrapped water has different properties compared to the bulk water. The differences affect polarity, viscosity and nucleophilicity [21].
Enzyme stability in reversed micelles depends on the cohesion of the micellar aggregates containing the enzyme, pH, temperature, ionic strength, shear forces and the relationship of enzyme/surfactant interactions [6].

In this context often the term “super activity” [6, 21] is used. This perception describes the very high activity in reversed micelles compared to the activity of the enzyme in a bulk aqueous phase.

1.1.3.4 Lyophilisation and additives

Lyophilised enzymes are mainly used in pure organic solvents. Although lyophilisation does not remove the structural water of an enzyme essential for catalysis, nevertheless it can case significant denaturation. To minimize this effect structural preserving lyoprotectants like sugar, PEG, inorganic salts, substrate-resembling ligands and crown ethers can be used [26].
Kilbanov [26] combined lyophilisation with a memory effect. He suggested that a lyophilized enzyme resuspended in an anhydrous solvent has the same properties as in the solution used for lyophilisation. A graphical description of this phenomenon is shown in Figure 2.

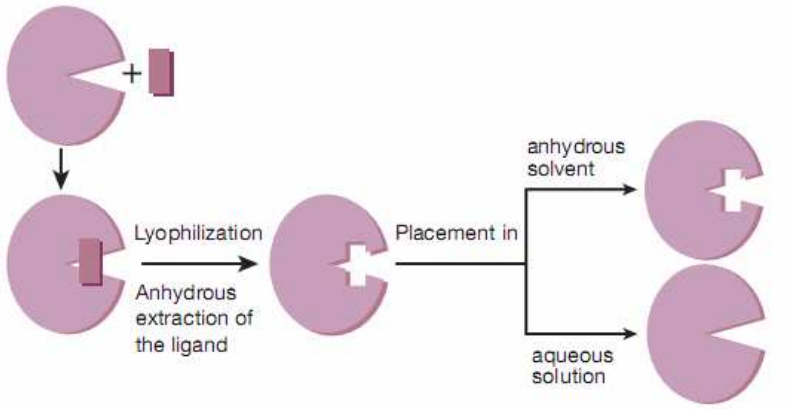

Figure 2: “Schematic representation of the ligand-induced imprinting of the enzyme active site” [26]**:** The enzyme is shown as shaded oval with an angular cleft representing the active site; the ligand molecule is shown as rectangle. When the ligand (in this case a substrate analogue) is bound to an enzyme a conformational change occurs. This altered conformation remains intact after lyophilisation and followed extraction of the ligand. The ligand induced imprint persists even after the suspension of the enzyme in anhydrous solvents. The reason therefore is the structural rigidity in such media. But the imprint disappears when the enzyme is dissolved in water.

Ogion and Ishikawa [21] proposed that improved stability of lyophilised enzymes is caused by a layer of denatured protein on the surface. This layer protects deeper layers against denaturation caused via contact with the organic solvent.

Additives can be divided into two groups [8]. The osmotic stabilizers (osmolytes) are uncharged and affect solvent viscosity and surface tension. Examples are polyols, polysaccharides and amino acids.
The second group are the ionic stabilizers; they are used to shelter surface charges.

1.1.3.5 Protein engineering

Protein engineering is a very powerful tool to improve enzyme properties at the genetic level.

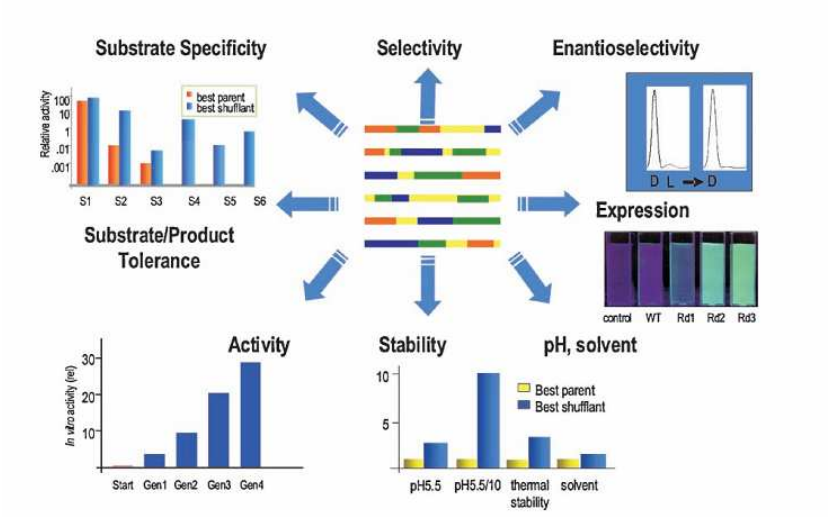

Figure 3: Possible applications for protein engineering [23]

Protein engineering can be performed by directed evolution and/or rational design.

1.1.3.5.1 Directed evolution

Directed evolution mimics the evolutionary process in the laboratory. As pioneers on this sector Stemmer and Arnold have to be mentioned [27].

The first step in directed evolution is gene diversification by the creation of a gene library. The most common random mutagenesis technique therefore is error-prone PCR or alternatively by recombination techniques like gene shuffling. Beside this also UV-light, chemical mutagens or mutator strains [28, 29] can be used.

In the case of error prone PCR a starting gene is amplified over a million fold, thereby uncontrolled errors are generated. There are two polymerases that could be used for the error-prone PCR, the Mutazyme polymerase (GeneMorph® kit, Stratagene) and the Taq polymerase. The difference of these two polymerases is their mutational spectrum [24].

When error-prone PCR is used the number of mutations has to be chosen very carefully. There are reports of substitution rates from 0.25 to 20 substitutions per 1,000 base pairs. If the mutation frequency is too high, the number of active enzymes will be low. Otherwise if the substitution rate is too low, most of screened mutants will be wild type [30].

Moore and Arnold [30] further reported that most variations in the amino acid sequence are neutral or deleterious and that the number of beneficial mutations is relatively rare.

Silvestre and co-workers [28] determined that starting from a codon (e.g. AAA) not all other codons can be obtained.

Randomized libraries can contain up to 10^{11} variants, these number is decreased to 10^{7} individual clones when the mutated gene must be inserted into a plasmid vector [28].

After transformation of the library into a host strain where gene diversity is further reduced, the developed mutants are cultivated and further used for screening. The conditions used for the screening assay should mimic the specific conditions of the final process as closely as possible [23, 30]. Beside this the screening method should also detect small enhancements of the desirable property.

After screening potentially positive variants are rescreened. The clones with the highest increase of the desired property can be further characterised and the beneficial mutations can be combined. The best variant can further be used as parental gene for the next mutagenesis round [7, 24, 30].

1.1.3.5.2 Rational design

In contrast to directed evolution rational design requires structural information of the enzyme. In addition the relationship between sequence, structure and mechanism/function [31] and the knowledge of the inactivation process [20, 27] is beneficial.

Molecular modelling is used to predict how an amino acid exchange influences the selectivity, activity or stability of an enzyme. If there is no structural data available for the enzyme of choice, the structure of a homologue enzyme can be taken as a model [31].

The integration of the desired mutations into the gene sequence is done by site-directed mutagenesis or by saturation mutagenesis.

1.1.4 Design rules for stabilisation of enzymes in organic (co-)solvents

In 1990 Arnold [1] recommended amino acid modifications to enhance the stability in organic solvents should either lower the free energy of the active form or raise the free energy of the inactive form. Based on these facts she developed a set of design rules and general suggestions for protein engineering. She divided her suggestions into mutations leading to a more hydrophobic surface and mutations regarding the conformational stability. Her proposals are summarized below.

Mutations for a more hydrophobic surface

- Remove surface charges

  Charged surface residues are well solvated by water, a dehydrogenation of these residues results in an increase in system free energy. The replacement of these changed groups can also decrease the required amount of water for the enzyme activation. Removing charged residues from the surface results in a more hydrophobic surface. The replacement of charged residues does not affect the protein as long as they are not required for the biological function. Altering surface charges can influence the pH dependence of the catalytic activity.

- Remove (or satisfy) unfulfilled surface hydrogen binding sites

  A protein is not able to form hydrogen bonds with the solvent. So hydrogen binding has to be fulfilled internally, which further could stabilize the enzyme.

Conformational stability

- Internal cross-links

  Internal cross-linking can be reached either by the introduction of new disulfide bonds or by introduction of new or improvement of existing hydrogen bonds and other electrostatic interactions. The integration of disulfide bridges can limit the aggregation and irreversible inactivation associated with protein unfolding.

- Increase van der Waals interactions

  Increasing van der Waals forces can maintain or improve the tight packing of the protein and may result in increased stability in organic solvent.

- Maximise internally fulfilled hydrogen bonds

  Fulfilled hydrogen bonds lead to a high degree of ordered secondary structure. To fulfil the main-chain hydrogen bond sites the side chains can be used.

- Electrostatic interactions

  Enzymes are likely stabilized by interactions in the protein interior.

Studies have shown that even multiple mutations do not lead to substantial alterations of the protein structure. As reported by Arnold [1], single mutations can increase the stability. A combination of such mutations can lead to an enzyme which can work under completely different conditions than the wild type.

Beside Arnold [1] also many others such as e.g. Yang and co-workers [22] have developed some rules for protein stabilisation in organic (co-)solvents. Their proposals are based on a

rational design approach, which they used to increase the co-solvent stability of a penicillin G acylase. The suggestions of Yang and co-workers are summarized below.

- Remove basic amino acids on the surface by mutation. Only amino acids that are not part of structural elements (i.e. α- helix, β-sheet and β-turn) should be mutated.
- Only residues that are not conservative in multiple sequence alignments of homologue enzymes shall be mutated.
- Mutate amino acids which are only involved in few interactions (no salt bridges or < 3 hydrogen bonds) to others.
- Only amino acids far away from the active site should be mutated. So the effect on the activity of the enzyme is minimized.
- To enhance the stability in non-aqueous media, basic amino acids are mutated into neutral amino acids.
- To minimize the conformation of the enzyme bulky amino acids should be mutated to small ones.

However, although this lead to success in some cases, results from directed evolution experiments demonstrated that useful mutations might also be found somewhere else (e.g. in structural elements). Therefore random mutagenesis combined with screening still remains one of the most useful methods to stabilize enzymes in the presence of organic solvents.

1.2 `Ene´ Reductases

1.2.1 Overview about `ene´ reductases

The term `ene´ reductase describes enzymes, that catalyse the asymmetric reduction α,β-unsaturated alkenes including enones, enals, maleimides and nitroalkanes [32, 33]. In this context also the name enone or enoate reductase is frequently used to describe this form of enzymatic activity. This class of enzymes has a high stereospecificity, strict regioselectivity and a rather broad substrate specificity [34].

`Ene´ reductases catalyse the asymmetric reduction of C=C bonds. Thereby up to two stereogenic centers can be created [35, 36, 37, 38, 39, 40].

In contrast to transition-metal-based homogeneous catalysts, which catalyze cis-hydrogenation, `ene´ reductases add the hydrogen to the double bond following an anti addition mechanism [35, 36, 40].

A schema of the catalysed reaction is illustrated in Figure 4. The reaction mechanism is described in a more detailed way in 1.2.4.

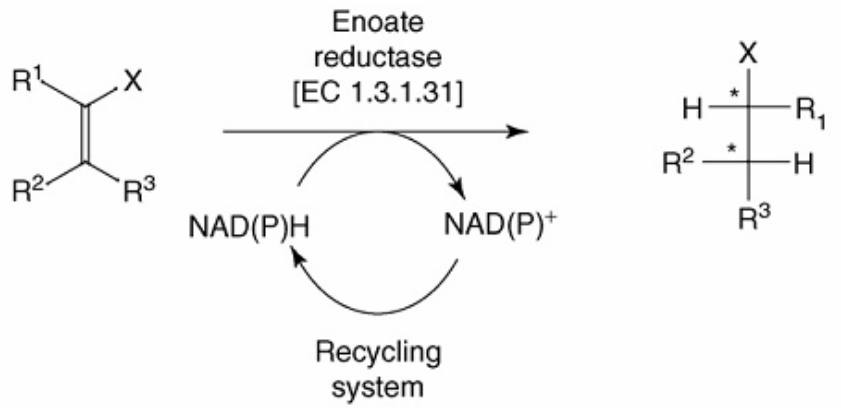

Figure 4: "Asymmetric reduction of activated C=C bonds using enoate reductases at the expense of NAD(P)H yields the corresponding alkane in non racemic form"[40]: * is used to indicate the chiral centres, X illustrates an activating group; different electron-withdrawing groups are described in 1.2.2.

The most common representative of the `ene´ reductases are the family of the `Old Yellow Enzymes´ (OYE). OYE was first isolated from *Brewer`s* bottom yeast by Warburg and Christian in 1933. The name OYE comes from the colour of the enzyme, which derived from the flavin cofactor [40].

The OYE family has grown in the last years and includes different yeast OYE's, XenA/XenB reductase, bacterial morphine reductase, bacterial nitrate reductase, etc. [37].

Sources for enzymes with `ene´ reductase activity are yeasts, fungi, (aerobic) bacteria and plants [40].

Examples for enzymes that also show `ene´ reductase activity are YqjM an old yellow enzyme homolog from *Bacillus subtilis* [39], NCR reductase form *Zymomonas mobilis* [36], OPR1 and OPR3 from *Lycopersicon esculentum* (tomato) [35] and different OPRs from *Arabidopsis thaliana* [41, 42]. While those all belong to the class of flavoproteins also other enzymes can catalyse double bond reductions for example see 1.3.

1.2.2 Substrates

`Ene´ reductases have a broad substrate specificity [34], interesting substrates have an electron-withdrawing group attached to the C=C bond (see Figure 4 and 5). The most common activating group is the carbonyl group. Examples therefore are aldehydes, ketones, carboxylic acids and derivates thereof like lactones, acid anhydrides and cyclic imides. Beside the carbonyl group also the nitro group can be used for the activation of the double bond. The reason therefore is the electronic similarity of these two groups [35, 40].

Typical substrates are α,β-unsaturated carbonyl compounds like conjugated enals and enones; α,β-unsaturated carboxylic acids and derivates thereof as well as α,β-unsaturated nitroalkanes [40].

1.2.3 Cofactor specificity

Hall and co-workers [36] determined that enoate reductases are less specific in this context. There are enzymes that only accept NADH or NADPH, whereas others can use both cofactors.

It was noticed, that the choice of the enone substrate seems to influence the NADPH/NADH specificity ratio [37].

1.2.4 Mechanism

The reaction mechanism for the reduction of activated alkenes by `ene´ reductases has been studied in detail. The mechanism can be described as a "ping-pong bi-bi mechanism" [35, 36] and can be divided into two half reactions, an oxidative and a reductive one, respectively.

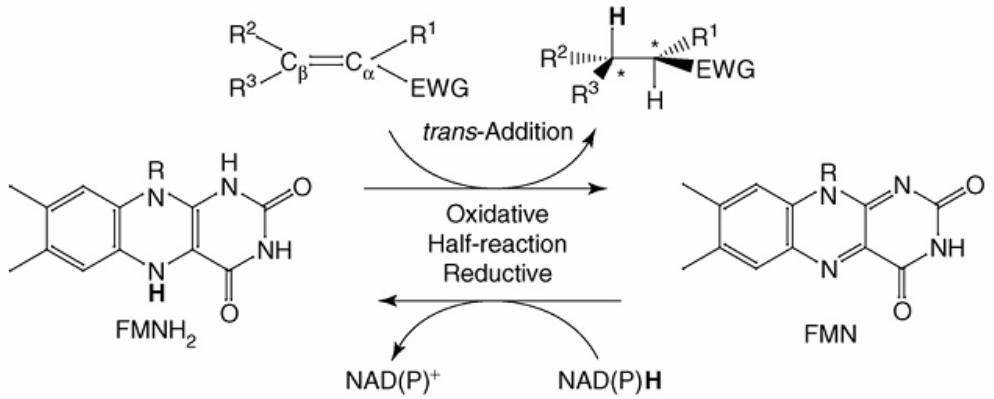

Figure 5: "Asymmetric reduction of activated alkenes by enoate reductases" [40]**:** The asterisks (*) indicate chiral centers, EWG stands for electron-withdrawing group. The hydride which is transferred from the flavin cofactor is indicated in bold.

The oxidative half reaction can be described by the stereoselective transfer of a hydride from the reduced cofactor $FMNH_2$ to the C_β of the substrate. At the same time a proton is added to C_α by a Tyr-196 [38]. This Tyr residue is conserved in all members of the OYE family. The proton added to C_α is derived from the solvent.

The catalytic cycle is completed with the reductive half-reaction. Thereby the oxidised flavin cofactor (FMN) is reduced at the expense of NAD(P)H.

The whole reaction can be summarized as addition of H_2 to a C=C bond and in addition this happens in trans-fashion with absolute stereospecificity [35, 36, 40].

1.2.5 Enantio-specificity

Müller and co-worker [38] determined that the enantio-specificity does not only depend on the enzyme but also on the substrate.

They found three factors, which are important for the enantio-specificity of a reaction:

- The position of the methyl group in α- or β-position, this admission is supported by the fact that OYE1 reduces α-methyl 2-cyclohexenone to the R-enantiomer and β-methyl 2-cyclohexenon to the S-enantiomer.
- The E-or Z-configuration of the double bond

- The rest of the substrate molecule, because they obtained different enantiomers when E-2-methyl-pentenal and E-3-phenyl-2-methyl-propenal was used as substrate. These two components differ only with regards to an ethyl versus phenyl rest.

1.2.6 Applications of `ene´ reductases

The potential of `ene´ reductases has been recognized long time ago, but till today their industrial applications are limited [35, 36, 37, 40].

Generally `ene´ reductases are used as whole-cell systems. The advantage of the whole-cell system is the internal NAD(P)H recovery, nevertheless undesired side reactions can occur [36].

The main problem regarding the side reactions are the alcohol dehydrogenases. Stürmer and co-workers [40] noticed that, "the rate of desired C=C- versus undesired C=O-bond reduction depends on the substrate and the involved enzyme". The reduction of the C=O bond leads to an alcohol, which cannot be further converted by the `ene´ reductase. The reduction of the carbonyl group to the corresponding alcohol can also lead to saturated alcohols as final product.

Beside alcohol dehydrogenases also carboxyl ester hydrolases [36] catalyses undesired side reactions.

Typical applications for `ene´ reductases are the production of products for biotechnological and pharmaceutical applications [33].

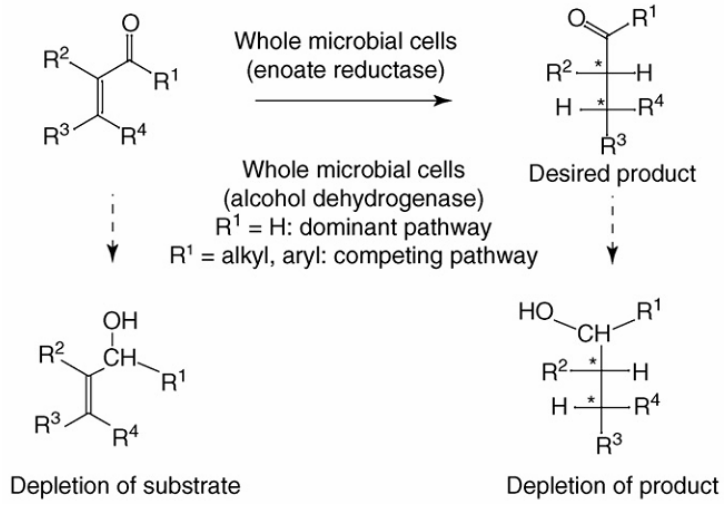

Figure 6:"Asymmetric bioreduction of α, β- unsaturated aldehydes and ketones using whole microbial cells often shows undesired carbonyl reduction"[40]: * indicates chiral centers.

1.3 P1-ζ-crystallin in *Arabidopsis thaliana*

The enzyme used during this diploma thesis was P1 from *Arabidopsis thaliana*. In contradistinction to the `ene´ reductases described in the previous chapter, P1 is a 2-alkenal reductase. Nevertheless, studies with purified fractions of P1 revealed, that P1 shows `ene´ reductase activity [52], although this it does not contain the flavin cofactor typical for `ene´ reductases.

1.3.1 Overview about *Arabidopsis thaliana*

Arabidopsis thaliana is a member of the Brassicaceae family. Although *Arabidopsis thaliana* is a small plant (only ~30 cm) it shows the same complex morphology as other plants [43]. The benefit of this plant was recognised in the 1980s, since this time *Arabidopsis* is used as a model system for plant development, physiology and molecular genetics [44]. Therefore it is not further amazing that *Arabidopsis thaliana* has been the first plant genome that was sequenced [45].

Arabidopsis has five chromosomes, the first correct propose of the chromosome number was done by Laibach in 1907 [43]. The size of the nuclear genome is 119 Mb and codes for 26,207 predicted genes and 3,786 pseudogenes, which include the transposable-element related gene models [45].

1.3.2 P1 a member of the ζ-crystallin family

The synthetic gene fragment used in this work codes for 345 amino acids and has a molecular mass of ~38 kDa. The NCBI Accession number of P1 is Z49768.

P1 was first described in 1995, during a study for plant genes involved in the defence against oxidative stress. The highest expression levels of P1 were found in leaves and less in stems of *Arabidopsis.* Also in flowers and roots weak expression occurred [46]. The reason for the high expression level in the leaves is that they are exposed to environmental influences (e.g. sunlight), which can cause oxidative stress.

The amino acid sequence of P1 has no apparent organelle-targeting signal and therefore it is most probably localized in the cytosol [47] .

The crystal structure (PDB acc. no. 2J3J) of the ternary complex has a resolution of 2.8 Å [48] and reveals that P1 is a non covalent homodimer [49].

Initially, the enzyme was classified to the plant ζ-crystallins (ZCrs) [46]. The classification was done based on the high homology to ZCR, a major lens protein of hystricomorph rodents and camelids. ZCrs can be found in a plethora of organisms, from bacteria to mammals and higher plants [50]. ζ-crystallins exert various physiological functions, some of them have been characterised as dehydrogenases or reductases [48, 50].

The overall fold of P1 indicates that this enzyme belongs to the zinc-independent MDR superfamily. As already described for other MDRs, P1 is composed of two domains: a substrate-binding and a nucleotide binding domain. [49].

ZCr homologs and alcohol dehydrogenases share a conserved three-dimensional structure. In contrast to alcohol dehydrogenases in ZCr homologs a Zn-binding domain is missing.

1.3.3 Reactions catalysed by P1

First of all Babiychuk and co-workers [46] described P1 in 1995. Based on the obtained results and the fact, that the expression of P1 is induced by oxidative stress they suggested that this enzyme may participate in oxidative stress response.

Subsequently, Mano and co-workers [48, 50] determined that P1 is involved in the detoxification of quinones and azo compounds. Based on the fact that quinones and azodicarbonyl compounds can serve as electron acceptors, they proposed P1 to be a NADPH: azodicarboyl/quinone reductase.

Further investigations of potential substrates for P1 have indicated that the enzyme can catalyse the reduction of 2-alkenals [51]. These α,β-unsaturated aldehydes are degradation products of lipid peroxides. The toxicity of these aldehydes comes from their ability to form Michael adducts with thiols and amino groups. An example for a reactive aldehyde is 4-hydroxy-(2E)-nonenal (HNE). HNE can inactivate the glucose-6-phosphat-dehydrogenase and the Cyt c oxidase or can further be converted to 2,3-epoxy-4-hydroxynonanal, a potential carcinogenic epoxide. P1 can reduce HNE and other α,β-unsaturated aldehydes, e.g. (2E)-nonenal, 3-buten-2-one and 4-hydroxy-(2E)-hexenal [47, 51].

Based on the fact, that P1 can catalyse the reduction of α,β-unsaturated bonds of 2-alkenals/alkenones, Mano et al [51] suggested P1 to be a “NADPH: 2-alkenal/one α,β-hydrogenase (ALH)”.

The AHL reaction is the fourth route to detoxify 4-hydroxy- and non-hydroxylated 2-alkenes. The other three routes are:

- Conjugation with glutathione with the glutathione-S-transferase (GST)
- Reduction to alcohols with the aldo-keto reductase (AKR)
- Oxidation to carboxylates with the alcohol dehydrogenase (ADH)

All four routes for the detoxification of HNE have been established in mammals, while in plants only AKR and ALH have been identified.

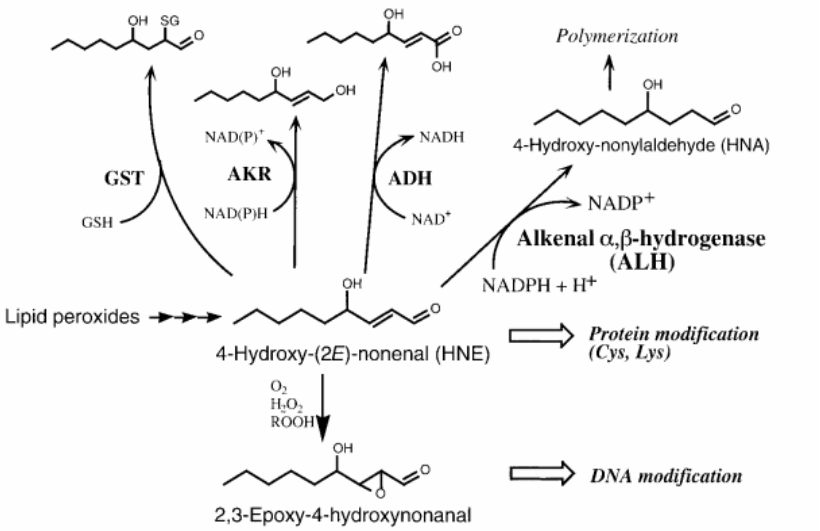

Figure 7: Formation, biological effects and scavenging of 4-hydroxy-(2E)-nonenal [51]

1.3.4 Reaction mechanism

Youn and co-workers [49] determined that P1 shows a striking similarity to 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (12-HD/PGR), an enzyme which can reduce different α,β-unsaturated aldehydes and ketones. For 12-HD/PGR a "ketoreductase reaction mechanism" with a conjugated enolate as intermediate has been proposed [49].

Youn and co-authors [49] suggested a similar mechanism for P1. They proposed that Tyr-260, Tyr-81 and Ser-287 might be involved in substrate binding. Furthermore they recommended that Tyr-53 participates in binding of phenolic substrates by π-π interactions.

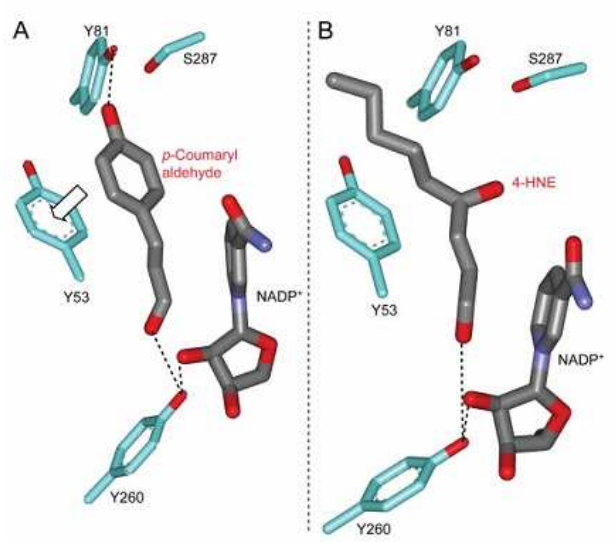

Figure 8: Proposed interactions of P1 with the bound cofactor NADP$^+$ and the substrates p-coumaryl aldehyde (A) and 4-hydroxy-(2E)-nonenal (B) [49]**:** The arrow indicates the π-π interaction between the phenol rings. Hydrogen bonds are shown as dashed lines.

Youn and co-authors [49] proposed that Tyr-260 serves as a "general acid/base" by stabilizing the enol form of the transition state. The stabilized intermediate enables the transfer of a hydride from C-4 of the NADPH nicotinamide to the substrate. The second proton added to the C=C bond probably comes from the solvent because there is no adjacent amino acid in the active site, that can perform the proton transfer.

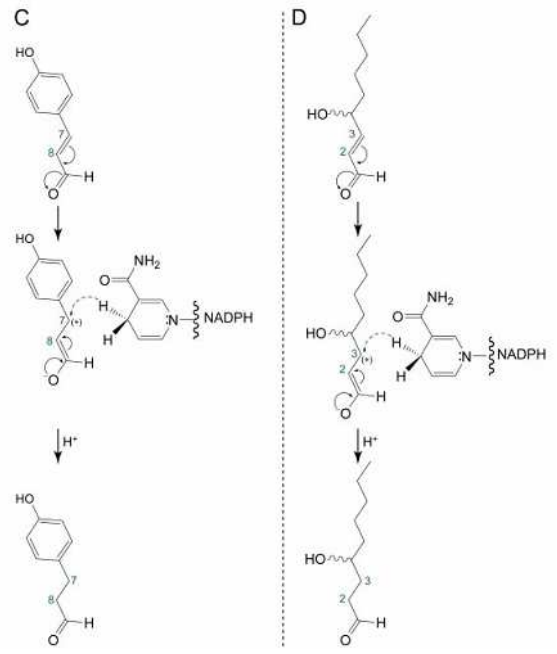

Figure 9: Schematic reaction mechanisms with p-coumaryl aldehyde (C) and 4-hydroxy-(2E)-nonenal (D) [49].

1.3.5 Recent developments

In the publication of Youn and co-workers [49] it is mentioned that P1 does not reduce cinnamyl aldehyde, a common substrate for other `ene´ reductases [37].

Nevertheless, activity measurements with purified fractions of P1 revealed that the P1 has `ene´ reductase activity. One of the used substrates for the characterisation of P1 was α-methyl-trans-cinnamaldehyde [52].
The model reaction of the substrate, α-methyl-trans-cinnamaldehyde is shown in Figure 10.

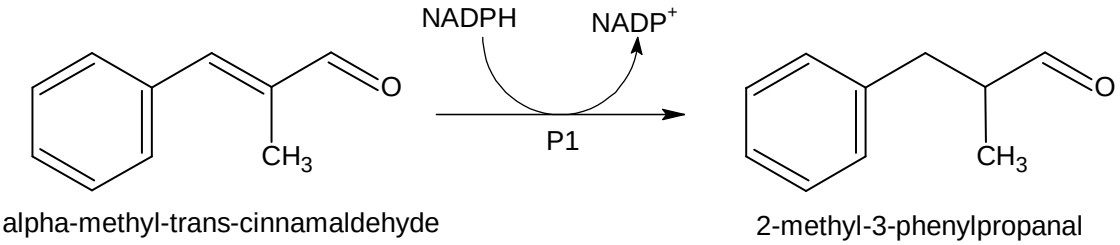

Figure 10: Model reaction: conversion of was α-methyl-trans-cinnamaldehyde into 2-methyl-3-phenylpropanal by P1

Investigations regarding the solvent stability of P1 revealed that the enzyme is rather unstable in the presence of organic co-solvents.
To satisfy the requirements of our industrial partner, who wants to use P1 in an industrial process, the stability of the enzyme had to be improved.

2 Objectives

As aforementioned, the low stability of natural enzymes under process conditions is often limiting their applicability.

In our case, the wild type enzyme of P1 provides an interesting alternative to the well known OYE type enzymes for `ene´ reduction. However, P1 is rather unstable in the presence of solvents and therefore the improvement of solvent stability to satisfy requirements of DSM for industrial applications was targeted.

My part of a larger project was to increase the stability of the 2-alkenal reductase P1 in the presence of organic solvents.

This study included the establishment of a screening system (solvent concentration, shaking rate and incubation time) and screening of mutants generated by error-prone PCR towards improved stability in organic co-solvents. For stability screening the organic solvents DMF, NMP and toluene were used.

Beneficial mutations found during screening should be combined. It was further planed to evaluate and confirm the stability enhancement using an activity improved variant from Brigitte Höller as template.

3 Material and Methods

3.1 Strain, plasmid and primers

3.1.1 Strain

E. coli Top10F' (F'{*lac*I^{q}*Tn*10(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G)

This expression host used throughout this study was purchased from Invitrogen.

3.1.2 Plasmid

As expression plasmid pMS470 harbouring P1syn was used during this diploma thesis. P1syn was ligated into pMS470 via *NdeI* and *HindIII* restriction sites. The insertion of P1syn from *Arabidopsis thaliana* into pMS470 has been carried out by Brigitte Höller.

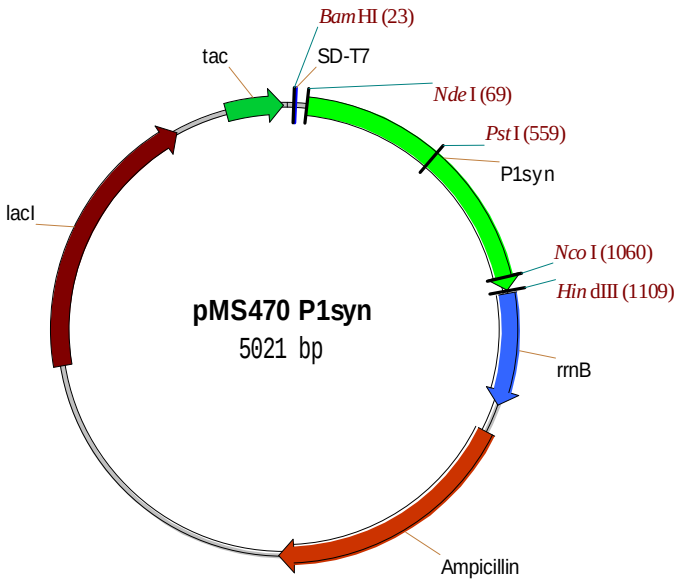

Figure 11: pMS470 P1syn

3.1.3 Primers

Table 5: Primers used during this work

Number	Name	Sequence (5'→3')
P08-257	pMSfw	gtgagcggataacaatttcacaca
P08-258	pMSrv	gttttatcagaccgcttctgcg
P09-373	13_G6fw	ccgcagcgctggcgcaggcttttactccgggtcagccg
P09-374	13_G6rv	cggctgacccggagtaaaagcctgcgccagcgctgcgg
P09-392	11_G9fw	ccggtctgctgggtatgcctggcatgaccgcgtacactggtttctacg
P09-393	11_G9rv	cgtagaaaccagtgtacgcggtcatgccaggcatacccagcagaccgg

3.2 Instruments and devices

3.2.1 Centrifuges

- **Centrifuge 5415 R:** Eppendorf AG, Hamburg, DE
- **Centrifuge 5810 R:** Eppendorf AG, Hamburg, DE
- **Avanti J-20 XP:** Beckman Coulter Inc, Fullerton, CA, US

3.2.2 Electrophoresis

- **PowerPac™ Basic power supply:** Bio-Rad Laboratories, Vienna, AT
- **Sub-cell GT:** Bio-Rad Laboratories, Vienna, AT
- **PowerEase 500 power supply:** Invitrogen Corporation, Carlsbad, CA, US
- **XCell SureLock Mini-Cell:** Invitrogen Corporation, Carlsbad, CA, US
- **NuPAGE® Novex 12 % Bis-Tris Gel 1.0 mm, 12 well:** Invitrogen Corporation, Carlsbad, CA, US

3.2.3 Microplates

- **PS-Microplate 96-well, flat bottom:** Greiner Bio.One GmbH, Frickenhausen, DE (Cat. Number 655161)
- **PS-Microplate 96-well, V-shape:** Greiner Bio.One GmbH, Frickenhausen, DE (Cat. Number 651161)

- **PS-Microplate 384 well, flat bottom:** Greiner Bio.One GmbH, Frickenhausen, DE (Cat. Number 781186)
- **UV-Star, Plate 96-well, flat bottom**: Greiner Bio.One GmbH, Frickenhausen, DE (Cat. Number 655801)
- **PP-Micoplate 96-well, flat bottom:** Greiner Bio.One GmbH, Frickenhausen, DE (Cat. Number 6552618)
- **PP-Deep-Well plate 96-well 2mL, U-shape:** VWR International, Wien, AT (Cat. Number 391-90-40)

3.2.4 Plate Reader and Photometer

- **Spectramax Plus 384:** Molecular Devices, Ismaning/München, DE
- **BioPhotometer:** Eppendorf AG, Hamburg, DE

3.2.5 Pipettes and Devices

- **Denville XL 3000i (XL2, XL10, XL20):** Denville Scientific Inc., Westbourne, UK
- **Gilson pipetman (P200, P1000):** Gilson Inc., Middleton, US
- **Biohit Proline® multichannel electronic pipettor, 8 ch 5-100 µl, 8 ch 50-1200 µl,:** Biohit Plc., Helsinki, FI
- **Biohit Proline® multichannel pipettor, 8 ch 5-50 µl:** Biohit Plc., Helsinki, FI
- **Easypet Pipetting Aid:** Eppendorf AG, Hamburg, DE
- **Pipette tips, micro P10:** Greiner Bio-One GmbH, Frickenhausen, DE,
- **Pipette tips 200:** Greiner Bio-One GmbH, Frickenhausen, DE
- **Pipette tips 1000:** Greiner Bio-One GmbH, Frickenhausen, DE
- **Biohit®Tips 300 µl:** Biohit Plc., Helsinki, FI
- **Biohit®Tips 1200 µl:** Biohit Plc., Helsinki, FI
- **Pipette with tip 5mL, 10mL, 25mL:** Greiner Bio-One GmbH, Frickenhausen, DE

3.2.6 Shaker

- **Thermomixer comfort:** Eppendorf AG, Hamburg, DE (3mm)
- **Titramax 1000:** Heidolph Instruments, Schwabisch, DE (1,5mm)

- **Multitron II:** Infors AG, Bottmingen-Basel, CH (25mm)

3.2.7 Thermocycler

- **GeneAmp® PCR System 2700:** Applied Biosystems, Foster City, CA, US

3.2.8 Additional Instruments and Devices

- **MicroPulser™:** Bio-Rad Laboratories, Vienna, AT
- **Vortex-Genie 2:** Scientific Industries Inc, Bohemia, NY, US
- **InoLab® pH720 pH-meter:** WTW, Weilheim, DE
- **µ-Fill Microplate Dispencer:** Bio Tek Instruments Inc., Winooski, US
- **Half-micro cuvettes:** Greiner Bio-One GmbH, Frickenhausen, DE
- **Electroporation cuvettes 2mm, blue cap:** Cell Projects, Kent, UK
- **Vivaspin 20 centrifugal concentrators:** Sartorius AG, Göttingen, DE
- **IKA® RCT basic safety control:** IKA ® Werke GmBH & Co. KG, DE
- **GP3202 Precision Balance:** Sartorius AG, Göttingen, DE
- **ABS 220-4 analytical balance:** Kern & Sohn GmbH, Balingen, DE
- **Branson Sonifier 250 Analog:** Branson Ultrasonics Corporation, Danbury, US
- **Clean Air *EN12469*:** Clean Air Techniek B.V., Woerden, NL

3.3 Buffers, Stocks and Media

Unless otherwise declared all components were autoclaved at 121°C for 20 minutes. Exempted from this were antibiotic stock solutions and stocks, which were filter sterilized.

3.3.1 Buffers

- **50 mM MES NaOH pH 6**

  9.76 g/L MES, pH was adjusted to 6.0 with 2 M NaOH.

- **50 mM PPB pH 7**

  3.40 g KH_2PO_4 and 4.35 g K_2HPO_4, respectively were dissolved in 500 mL of ddH_2O. These two solutions were than mixed to give a final pH-value of 7.

3.3.2 Stocks

- **Ampicillin stock (100 mg/mL)**

2 g Ampicillin were dissolved in 20 mL ddH_2O and filter sterilised. Sterilised aliquots were stored at -4°C.

- **Lysozyme stock (50 mg/mL)**

500 mg Lysozyme were dissolved in 10 mL ddH_2O and filter sterilised. Sterilised aliquots were stored at -4°C.

- **IPTG stock (1 M)**

2.38 g IPTG were dissolved in 10 mL ddH_2O and filter sterilised. Sterilised aliquots were stored at -4°C.

- **DNAseI stock (1 mg/mL)**

10 mg DNAseI were dissolved in 10 mL ddH_2O and filter sterilised. Sterilised aliquots were stored at -4°C.

- **NADPH stock (2 mM)**

15 mg NADPH were dissolved in 10 mL of 50 mM MES buffer pH 6.

3.3.3 Media

- **LB (Low Salt Luria Bertani)**

20 g/L LB-medium (Lennox) were dissolved in ddH_2O and autoclaved. The addition of the antibiotic was done after autoclaving. Before the addition of the antibiotic the solution was cooled down to ~55°C. The final concentration of Ampicillin was 100 µg/mL.

- **LB Agar**

35 g/L LB-agar (Lennox) were dissolved in ddH_2O and autoclaved. If needed, antibiotics were added as described before.

- **2xTY**

10 g/L Bacto™ Yeast Extract, 16 g/L Bacto™ Peptone and 5 g/L NaCl were dissolved in ddH_2O and autoclaved and if required antibiotics were added as described before.

- **TB (Terrific Broth)**

24 g/L Bacto™ Yeast Extract, 12 g/L Bacto™ Peptone, 4 mL/L Glycerol, 2.13 g KH_2PO_4, 12.54 g K_2HPO_4

For the preparation of the TB media yeast extract, peptone and glycerol were dissolved in 900 mL ddH_2O and autoclaved. Required amounts of KH_2PO_4 and K_2HPO_4 were dissolved in 100 mL ddH_2O and even autoclaved. After the autoclaving the two solutions were combined and if required antibiotics were added like described before.

3.4 Organic Solvents

Organic solvents were purchased from Roth if not specifically marked.

Table 6: Organic solvents used during this study

Solvent	Level of purity
DMF	ROTIPURAN® ≥99.8%, p.a., ACS, ISO
NMP	≥99.8%
Toluene	ROTISOLV® HPLC

3.5 Enzymes and Solutions

3.5.1 Restriction enzymes

The conventional restriction enzymes used during this work were purchased from MBI Fermentas. Digestion was performed as recommended by the producer using the unique five buffer system with colour coded tubes

Table 7: Restriction enzymes

Enzyme	Concentration [U/µL]	Recommended buffer	Recognition site
*Hind*III	10	Red	5'-A^A G C T T-3'
*Nde*I	10	Orange	5'-C A^T A T G-3'
*Dpn*I	10	Yellow	5'-G A^T C-3' cuts only if A is methylated

1 unit (U) is defined as the amount of enzyme which is required to digest 1 µg of λ DNA in one hour at 37°C.

3.5.2 DNA Polymerases

Table 8: DNA polymerases

Enzyme	Concentration [U/µL]	Recommended buffer	Producer
Pfu Ultra High Fidelity DNA polymerase	2.5	10x Pfu Ultra HF reaction buffer	Stratagene

The polymerase and the recommended puffer were used according to the supplied instructor manual [53].

3.5.3 Solutions

The final concentration of each nucleotide in the dNTP mixture was 10 mM. dNTPs were purchased from Fermentas.

3.6 Software and web tools

3.6.1 Software

- **SoftMax Pro 4.8:** Molecular Devices, Ismaning/München, DE
- **SeqMan 5.01:** DNASTAR Inc., Madison, WI, US
- **EditSeq 5.02:** DNASTAR Inc., Madison, WI, US
- **PyMOL:** DeLano Scientific LLC, Palo Alto, CA, US
- **Vector NTI 8.0:** Invitrogen Corporation, Carlsbad, CA, US
- **ChemSketch:** Advanced Chemistry Development, Inc., Toronto, Canada

3.6.2 Web tools

- **Translation:** http://www.ebi.ac.uk/Tools/emboss/transeq/index.html
  http://www.expasy.org/tools/dna.html
- **Multiple sequence alignment:** http://www.expasy.ch/tools/sim-prot.html
  http://genome.cs.mtu.edu/map.html
- **Calculation of the MW:** http://www.expasy.ch/tools/pi_tool.html
- **Literature search:** http://www.scopus.com/home.url
  https://scifinder.cas.org/scifinder/

3.7 Kits and Protocols

3.7.1 GeneJET™ Plasmid Miniprep Kit (Fermentas Inc, Glen Burnie, MA, US)

For the plasmid isolation an *E. coli* colony was streaked out on at least one quarter of a selective media plate. Afterwards the plates were incubated over night at 37°C. The developed cell material was scraped off with a sterile toothpick and resuspended in the resuspension solution of the kit. Further steps were performed according to the supplied instructor manual [54, 55], with two exceptions. After the addition of the neutralization solution, centrifugation time was prolonged to 10 min at 13,200 rpm. For the elution of the plasmid DNA, the volume of water was varied according to the required DNA-concentration and the preferred end volume.

3.7.2 Pierce® BCA Protein Assay Kit (Thermo Fischer Scientific Corp., Waltham, MA, US)

This assay was used to determine the total protein concentration and uses bovine serum albumin (BSA) as standard. For determination of the protein concentrations the microplate procedure was performed according to the supplied instructor manual [56].
The absorption was determined at 562 nm using a Spectramax Plus 384 plate reader. If necessary, samples were diluted with ddH_2O prior to the BCA assay.

3.7.3 SDS-PAGE

SDS-PAGES were performed using an electrophoresis system from Invitrogen Corporation. Electrophoresis was performed according to the instruction manual.
Protein samples with concentrations of 5 µg or 10 µg were loaded onto a NuPAGE®12%Bis-Tris Gel. Electrophoresis was performed with MOPS buffer for at least 45 min using the NuPageGel program (200 V, 120 mA and 25 W). For staining SimplyBlue™ SafeStain was used.

3.7.4 Site-Directed Mutagenesis

Site-directed mutagenesis was performed by following the manual for Two-stage PCR protocol for Site-Directed Mutagenesis (AA.04) [57]. The polymerase of choice was *Pfu*Ultra^{TM} purchased from Stratagene.
After the amplification 25 µL of the PCR reaction were digested with 1 µL *Dpn*I for 2 hours at 37°C to remove the methylated template DNA.

3.7.5 Preparation of electrocompetent *E. coli* cells

For preparation of electrocompetent *E. coli* cells two days were required. The preparation was done according to a manual of Christoph Reisinger.

First day:

- Incubation of a shaking flask containing 20 mL TB media without any antibiotics with a single culture of the required strain
- Incubation overnight at 37°C and a shaking rate of 200 rpm

Second day:

- Incubation of 330 mL TB media (also without antibiotics) with 3 mL of the overnight culture
- Incubation at 37°C and a shaking rate of 200 rpm
- When the cell solution reached an OD_{600} of 1.1; the flask was cooled for one hour at 4°C
- Spin the cells down with 3,000 g, at 4°C for 10 min and discard the supernatant

- Resuspend the cells in pre cooled 1mM HEPES buffer
- Spin the cells down with 4,000 g, at 4°C for 10 min and discard the supernatant
- Resuspend the cells in pre cooled HEPES buffer/10% glycerol solution
- Spin the cells down with 4,500 g, at 4°C for 20 min and discard the supernatant
- Resuspend the cells in pre cooled 10% glycerol
- Spin the cells down with 4,500 g, at 4°C for 20 min and discard the supernatant
- Repeat the resuspension step in 10% glycerol and the following centrifugation step
- Resuspend the cells in pre cooled 10% glycerol (~3 mL)
- Divide the cell solution in aliquots
- Deep-freeze them with liquid nitrogen
- Storage at -80°C

3.7.5.1 Determination of the transformation efficiency

For the determination of the transformation efficiency 1 pg of pUC19 DNA was used. After regeneration the cell solution was diluted and plated out.
The transformation efficiency was calculated with Formula 1.

Formula 1: Determination of the transformation efficiency

$$\frac{Number_{Colonies}}{Volume_{plated}[\mu L]} \times \frac{Volume_{total}[\mu L]}{DNA_{transformed}[\mu g]} \times Factor_{Dilution} = \frac{Transformants}{\mu g DNA}$$

Number $_{Colonies}$	Number of colonies
Volume $_{plated}$[µL]	plated volume [µL]
Volume $_{transformation}$[µL]	total volume of the transformation reaction [µL]
DNA $_{transformed}$[µg]	amount of transformed DNA [µg]

3.7.6 *E. coli* transformation

For library creation electrocompetent Top10F' cells purchased from Invitrogen [58] were taken. For retransformation self prepared electrocompetent *E. coli* cells were used for the transformation.
For the transformation of high DNA amounts, the DNA has to be desalted before transformation. Therefore the DNA was pipetted onto a filter membrane (Millipore MF^{TM} membrane filter 0.025µm VSWP) which was floating on ddH_2O in a petri-dish. The desalination was done for 30 min to 1 hour.

For the transformation the deep frozen electrocompetent cells were thawed on ice and mixed with the desired amount of DNA. The mixture was transferred to a pre chilled electrotransformation cuvette and pulsed at 200 Ω, 25 µF and 2.0 kV (MicroPulser^{TM}). Furthermore 500 µL of SOC medium were added to the cuvette and the cell solution was transferred to a sterile microcentrifuge tube. Afterwards the cell solution was incubated for 30 min at 37°C, during the incubation time the tube was shaken with 800 rpm. After the regeneration phase aliquots were plated onto selective media and incubated overnight at 37°C.

3.7.7 Library creation

The first generation of error-prone libraries was created by Brigitte Höller using the GeneMorph® II Random Mutagenesis Kit (Stratagene). The instruction can be found in detail in the dissertation of Brigitte Höller [52].
The mutant library L2g1 was transformed into purchased electrocompetent TOP10F' cells and transformation as well as the subsequent steps were performed like described in chapter 3.7.6.
The developed colonies were used for inoculation of 384 well plates, containing 50 µL of LB media with a final concentration of 100 µg/mL of ampicillin. For the inoculation a picking robot (Q-Pix, Genetix) was used. Afterwards the plates were incubated over night at 37°C and ~ 50% air humanity. To reduce the evaporation the plates were incubated in a covered glass reservoir. Subsequently, the clones were preserved with glycerol (~14%) at -80°C.

3.7.8 Cultivation

3.7.8.1 Pre-culture

3.7.8.1.1 Pre-culture for plate cultivation

For the pre-culture 384 well glycerol stocks were thawed and used for the inoculation of 96 well microplates (flat bottom) containing 150 µL of LB Amp_{100} or TB Amp_{100}. Inoculation was performed with a 96 well pin replicator. After inoculation the plates were incubated for about 16 hours in a 37°C room with ~50% air humidity. In addition the plates were incubated in a covered glass reservoir to reduce evaporation.

3.7.8.1.2 Pre-culture for flask cultivation

For flask cultivation a shaking flasks containing 50 mL of TB Amp_{100} were inoculated with a single colony. Afterwards, the flasks were incubated at 37°C and 80% air humidity. During overnight incubation the flasks were shaken with 120 rpm.

3.7.8.2 Main-culture

3.7.8.2.1 Main-culture for microplate cultivation

For main-culture a 96 well microplates (V-shape) were filled with 150 µL TB Amp_{100} with a final concentration of 0.5 mM IPTG per well. For the inoculation a 96 well pin replicator was used. After the inoculation the plates were incubated for another 20 hours at 30°C and 50% air humidity. The incubation was performed in a in a covered glass reservoir to reduce evaporation.

3.7.8.2.2 Main-culture for deep well plate cultivation

Deep well plates containing 250 µL TB Amp_{100} with a final concentration of 0.5 mM IPTG per well were inoculated with a pre-culture, therefore a 96 well replicator was used. The plates were incubated at 30°C, 80% air humidity and shaken with 250 rpm. The total incubation period in this case was 20 hours.

3.7.8.2.3 Main-culture for flask cultivation

Shaking flasks containing 550 mL TB Amp_{100} were inoculated by the pre-culture to a final OD_{600} of 0.1 and incubated at 37°C and 80% air humidity. During the incubation the flasks were shaken with 120 rpm. At an OD_{600} of 0.8 the cells were induced by addition of IPTG. The final IPTG concentration in the flasks was 0.5 mM. After induction the cells were further incubated for another 20 hours. For this incubation period temperature was reduced to 30°C.

3.7.9 Harvest

3.7.9.1 Cell harvest from micro/deep well plates

The cells were harvested by centrifugation at 4°C, with 3,000 rpm for 12 min. After the removal of the supernatant the pellet was resuspended in 100 µL ddH_2O in the case of the microplate cultivation and in 250 µL ddH_2O in the case of the deep well plate cultivation,

respectively. To resuspend the cell pellets the plate were shaken for about 10 min with 1,050 rpm, therefore a Titramax was used.
Afterwards the cell solution was centrifuged for 15 min with 3,500 rpm at 4°C. The supernatant was discarded and the pellets were stored at -20°C for at least 2 hours.

3.7.9.2 Cell harvest from flask cultivations

To harvest the cells from flask cultivations the bacterial solution was centrifuged for 20 min with 5,000 rpm at 4°C. After the removal of the supernatant the pellet was resuspended in required amount of 50 mM MES-buffer pH 6. The cell suspension was further filled into 50 mL greiner tubes and centrifuged with 4,000 rpm, at 4°C for 20 min. Then the supernatant was discarded again and the pellets were stored at -20°C.

3.7.10 Cell lysis

3.7.10.1 Lysis solutions

For lysis of bacterial cells in microplates and deep well plates, respectively a lysis buffer was used. Lysis buffer compositions are shown in Table 9.

Table 9: Composition of the lysis solutions

Components	Lysis buffer for microplates	Lysis buffer for deep well plates
Lysozyme	1 mg/mL	1 mg/mL
DNAseI	-	0.5 µg/mL
$MgCl_2$	-	10 mM
Tween20	0.1%	0.1%

In Table 9 the final concentrations of the indicated compounds dissolved in 50 mM PPB pH 7 are given.
After thawing the cell pellets were lysed with 40 µL of lysis solution (microplates) and 100 µL of lysis solution (deep well plates) per well, respectively.
The microplates were then sealed with a plastic film, while the deep well plates were shut with the corresponding lids.
To resuspend the pellet the plates were shaken for 10 min with 1,050 rpm using the Titramax 1000. Subsequently for the following incubation the plates were shaken with 600 rpm

(Titramax) in a 30°C room for 1 hour. If required the lysate was diluted with 50 mM MES buffer pH 6 and centrifuged with 3,500 rpm for 15 min at 4°C. The supernatant was further examined.

3.7.10.2 Ultrasonic treatment

The cells pellets were thawed and resuspended in 25 mL of 50 mM MES buffer pH 6. The cell solutions were then lysed by ultrasonic treatment with a Branson Sonifier 250 Analog. For the cell disruption the following adjustments were made: DUTY cycle was ~80, output control was ~8 and disruption time was 6 min.
After ultrasonic treatment the solutions were centrifuged for 1 hour with 20,000 rpm at 4°C. Afterwards, the supernatant was used for further experiments. If required, the lysates were diluted with 50 mM MES buffer pH 6 or concentrated using ultrafiltration devices (Vivaspin 20 centrifugal concentrators, 10 kDa MWCO).

3.7.11 NADPH depletion assay

A detailed description of the sample composition is given in the chapter 4 assay development. The enzyme activity was determined by measuring the NADPH depletion at 340 nm. Therefore the reactions were started by the addition of NADPH, the plates were placed into a plate reader and mixed once, before the oxidation of NADPH to $NADP^+$ was measured.
The measurements were performed for 4 min, during this time span each sample was measured 21 times. Analysis of the obtained results was done with SoftMax Pro 4.8.

3.7.12 Sequencing

Sequencing was performed by AGOWA genomics (Berlin, Germany). The sample composition was as recommended by AGOWA.
Sequencing results were analysed with SeqMan 5.01.

4 Assay development

For stability screening a screening assay in microplate-format had to be established. The substrate used in this context was α-methyl-trans-cinnamaldehyde. A reaction model of the conversion is shown in Figure 10. For measuring the conversion of α-methyl-trans-cinnamaldehyde into 2-methyl-3-phenylpropanal 5 mM of the substrate was used. The substrate concentration was already determined in previous experiments by Brigitte Höller. At higher concentrations α-MCA is poorly soluble in aqueous solutions. Therefore the presence of an organic co-solvent increases the solubility of the substrate and in addition more stable variants can be selected.

All experiments for assay establishment were performed with lysates of *E. coli* TOP10F' [pMS470_P1syn].

For activity detection the NADPH depletion assay described in 3.7.11 was used. For the assay α-MCA was first dissolved in pure organic solvent. The concentration of the stock solution depended on the final amount of organic co-solvent present in the samples. This stock solution was further mixed with 50 mM MES buffer pH 6. The final concentration of α-MCA was 5 mM, the concentration of the organic co-solvent was varied during the assay development. Sample compositions depended on the experiment and were described in the corresponding chapters. For all experiments enzyme and substrate solution was propound in the screening plate. The conversion was started by the addition of the NADPH solution.

4.1 Single-phase systems

The solvents used during this study for single-phase systems were N,N-dimethylformamide (DMF) and N-methyl-2-pyrrolidone (NMP). The minimal amount of co-solvent was 2%, which was required to dissolve the substrate.

The sample composition used for determination of the appropriate assay conditions was as follows:

- Substrate solution: The substrate solution contained a final concentration of 5 mM α-MCA, MES-buffer and co-solvent. Therefore a stock solution containing different amounts of α -MCA dissolved in pure organic solvent was prepared. The final amount of co-solvent in the sample was varied between 2% and 25%.

- Enzyme solution: If necessary, enzyme solution was diluted for the measurement.
- NADPH solution: The final concentration was 2 mM. The measurements were started by adding NADPH to the samples.

Table 10: Sample composition (single-phase systems)

Compound	Volume [µL]
Substrate solution	170
Enzyme solution	10
NADPH solution (added before the measurement)	20
Final volume	200

4.1.1 Variation of the solvent concentration

The solvent concentrations were varied between 2% and 25%. The composition of the samples was like described in Table 10. Measurement was performed by the use of the NADPH depletion assay described in 3.7.11.

The graphical evaluations of the results are shown in Figure 12 and 13.

For the calculation of the relative average activities and the standard deviations 32 values obtained from samples treated under the same conditions were used.

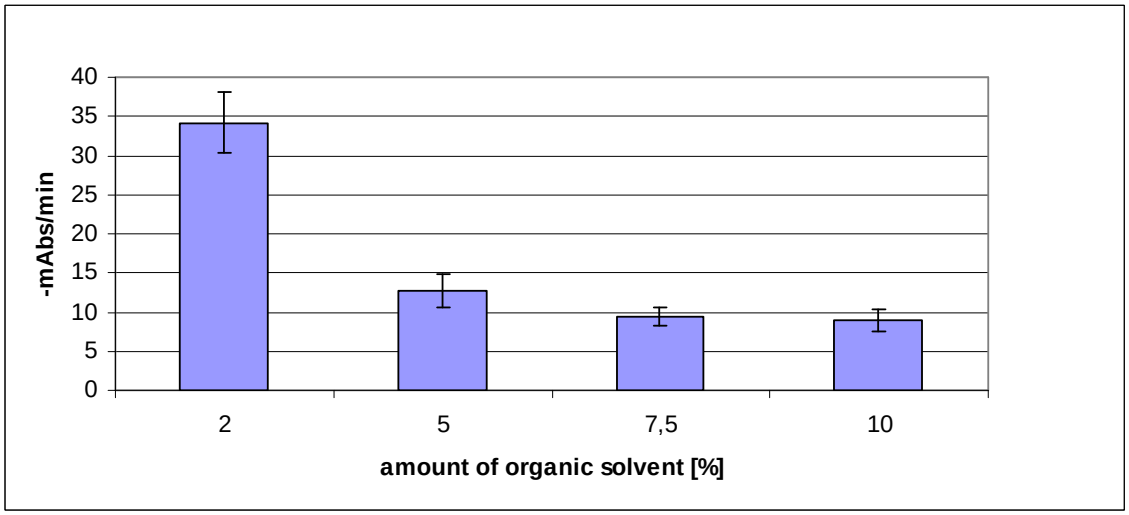

Figure 12: Variation of DMF concentration: Reaction mixtures containing between 2 and 10% co-solvent are shown. The bars illustrate the average of the activity; the standard deviation is illustrated by error bars.

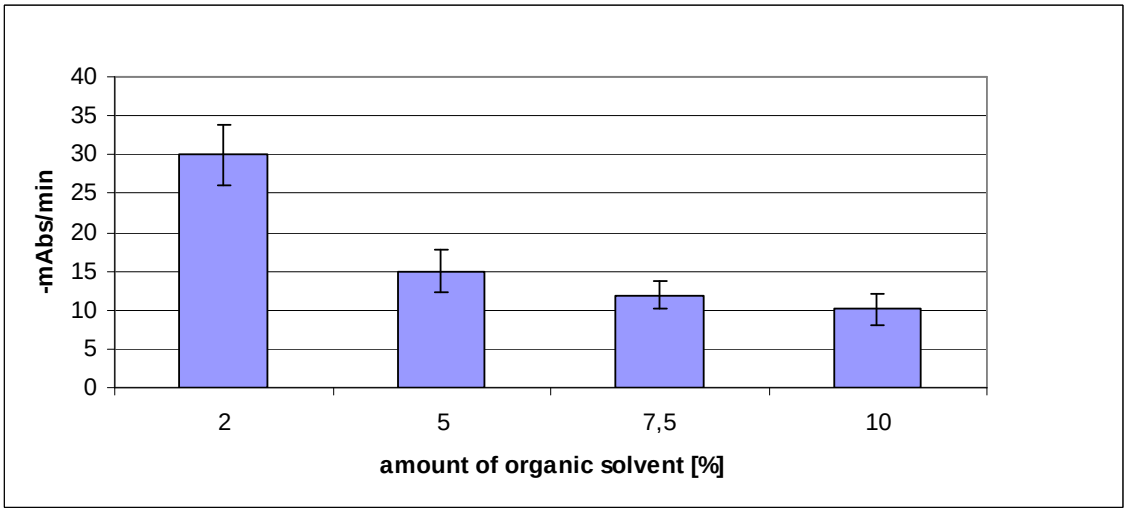

Figure 13: Variation of NMP concentration: Reaction mixtures containing between 2 and 10% co-solvent are shown. The bars illustrate the average of the activity; the standard deviation is illustrated by error bars.

Enzymatic activity dropped with increasing co-solvent concentration. Although solvent concentration was varied between 2% and 25%, only reaction mixtures containing between 2% and 10% co-solvent are shown in the Figures 12 and 13. The reason therefore was that no activity could be detected when the solvent concentration was increased above 10%.

The variation of the co-solvent concentration revealed the threshold behaviour [9, 11, 13] of the enzyme. Based on the obtained results the threshold concentration [9, 10, 11] could be determined. For further information, the required diagrams are represented in the appendix. C_{50} is the concentration of organic solvent, at which the enzymatic activity dropped to 50% of the initial activity. In case of DMF C_{50} was under 5%, in case of NMP it was ~5% of organic solvent. The different C_{50} values result from different solvent properties, beside this also the different concentrations of the enzyme solutions could affect the results. Enzyme solutions for the measurement with NMP were always higher concentrated, what results in a higher protein amount, which could further stabilise the enzyme by protein-protein interactions.

4.1.2 Variation of the incubation time

For the determination of the ideal incubation time the lysate/substrate mixture was incubated and measured at different time points. During incubation plates were sealed with a plastic film to minimize the evaporation.

The solvent concentration used during this test series was 5% (DMF, NMP) and incubation time was varied from 0 min to 24 hours. During incubation the plates were shaken with 600 rpm (Titramax 1000). After the examination of the incubation time the samples were measured in the presence of the organic co-solvent (5%).

The average activity measured at 0 min was set to 100% and the values of all other time points were related to this value. For calculation of the residual activity after a certain time of incubation Formula 2 was used.

Formula 2: Determination of the Stability [%]

$$Stability[\%] = \frac{Activity_{x\,\min}}{Activity_{0\,\min}} \times 100$$

Stability [%]	Percent of activity remaining after the incubation
$Activity_{xmin}$	Remaining activity after *x* min of incubation
$Activity_{0min}$	Initial activity

The graphical evaluations of the results are shown in the Figures 14 and 15. The average activities and the standard deviations were calculated from 48 values obtained from samples treated under the same conditions.

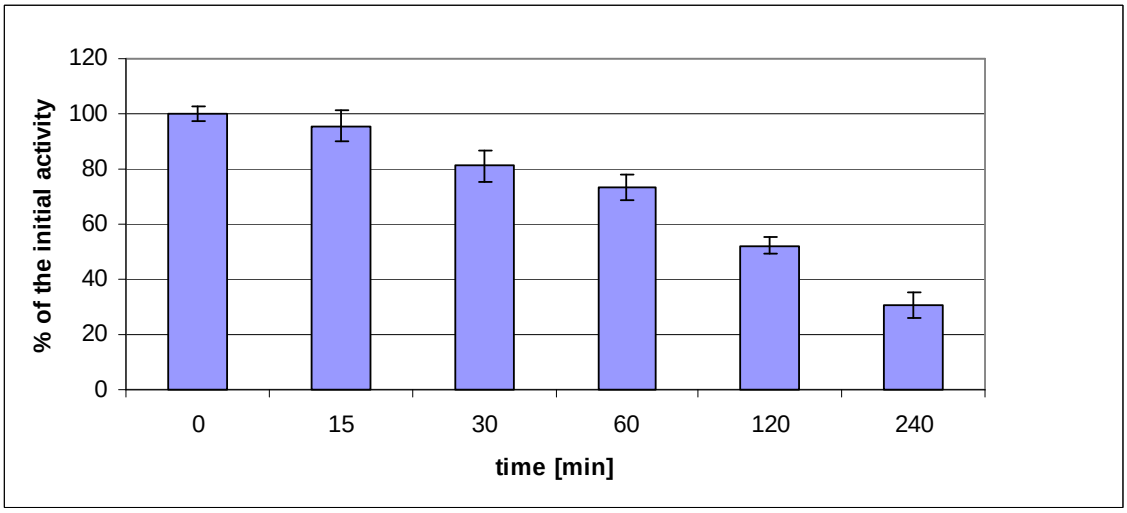

Figure 14: Variation of the incubation time with DMF as organic co-solvent: Samples incubated between 0 min and 240 min are shown. The bars illustrate the average of the activity; the standard deviation is shown by error bars.

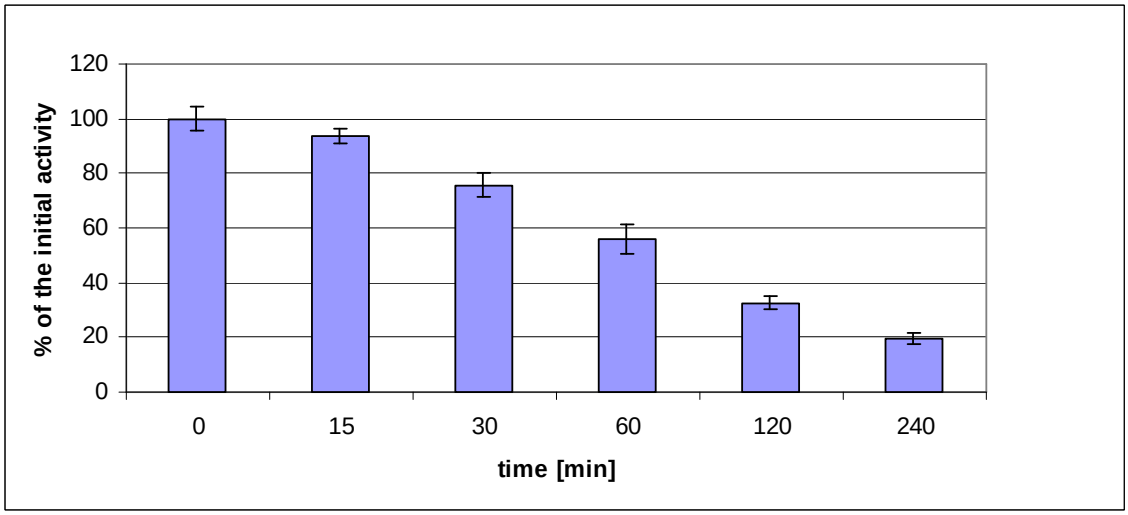

Figure 15: Variation of the incubation time with NMP as organic co-solvent Samples incubated between 0 min and 240 min are shown. The bars illustrate the average of the activity; the standard deviation is shown by error bars.

Measured activities dropped with increasing incubation time. After 4 hours of incubation the remaining activities decreased to ~30% in DMF and to ~20% in NMP, respectively. Although incubation time was varied from 0 min to 24 hours, only the results until 240 min of incubation are shown in Figure 14 and 15. The reason therefore was that no residual activities were found after 24 hours of incubation, a reason therefore could be the evaporation of the solvent and the associated drop formation on the seal. Drop formation also occurred before; in this case drops were only observed in the border areas of the microplates. Problems associated with drop formation were the risk of cross-contamination and that the reduction of the liquid volume, leads to a change of the photometric properties.

4.2 Two-phase systems

The solvent used for two-phase systems was toluene. The use of toluene made it unavoidable to change the used screening plates. Determining therefore was that the UV-Star plates are made out of polystyrene, a material not resistant against toluene.

A comparison of the transmission spectra of UV-Star and PP-plates used for the experiments is shown in Figure 16.

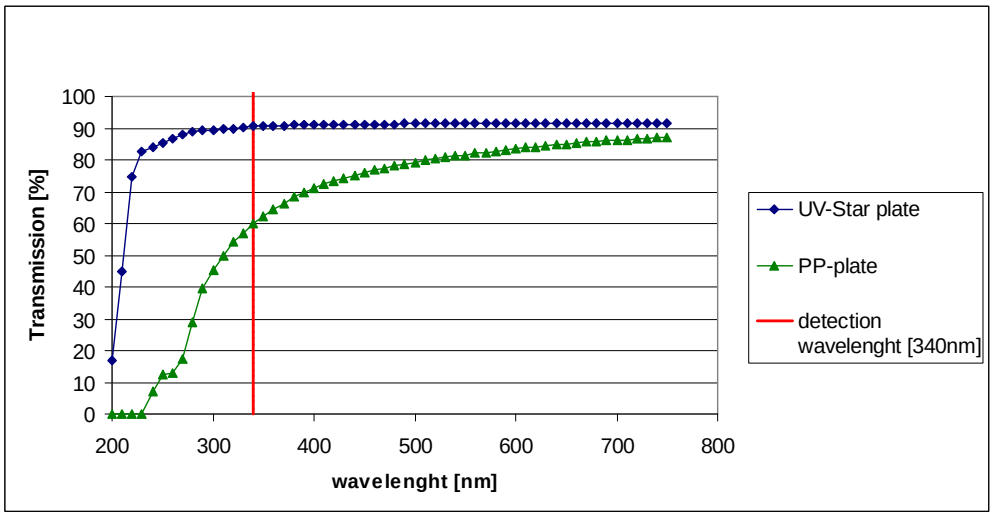

Figure 16: Comparison of the transmission spectra of a UV-star and a PP-plate: The red vertical line indicates the detection wavelength

The comparison of the transmission spectra indicated that the amount of light which shines through was reduced in the case of the PP-plates. Measurements revealed that the reduced transmission of the PP-plates had no effect on the obtained results.

Toluene was always added directly to the wells because otherwise a homogeneous distribution of the organic solvent was not guaranteed. The applied amount of toluene was 20 µL, this amount allowed the formation of a film, which covered the whole surface of the well. Measurements with lower amounts of toluene indicated that the obtained results were not reproducible, when the ray of the photometer did not always pass through the toluene film.

Samples measured with toluene always contained a further organic co-solvent (DMF) to dissolve α-MCA.

The substrate solution used for these experiments contained a final concentration of 5 mM α-MCA, MES-buffer and 2% of DMF.

An overview of the sample composition is shown in the Table 11.

Table 11: Sample composition (two-phase systems)

Compound	Volume [µL]
Substrate solution	150
Enzyme solution	10
Toluene	20
NADPH solution (added before the measurement)	20
Final volume	200

4.2.1 Variation of the shaking rate

Samples with the composition described in Table 11 were incubated for 5 min. During the incubation the plates were shaken with a Titramax, thereby the shaking rate was varied between 0 rpm and 750 rpm.

The graphical evaluations of the results are shown in Figure 17. The average activity obtained without shaking was set to 100%. The average activities and the standard deviations were calculated from 96 values obtained from measurements of samples treated under the same conditions. The obtained results were related to the activity without shaking.

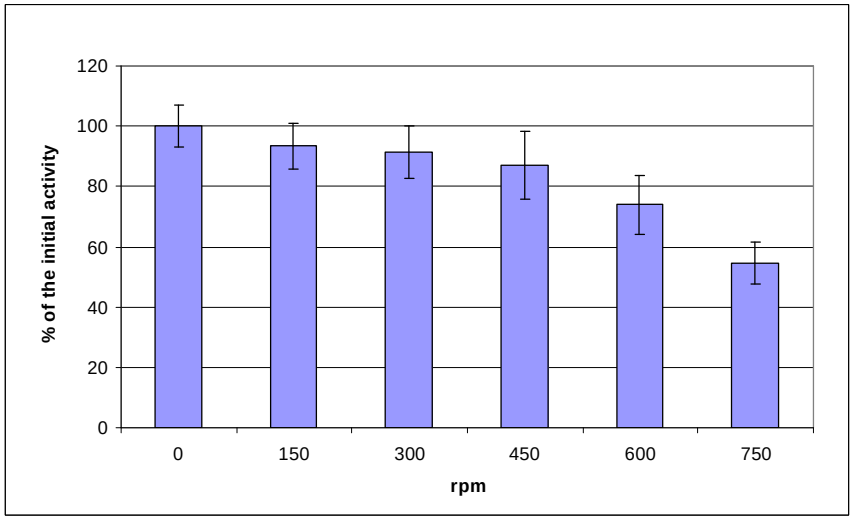

Figure 17: Variation of the shaking rate between 0 rpm and 750 rpm: The obtained activities are shown as blue bars. The obtained standard deviation is illustrated by error bars.

Activity dropped down with increasing shaking rate, a further increase of the shaking rate was limited by the detectability limit of the residual activity. The decrease of activity with increasing shaking rate could be explained by the denaturation caused by interactions of the enzyme at the liquid-liquid interface. This phenomenon was already described by Asakura and co-workers [18], they determined that even small amounts of toluene could case denaturation in shaken samples.

For further experiments a shaking rate of 450 rpm was used. The reason to choose this shaking rate was that a fast decrease of activity would result in a reduction of incubation time.

4.2.2 Variation of the incubation time

For the determination of the ideal incubation time plates were measured at different time points. During the incubation the sealed samples were shaken with 450 rpm.
The obtained results are shown in Figure 18, for this diagram the activity obtained without incubation was set to 100% and all other values were related to this value. The average activities and the standard deviations were calculated from 96 values obtained from measurements of samples treated under the same conditions.

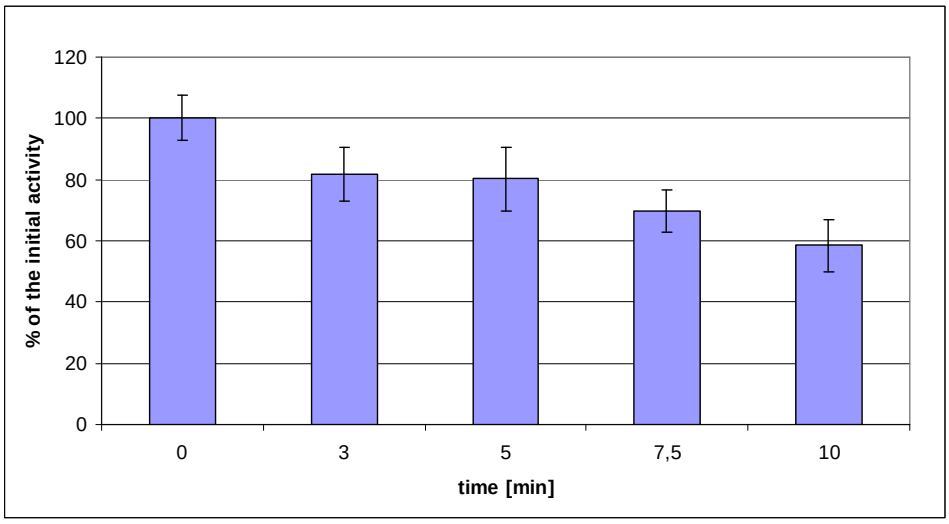

Figure 18: Variation of the incubation time between 0 min and 10 min: The residual activities are shown as blue bars. The obtained standard deviation is shown by error bars.

A decrease of the activity was even detected after 3 min of incubation. Samples incubated for 10 min only showed about 60% of the initial activity. Also in this case the limitation for a further increase of the incubation time was the detectability limit.
Experiments with lysates obtained after deep well plate cultivation showed that even an incubation time of 15 min was possible with this setup.

4.3 Incubation conditions for screening

4.3.1 Single-phase systems

The substrate solution for experiments with water miscible solvents contained a final concentration of 5 mM α-MCA, MES-buffer and 5% of the respective organic co-solvent. A summary of the incubation parameters is given in Table 12.

Table 12: Incubation parameters for single-phase systems

Incubation parameters	
Amount of organic solvent	5% DMF
	5% NMP
Shaking rate	450 rpm
Incubation time	Varied between 0 min and 240 min

4.3.2 Two-phase systems

In the case of two-phase systems the substrate solution contained 5 mM α-MCA, MES-buffer, 2% of a water miscible organic co-solvent and 10% of toluene. The incubation parameters were as follows:

Table 13: Incubation parameters for two-phase systems

Incubation parameters	
Amount of organic solvent	2% DMF and 10% toluene
	2% NMP and 10% toluene
Shaking rate	450 rpm
Incubation time	Varied between 0 min and 15 min

4.3.3 Screening assay

For the screening a two-phase system was used, the sample composition and the incubation parameters were summarized in Table 14.

Table 14: Parameters for the screening assay

Parameters for the screening assay	
Sample composition	10µL Lysate 10% Toluene 2% DMF 50 mM MES buffer pH 6
Shaking rate	450 rpm
Incubation time	15 min

4.4 Determination of the expression level

The expression level of *E. coli* TOP10F' [pMS470_P1syn] was measured under different cultivation methods (mico-, deep well plates and flasks) and cultivation media (LB, TB and 2xTY).

After cell lysis and determination of the protein concentration, an amount of 10 µg protein was used for a SDS-PAGE.

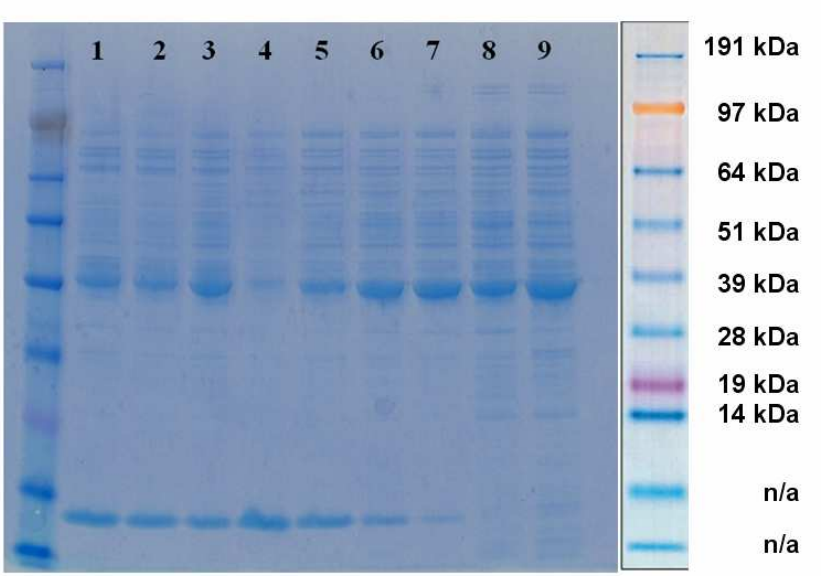

Figure 19: SDS page for the determination of the expression level: the applied protein amount was 10µg. The SeeBlue® Plus2 Pre-Stained standard from Invitrogen Corp. is shown on the right. 1: MTP cultivation using LB media, 2: MTP cultivation using 2xTY media, 3: MTP cultivation using TB media, 4: DWP cultivation using 2xTY media not shaken, 5 DWP cultivation using TB media not shaken, 6: DWP cultivation using 2xTY media, 7: DWP cultivation using TB media. 8: Flask cultivation using 2xTY media, 9: Flask cultivation using TB media.

The determination of the expression level (Figure 19) shows that not only the cultivation method but also the used media influenced the expression level. The expression level increased with rising cultivation volume and media complexity. A possible explanation for the increased expression level after cultivation with TB media might be the buffer and the glycerol contained in this media.

5 Results and discussion

5.1 Screening

For stability screening gene library L2 was used. Details about the library creation and further cultivation steps are summarized in chapter material and methods. The cultivation was done in deep well plates, after harvest and cell lysis the enzyme solution was used for measurements. For the measurement the cell solution was mixed with a substrate solution containing 5 mM α-MCA, MES-buffer and the required amount of organic co-solvents (2% DMF/10% toluene). Further details about the sample composition are summarized in Table 14.

After incubation the activity was measured with the NADPH depletion assay described in 3.7.11.

About 8,400 mutants were screened for improved stability in the organic co-solvent/water mixture. After the screening the obtained results were analyzed and are shown as landscape in Figure 20.

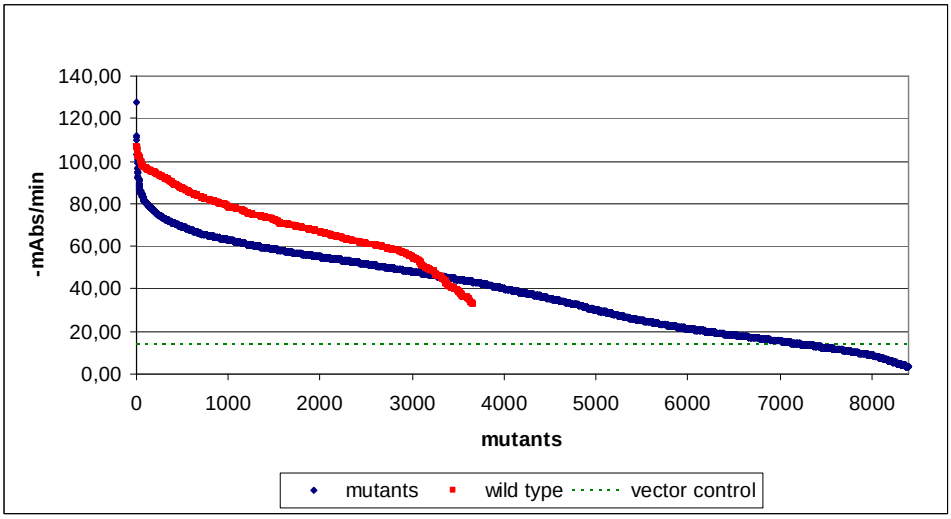

Figure 20: Landscape of the mutant library: measured mutants are shown as blue rhombuses; the wild type is shown as red squares; the vector control is indicated by a broken green line.

Beside the obtained data from the mutants also the landscape of the wild type and average of the vector control are shown in Figure 20. To compare the activities from mutants, wild type and vector control, they were cultivated on the same plate.

The average of the wild type was -69 mAbs/min, this value had a standard deviation of ~9 mAbs/min. The landscape is based on the results of about 350 measurements. For a better

comparability with the mutant landscape each obtained value of the wild type is shown 10 times in Figure 20.

As vector control *E. coli* TOP10F' [pMS470] was used. The average of the vector control was -14 mAbs/min and had a standard deviation of ~7 mAbs/min.

The screening revealed that the developed assay was not as precise and reliable as expected. The high standard deviation did not only depend on the assay, it was further noticed, that the properties of the substrate solution changed, when it was exposed to light and oxygen. To avoid this source of error, the substrate solution was further stored at dark places and sealed with Parafilm.

After the screening all measurements were analysed and the most promising mutants were taken into the rescreening to avoid false positives. As selection criterion the activity after 15 min of incubation was used. Only mutants with a residual activity higher than the average of the wild type and its 2-fold standard deviation (-86 mAbs/min) were taken into the rescreening.

5.1.1 Rescreening

39 mutants were taken into the rescreening. Therefore each mutant was streaked out to get single colonies. The single colonies were further used for the inoculation of the pre-culture (microplate). Details about cultivation were summarized in 3.7.8.

After cultivation the plates with the stability increased mutants were measured again.

The rescreening was performed like described in Table 14. In the course of rescreening also an approach using NMP instead of DMF was performed.

A further difference to the screening was that each sample was measured twice. The results obtained after 0 min and 15 min were used for the calculation of the residual activity, therefore Formula 2 was used.

The best mutations were further taken into a second rescreening round.

5.1.2 Re-rescreening

16 stability improved mutants were taken into the re-rescreening. Therefore they were cultivated and measured again.

Mutants were named as followed: 11_G9: plate 11 (384 well plate)_well G9

The measurement was performed in the same way as it was done during the rescreening. The best hits of the re-rescreening were shown in Figure 21 and 22. For the calculation of the average and the standard deviation at least 6 measurements of each mutant were used.

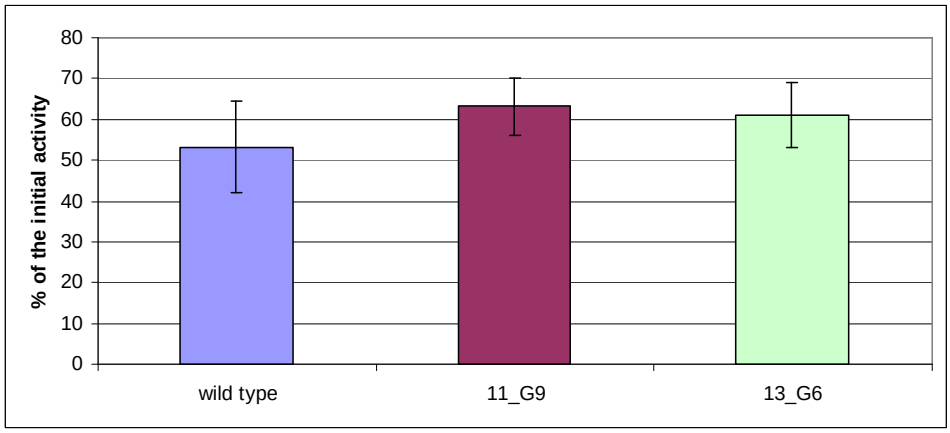

Figure 21: Hits of the Re-rescreening (2% DMF/10% toluene): Wild type (blue) and mutants with increased stability (violet and bright green) are shown. The standard deviation of the individual samples is indicated by error bars.

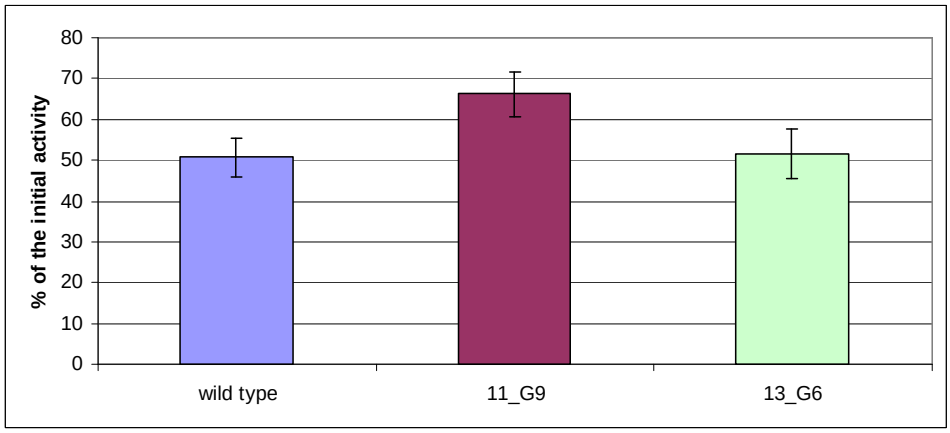

Figure 22: Hits of the Re-rescreening (2% NMP/10% toluene): Wild type (blue) and mutants with increased stability (violet and bright green) are shown. The standard deviation of the individual samples is indicated by error bars.

In both co-solvent mixtures (DMF/toluene and NMP/toluene) the mutants 11_G9 and 13_G6 showed the best stability. The high standard deviations of the remaining activities made it

unavoidable to perform further experiments to confirm the increased stability of these mutants.

5.1.3 Sequencing results

The results of the sequencing are summarized in the table below.

Table 15: Sequencing results

Mutant	Mutation (s)	aa- exchange	Position
11_G9	1	1	A145T
13_G6	1	1	Y72F

Both mutants have one mutation; this mutation leads in an amino acid exchange. In Figure 23 the localisation of the mutations are shown. The figure was created with the program PyMol and is based on the pdb file with the number 2J3J. The resolution of the used protein structure is 2.8 Å.

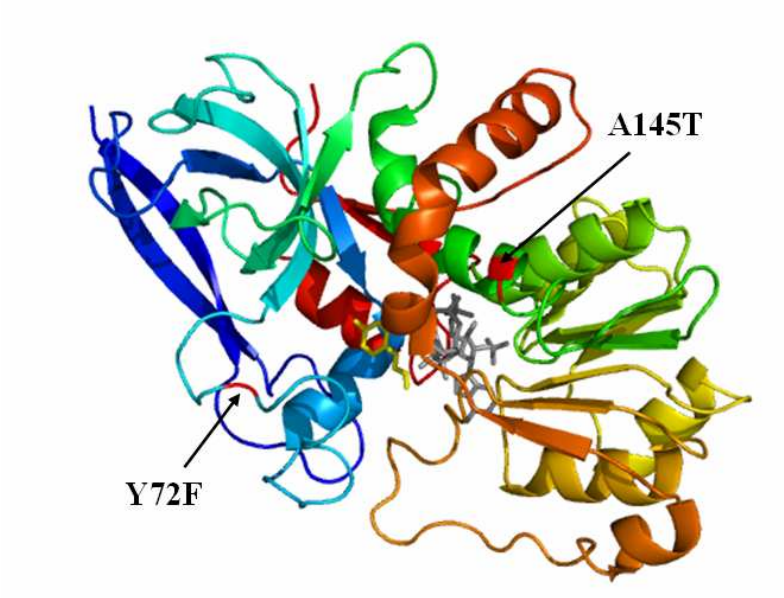

Figure 23: Localisation of the obtained mutations: The mutations of 11_G9 and 13_G6 are shown in red and indicated by arrows. Beside the mutations also the bound co-factor NADPH (grey) and the substrate used for crystallization p-coumaryl aldehyde (yellow) are shown in the image.

The mutations are described in a more detailed way after the verification of the screening results.

5.2 Verification of the screening results

To confirm the obtained results further experiments were performed. All experiments made in this context were done with lysates obtained after flask cultivation.
The stability of the *E. coli* TOP10F' [pMS470 P1], TOP10F' [pMS470 11_G9] and TOP10F' [pMS470 13_G6] was determined in single-phase and in two-phase systems. In all experiments it was attempted that the initial activity of the wild type and the mutants differed as little as possible.

5.2.1 Two-phase systems

The parameters used for this test series are described in Table 13. The high start activity allowed the determination of the residual activity in a time interval of 45 minutes.
The graphical evaluations of the results are shown in Figure 24 and 25. In both diagrams the average of the activity measured without incubation was set to 100%. All other values were related to this value.

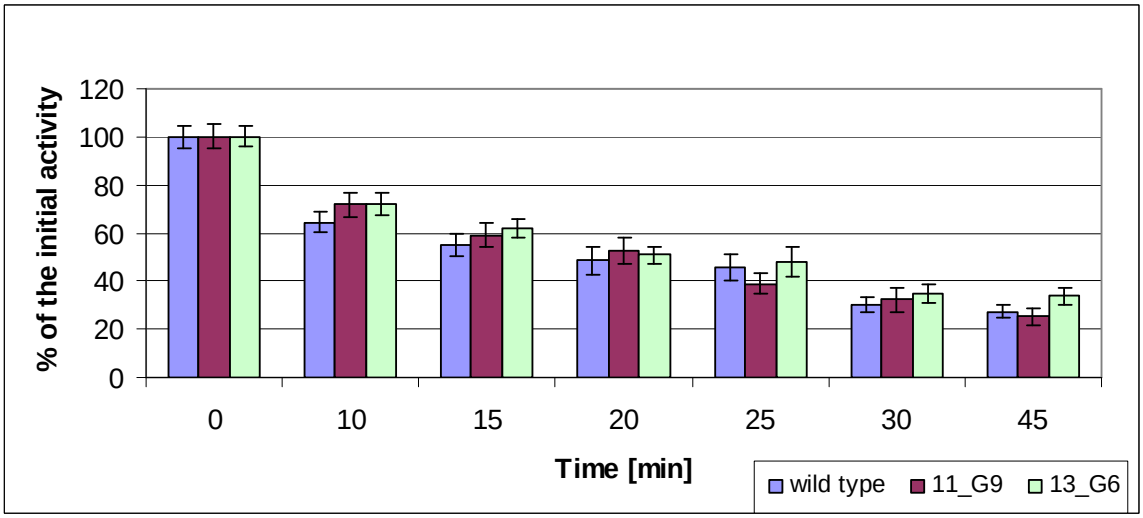

Figure 24: Verification of the re-rescreening results (2% DMF/10% toluene): The average activities of the wild type (blue), 11_G9 (violet) and 13_G6 (bright green) are shown. The standard deviation is shown by error bars.

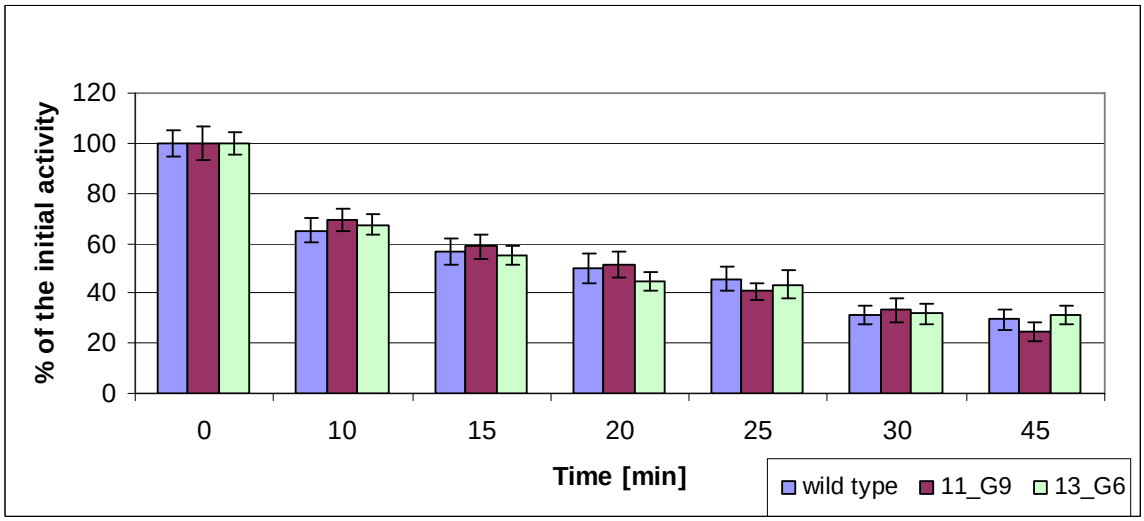

Figure 25: Verification of the re-rescreening results (2% NMP/10% toluene): The average activities of the wild type (blue), 11_G9 (violet) and 13_G6 (bright green) are shown. The standard deviation is shown by error bars.

As indicated by Figure 24 and 25 the standard deviation in the two-phase systems was too high to confirm the increased stability of the selected mutants.

5.2.2 Single-phase systems

The mutants 11_G9 and 13_G6 were also characterised in single phase systems. The parameters used for these experiments are described in Table 12.

The results of this test series are shown in Figure 26 and 27. In both diagrams the average of the start activity was set to 100% and all other values were related to this value.

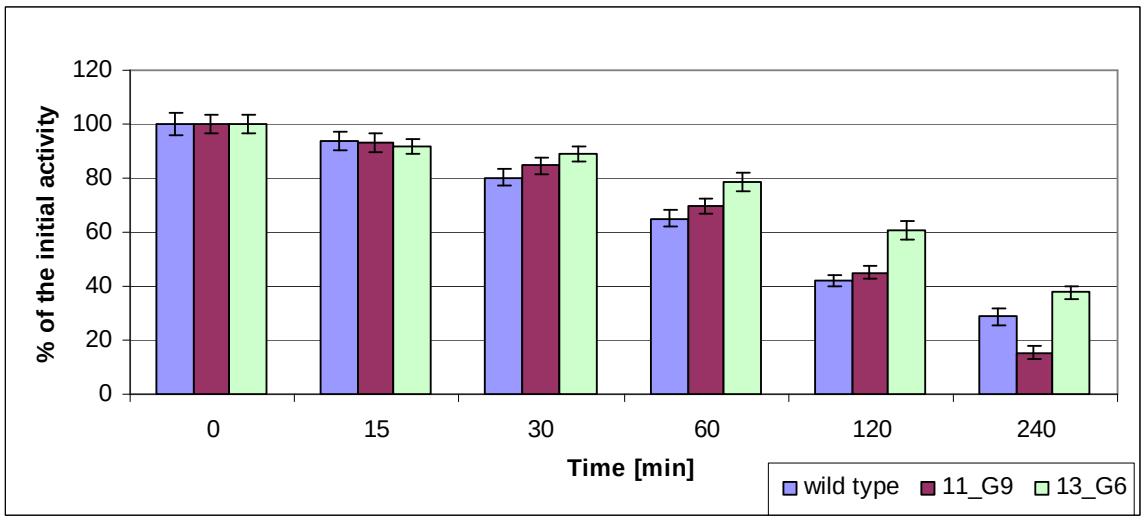

Figure 26: Analysis of stability with 5% DMF as organic co-solvent: The average activities of the wild type (blue), 11_G9 (violet) and 13_G6 (bright green) are shown. The standard deviation is indicated by error bars.

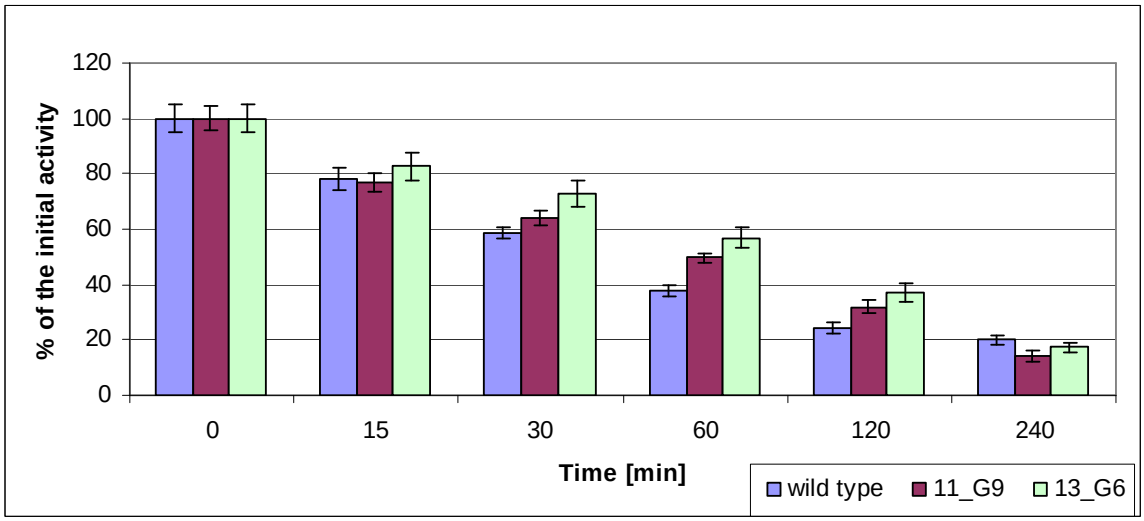

Figure 27: Analysis of stability with 5% NMP as organic co-solvent: The average activities of the wild type (blue), 11_G9 (violet) and 13_G6 (bright green) are shown. The standard deviation is indicated by error bars.

The obtained standard deviations for measurements in single-phase systems were not as high as in the case of the two-phase systems.

The mutant 11_G9 showed increased stability especially till 120 min. After 120 min the stability was ~1.1-fold improved in DMF and ~1.3-fold improved in NMP. Later the activity dropped down and could not reach the level of the wild type.

The remaining activity of 13_G6 was always higher in DMF. When NMP was used as co-solvent, 13_G6 showed increased stability till 120 min afterwards the stability of the wild type was not reached. After 120 min the stability was ~1.4-fold improved in DMF and ~1.5-fold improved in NMP.

Based on the results obtained during the characterisation of 11_G9 and 13_G6 in the single phase systems the included mutations were combined. The combination was done by site-directed mutagenesis.

The combination of 11_G9 and 13_G6 was named 11_G9/13_G6; the presence of the required mutations was confirmed by sequencing.

5.3 Characterisation of the combined mutant 11_G9/13_G6

For the characterisation of the mutant 11_G9/13_G6 the same experiments as for the verification of the screening results were performed. Also in this case it was attempted to have the same initial activity for all tested variants of the enzyme.

5.3.1 Two-phase systems

The results of the characterisation of 11_G9/13_G6 regarding its stability in two-phase systems with toluene are shown in Figure 28 and 29.

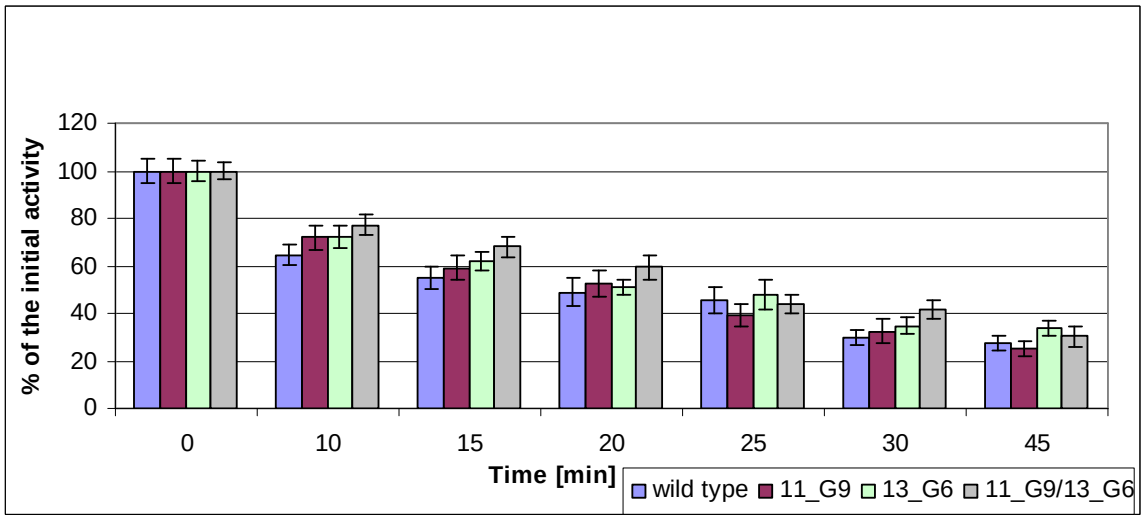

Figure 28: Characterisation of 11_G9/13_G6 (2% DMF/10% toluene): The average activities of the wild type (blue), 11_G9 (violet), 13_G6 (bright green) and their combination 11_G9/13_G6 (grey) are shown. The standard deviation is indicated by error bars.

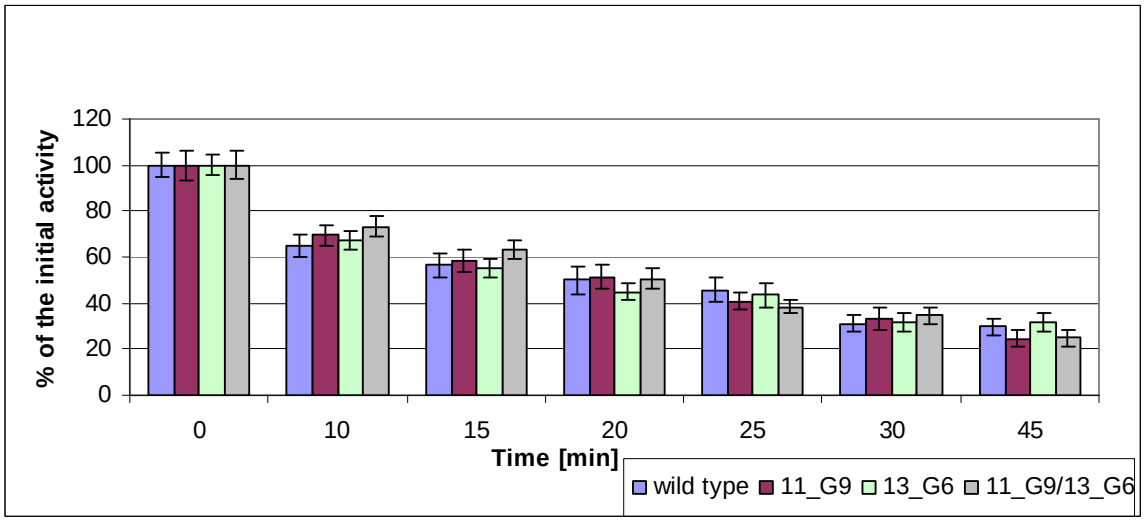

Figure 29: Characterisation of 11_G9/13_G6 (2% NMP/10% toluene): The average activities of the wild type (blue), 11_G9 (violet), 13_G6 (bright green) and their combination 11_G9/13_G6 (grey) are shown. The standard deviation is shown by error bars.

The results of the characterisation of the combined mutant in the two-phase systems showed that the standard deviations made it impossible to predict the behaviour of 11_G9/13_G6 in two-phase systems. A reason therefore could be the sensitivity of the developed screening assay.

5.3.2 Single-phase systems

The co-solvent stability of 11_G9/13_G3 was further determined in single-phase systems. The results of the stability tests are summarized in Figure 30 and 31. For a better comparability of the obtained results, the mutants 11_G9 and 13_G6 are not shown. The diagrams, which also contain the results of these mutants, can be found in the appendix.

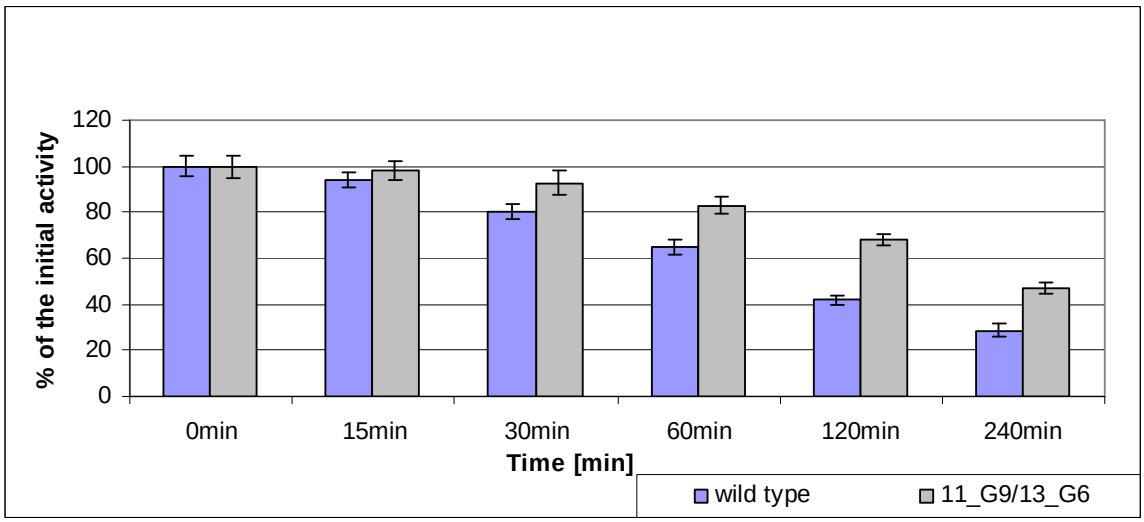

Figure 30: Characterisation of 11_G9/13_G6 with 5% DMF as organic co-solvent: shown are the average activities of the wild type (blue) and 11_G9/13_G6 (grey). The standard deviation is indicated by error bars.

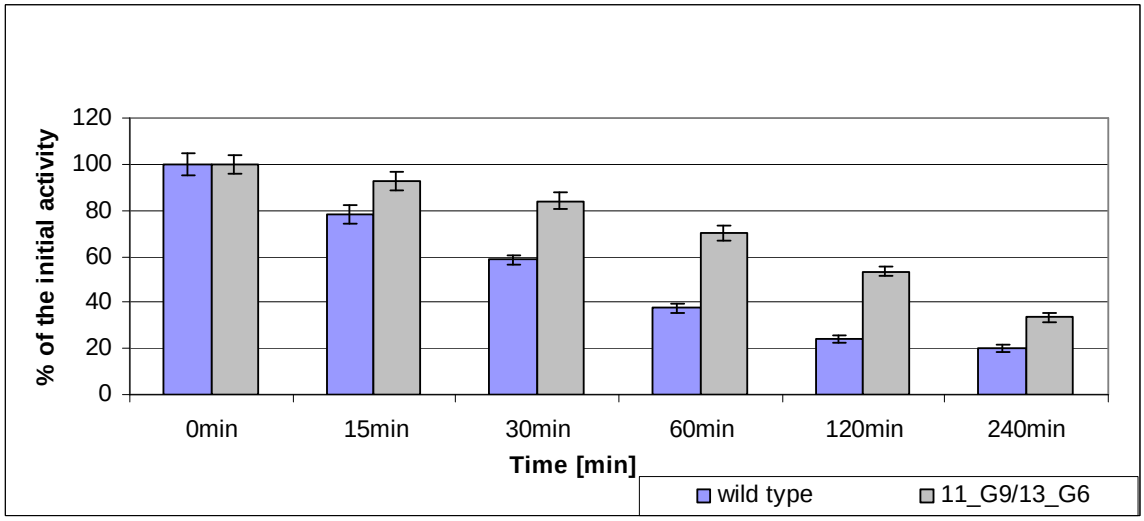

Figure 31: Characterisation of 11_G9/13_G6 with 5% NMP as organic co-solvent: shown are the average activities of the wild type (blue) and 11_G9/13_G6 (grey). The standard deviation is shown by error bars.

11_G9/13_G6 showed increased stability compared with the wild type. The residual activity of the combined mutant was in both co-solvents higher than the remaining activity of the wild type. The improvement was more pronounced than with the single mutations.
A calculation of the stability of the combination showed that the activity was ~1.4-fold improved in DMF and ~1.7-fold improved in NMP. These values represent the average increase in stability of the combined mutant.

Since a further combination was made, the combination 11_G9/13_G6 is named “stab” in this context.

5.4 Details about the mutations

As mentioned before in both stability improved mutants one mutation was found. This mutation resulted in an amino-acid exchange.
In the following section the occurred amino-acid exchanges are described in detail. To determine the reasons, responsible for the increased solvent stability, the obtained mutations were compared with the proposals of Arnold [1] and Yang and co-workers [22]. Their proposals for mutations that increase the solvent stability of enzymes are summarised in 1.1.4. The exchanged amino-acids are implemented in a way that the orientation of their side chain shows a maximum of correlation to the conformation of the native residue.

5.4.1 Mutant 11_G9 (A145T)

In 11_G9 the alanine at position 145 is replaced by a threonine. This mutation leads to an exchange of a neutral non-polar amino-acid to a neutral polar amino acid.

5.4.1.1 Localisation

For the localisation of the exchanged residue PyMole was used. The image of the localisation is shown in Figure 32.

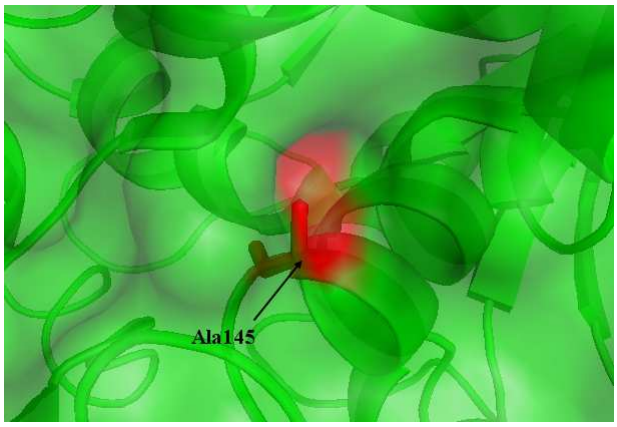

Figure 32: Localisation of Ala145: Ala-145 is shown in red; the rest of the protein is presented in green. Beside the protein structure also the surface is shown.

In Figure 32 the surface of the protein is shown as green shell, with a red spot, that confirmed that Ala-145 is part of the surface, what indicates that this residue is exposed to the solvent.

5.4.1.2 Interactions of residue 145 before and after the mutation

Details about the interactions of the amino-acid residue present at position 145 are presented in Figure 33.

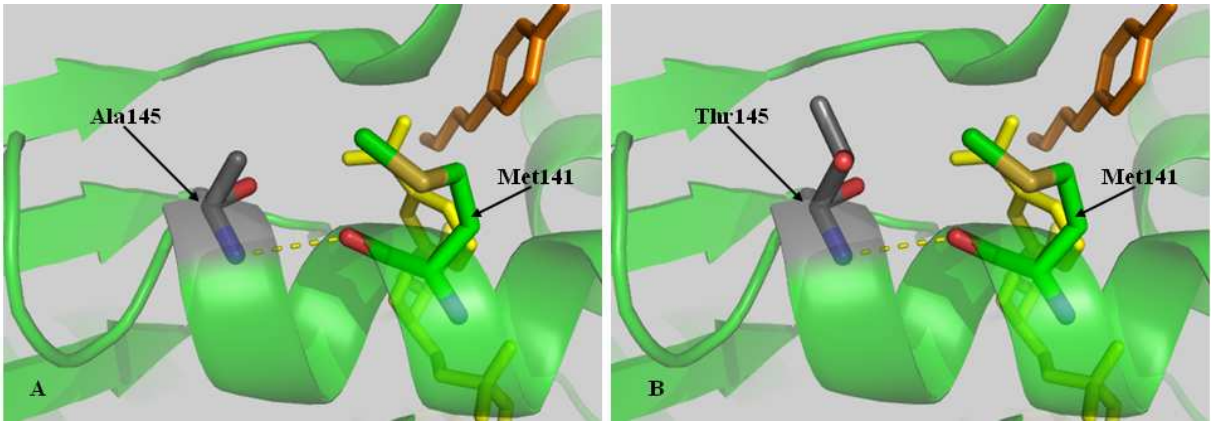

Figure 33: Interactions of amino-acid residue 145: A wild type residue; B mutated residue; hydrogen bonds are indicated as dashed yellow lines

In Figure 33 the interactions of the amino-acid at position 145 are shown. The wild type enzyme had an Ala at this position; this Ala can form a linkage to Met-141. The linkage is formed by main-chain interactions and represents the typical hydrogen bond (n+4) configuration shown in α-helixes.

Also the introduced Thr forms no additional H-bond linkages within the peptide chain but might show stronger interactions with the major solvent H_2O of the buffer.

Beside the amino-acids also the bound co-factor NADPH (yellow) and the substrate used for crystallization p-coumaryl aldehyde (orange) are illustrated in Figure 33.

5.4.1.3 Comparison of the obtained results with the design rules for enzyme stabilisation in organic solvents

Both authors [1, 22] recommended the exchange of charged surface residues. In this case both amino-acids found in position 145 are uncharged, but Thr is more hydrophobic than Ala.
They also proposed to remove unfulfilled hydrogen binding sides. The wild type Ala can create no hydrogen-bonds with its side-chain. As opposed to this Thr-145 has a free polar hydroxyl group, which points to the surface. This OH-group would allow the Thr to form further linkages to stabilize the enzyme. A rotation of the side chain by 180° would bring the hydroxyl group in binding distance with Met-141 as indicated in the Figure 34.

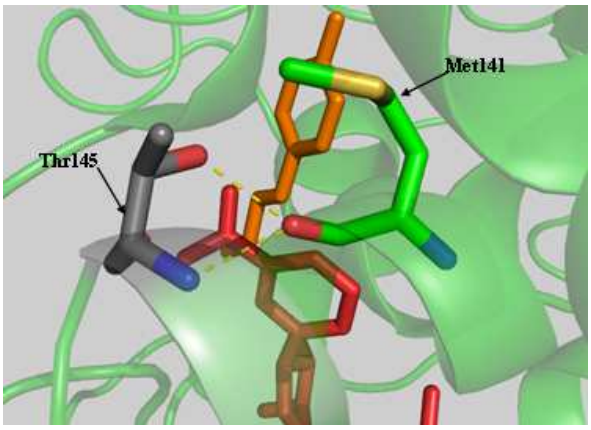

Figure 34: Possible polar contacts after the rotation of Thr145: polar contacts are presented as dashed yellow lines

Figure 34 shows that the rotation of the side chain results in the formation of a further hydrogen bond between Thr-145 and Met-141. In contrast to this observation Thr-145 formed only one hydrogen bond before the rotation (see Figure 33B). This further hydrogen bond allows Thr-145 to fulfil the hydrogen binding capacity, what could be a possible reason for the increased stability of 11_G9.
Yang and co-workers [22] also recommended the exchange of bulky amino-acids into small ones. A comparison of the van der Waals volumes revealed that in this case a small amino-acid was exchanged by another one. A table containing different properties of amino-acids [59, 60] is shown in the appendix.

Yang and co-authors also suggested to exchange only amino-acids far away from the active site. Figure 33 and 34 illustrate that position 145 is relatively close to the active site of the protein. The measurement of the distance indicates that the minimal distance to the co-factor bound in the active site is ~8 Å. However this enzyme is rather small and as discussed above, position 145 is on the surface of the protein.

A further explanation why the exchange A145T could lead to increased stability was given by Arnold [1]. She proposed increased van der Waals forces could result in tight packing, which may result in increased stability. A summary of "van der Waals distances for interatomic contacts" [61] is shown in the appendix. In Figure 35 possible interactions from Thr-145 are shown.

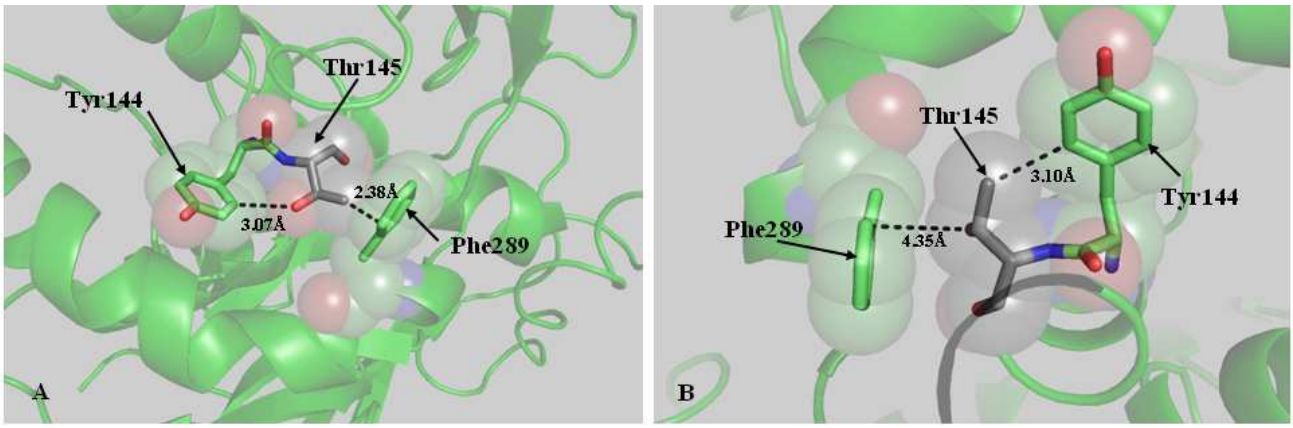

Figure 35: Possible spherical interactions of Thr-145: A before the rotation of the side chain; B after the rotation of the side chain. Distances are represented as black dashed lines.

Figure 35A reveals that the sphere of Thr-145 interacts with the spheres of Tyr-144 and Phe-289. The measured distances of 3.07 Å and 2.38 Å lie in the recommended range of van der Waal distances proposed from Ramachandran and Sasisekharan [61].

After the rotation of the side chain (Figure 35B) the distances between the amino-acid residues increased and the spherical contacts are reduced. The rotation of the side chain by 180° resulted in a complete loss of spherical interactions of Thr-145 and Phe-289.

In summary, it can be stated that the rotation of the side chain reduces the interactions of the spheres but on the other hand only in this conformation the formation of a further hydrogen bond (see Figure 34) is possible. The exact reasons for the stabilising effects of this mutation still remain unclear.

5.4.2 Mutant 13_G6 (Y72F)

In 13_G6 the tyrosine at position 72 is replaced by a phenylalanine. The mutation of a Tyr to a Phe can be described as the exchange of a neutral polar to a neutral non-polar amino acid.

5.4.2.1 Localisation

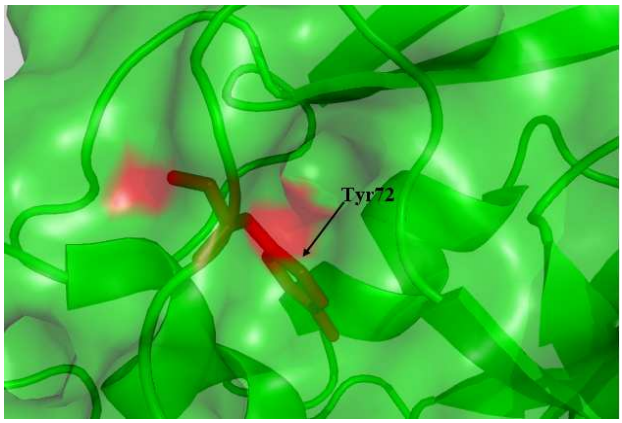

Figure 36: Localisation of Tyr-72: Tyr-72 is shown in red; the rest of the protein is presented in green. Beside the protein structure also the surface is shown

In Figure 36 the surface of the protein is shown as green shell, with two red spots, that confirm that Tyr-72 participates in the surface formation and therefore is exposed to the solvent.

5.4.2.2 Interactions of residue 72 before and after the mutation

Details about the interactions of the amino-acid residues present at position 72 are present in Figure 37.

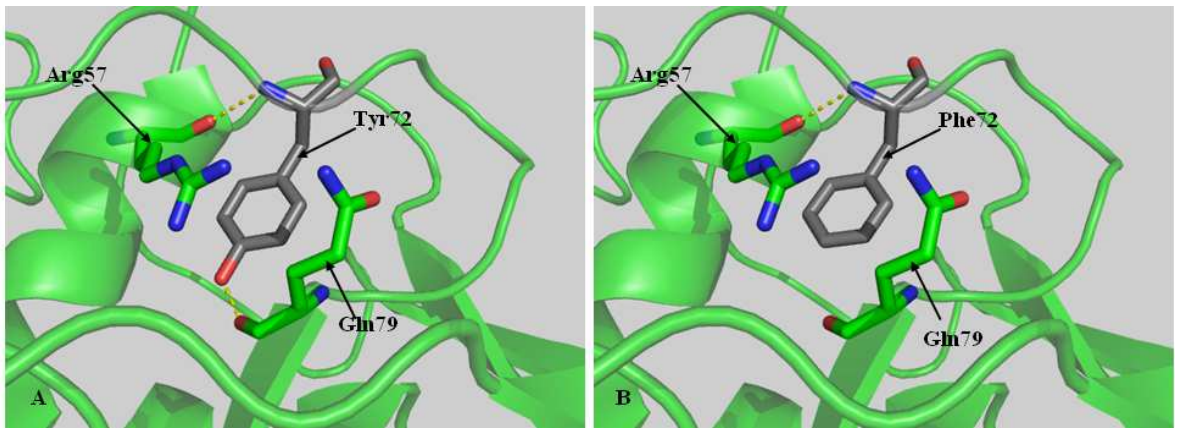

Figure 37: Interactions of amino-acid residue 72: A wild type residue; B mutated residue; hydrogen bonds are indicated as dashed yellow lines

In Figure 37 the interactions of the amino-acid at position 72 are shown. The wild type has a Tyr residue at this position; this Tyr residue is linked to Arg-57 and Gln-79. The hydrogen bond between the Tyr and Arg-57 is formed by their main chains and remains unchanged after the mutation. The linkage to Gln-79 is formed from the hydroxyl group of the Tyr side chain.
The mutation of the Tyr to a Phe results in the loss of the OH-group of the side-chain, so that the formation of a hydrogen bond to Gln-79 is not possible any more.

5.4.2.3 Comparison of the obtained results with the design rules for enzyme stabilisation in organic solvents

The recommended exchange of charged residues [1, 22] into neutral ones was not found here. Both residues present at position 72 are uncharged and have about the same size. The fact that these residues have similar van der Waals volumes contradicts the suggestion of Yang and co-workers [22], who purposed to exchange bulky into small amino-acids.
Arnold [1] and Yang and co-authors [22] proposed to remove unfulfilled hydrogen binding sides. As shown in Figure 37 the Tyr present in the wild type can form two hydrogen bonds, while the introduced Phe residue can not from a hydrogen bond with the side-chain. This observation stands in complete contrast to the suggestions of Arnold [1] and Yang and co-workers [22].
The analysis of position 72 regarding possible van der Waals contacts indicated that there are no residues within the recommended binding distance [61]. A rotation of the side chain by 180° does not alter that observation because both side chains are rotationally symmetric.

5.4.3 Conclusion

For both mutations the reason for their positive effect on the stability of P1 could not be determined exactly. However interactions with the solvent including H_2O was not taken in consideration in more detail. In the case of A145T, the formation of a further hydrogen bond seems to be the most promising effect for increased stability. Exactly the opposite phenomenon was found in Y72P, the exchange of the Tyr to a Phe residue results in the loss of one hydrogen-bond.

Also the exchange of charged to uncharged or bulky to small amino acids was not obtained. A further reason for the increased stability might be increased van der Waals forces, this kind of interaction was hard to determine. Van der Waals forces were demonstrated in form of atomic spheres and distance measurements. A prediction about their effects on the stability of P1 remained unclear. A comparison of hydrophobic interactions within the protein structure before and after the mutagenesis could be interesting as well. But therefore well calculated homology models would be required.

A reason, which restricted precise models and the prediction of the effects based on the obtained mutations, was the resolution of the crystal-structure. A resolution of 2.8 Å does not exclude that some side chains are represented by the wrong rotamer.

5.5 Characterisation of the combined mutant 3_L6/10_K5/stab

3_L6/10_K5 is a mutant, which shows increased activity in terms of the conversion of the business relevant substrate synthon xy into the corresponding saturated aldehyde. More details about the enzyme 3_L6/10_K5 and the substrate synthon xy can be found in the dissertation of Brigitte Höller [52].

For all experiments performed with 3_L6/10_K5, synthon xy was used as substrate. The final concentration of synthon xy in the assay was 0.5mM.

The mutant 3_L6/10_K5/stab (combination of 3_L6/10_K5 with the stability improved mutant 11_G9/13_G6) was only characterised in single phase systems, the incubation parameters are summarized in Table 12.

The obtained results are shown in Figure 38 and 39.

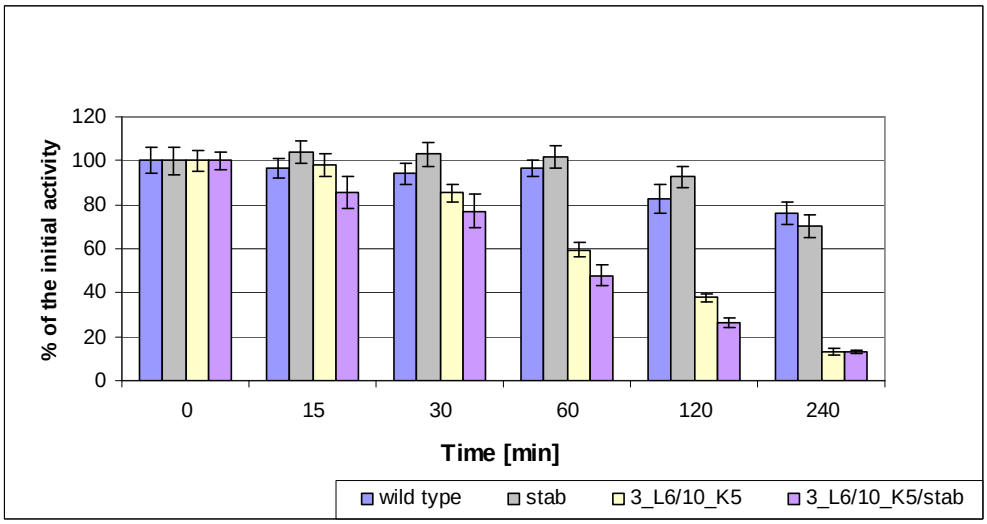

Figure 38: Stability check of 3_L6/10_K5/stab with 5% DMF as organic co-solvent: The average activities of the wild type (blue), stab (grey), 3_L6/10_K5 (yellow) and 3_L6/10_K5/satb (purple) are shown. The standard deviation of the samples is indicated by error bars.

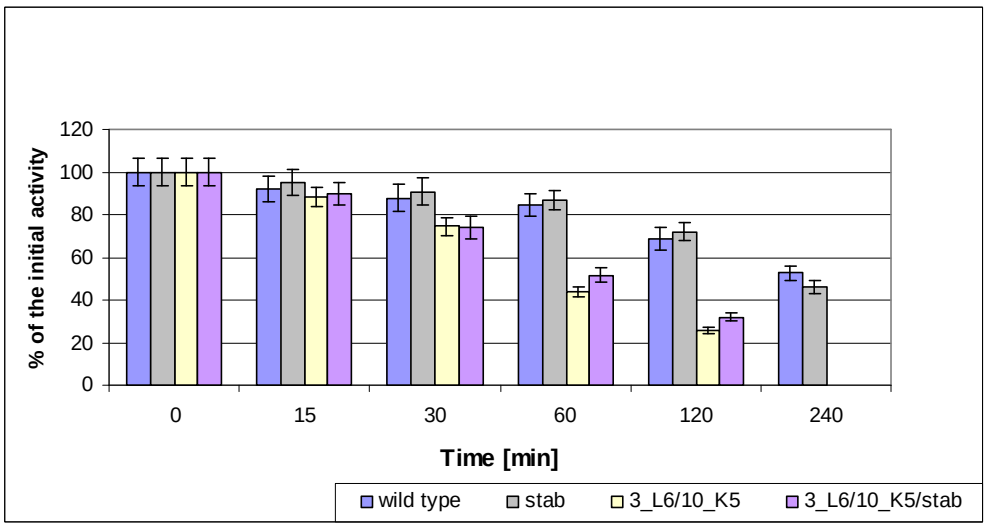

Figure 39: Stability check of 3_L6/10_K5/stab with 5% NMP as organic co-solvent: The average activities of the wild type (blue), stab (grey), 3_L6/10_K5 (yellow) and 3_L6/10_K5/satb (purple) are shown. The standard deviation of the samples is indicated by error bars.

Figure 38 and 39 reveal that the combination 3_L6/10_K5/stab is not as stable as the wild type. In the measurements with DMF, the obtained activity after 240 min of incubation was about one sixth of the residual activity of the wild type. Also the mutant stab (11_G9/13_G6) could not confirm the results obtained with α-MCA. However the stability of this enzyme variant was in the range of the wild type.

When NMP was used as co-solvent, no residual activity for 3_L6/10_K5 and 3_L6/10_K5/stab was measured after 240 min.

The integration of the mutations responsible for the improved stability into 3_L6/10_K5 could enhance the stability in 5% NMP (see Figure 39). Nevertheless the integrated mutations were not able to compensate the stability decrease caused by the mutations responsible for activity enhancement.

A reason for the different results might be the different substrates, because the substrate synthon xy contains further side-chains. The presence of these side chains could result in a particular unfolding of the enzyme, which could further result in a decrease of the stability.

Another reason for the decreased stability could be the required amount of enzyme solution used for the measurements. The needed volume of 3_L6/10_K5 and 3_L6/10_K5/stab was about one third of the required amount of the wild type and stab. Different amounts of enzyme solution implied that different protein concentration were present in the samples. A higher protein concentration could further stabilize the enzyme by increased protein-protein interactions.

Beside 3_L6/10_K5 also other activity improved variants of P1 (4_C2, 2_O17 and 2_O7 SSMB) were tested for their stability in aqueous solutions (5% DMF or 5% NMP). Details about the tested mutants can be found in the dissertation of Brigitte Höller [52].

Due to the fact that stability enhancement was only partially transferable, no further combination of activity improved mutants and the stability improved variant of P1 was done. The results of the activity improved variants regarding there stability in the presence of organic co-solvents are shown in Figure 40 and 41.

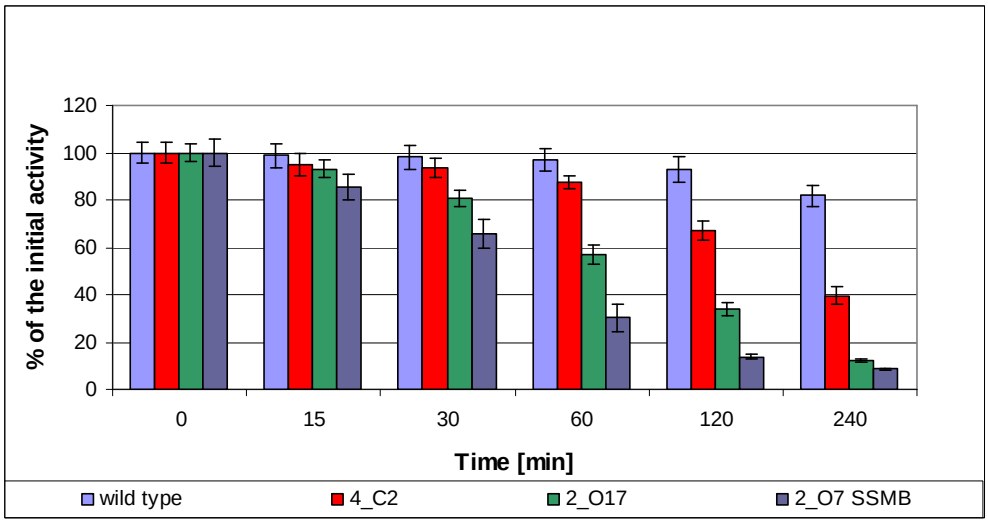

Figure 40: Stability analysis in an aqueous solution containing 5% DMF: the standard deviation is indicated by error bars.

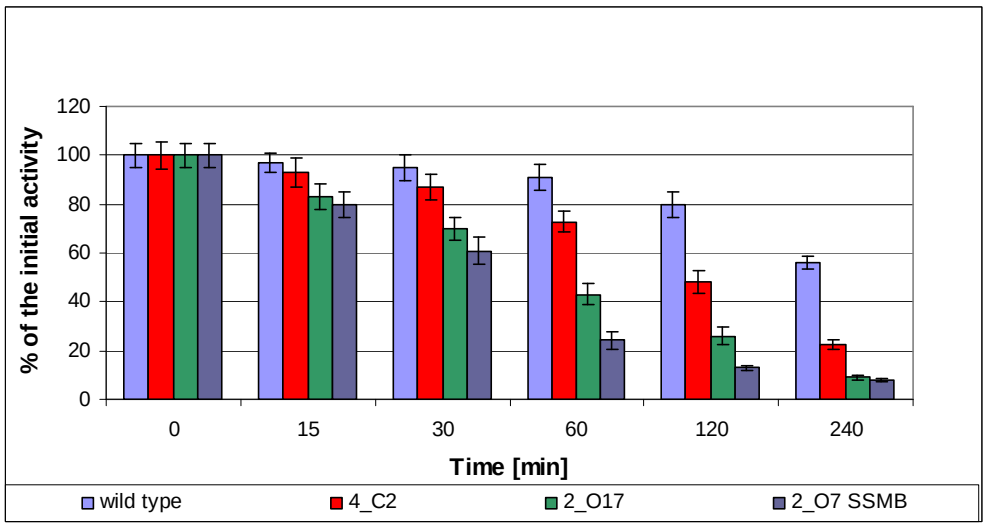

Figure 41: Stability analysis in an aqueous solution containing 5% NMP: the standard deviation is indicated by error bars.

6 Conclusion

6.1 Assay development

Experiments revealed that P1 is quite unstable in the presence of organic co-solvents. Experiments for the determination of the C_{50} showed that the activity of P1 decreases with increasing solvent concentration. The calculated C_{50} value was about 5% for both used solvents (DMF and NMP). Therefore the co-solvent concentration for single-phase assays was set to 5%. The determination of the incubation time revealed that the enzyme could be incubated for 4 hours in the presence of 5% of organic solvent. After the incubation the remaining activity was always higher for samples containing DMF.

Also experiments with two-phase systems were made. The presence of 10% toluene and 2% of DMF or NMP decreases the maximum incubation time from 4 hours to 15 min. A reason therefore might be the denaturation of the enzyme cased via contact with the liquid-liquid interphase.

The established screening assay was a two-phase system containing 10% toluene and 2% DMF. For the screening the plates were shaken with 450 rpm for 15 min.

Screening revealed that the assay was not reliable enough to find mutants with slightly improved stability. The reason therefore was the high standard deviation of the wild type.

A further problem of the screening assay might be the low shaking rate (450 rpm), which did not result in well mixed samples.

To improve the screening assay it might be better to measure each probe twice (with and without incubation) and use the obtained data to calculate the stability.

6.2 Screening

Screening revealed that the assay was not as reliable as required. Therefore each sample was measured twice (without incubation and after 15 min) during the two rescreening rounds. Two mutants with increased stability were found and further characterised in single-phase systems. Characterisation in 5% DMF/NMP revealed that the mutant 11_G9 was ~1.1-fold improved in DMF and ~1.3-fold improved in NMP after 120min of incubation. The second mutant found during screening 13_G6 was ~1.4-fold improved in DMF and ~1.5-fold improved in NMP after the same time of incubation.

The beneficial mutants were combined and the obtained mutant was characterised. The combined mutant 11_G9/13_G6 revealed a ~1.4-fold improvement in DMF and ~1.7-fold improvement of the stability in NMP. These results indicate that the improvement was additive in case of NMP, in the case of DMF the obtained improvement was about the same as in mutant 13_G6.

The characterisation of the contained mutations indicated that the reasons for the increased stability were difficult to find. In the case of A145T the reason for the improved stability could be the formation of a further hydrogen bond. For the amino acid exchange Y72F no plausible reason for the enhanced stability could be found.

Mutations responsible for improved stability were further combined with an activity improved variant of P1. The characterisation of this mutant was done with a business relevant substrate and revealed that stability was only partially transferable. A reason therefore might be the difference of the used substrates and destabilising effects cased by the mutations.

For further stability improvements additional error-prone libraries have to be created and screened. To improve the stability of P1 regarding the conversion of the industrial substrate, it would have been better to use the business relevant substrate itself for the screening. The reason therefore is that the screening conditions should mimic the specific conditions of the final process as closely as possible, because “you get what you screen for” [62]. However, at the time when this study started, this substrate was not yet available.

7 References

7.1 Literature

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7.2 Lab-book

441/GMA/1/BER

8 Figures

9 Tables

10 Formulas

11 Appendix

11.1 Determination of C_{50}

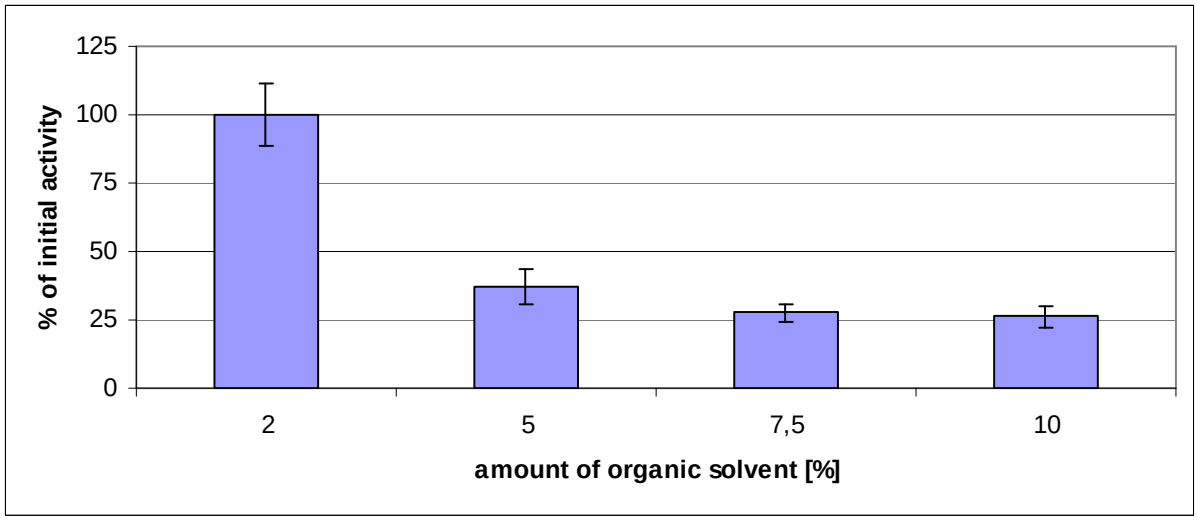

Figure 42: Determination of the C_{50} in DMF: the obtained activity with 2% co-solvent is set to 100% and all other values are related to this worth. The standard deviation is indicated by error bars.

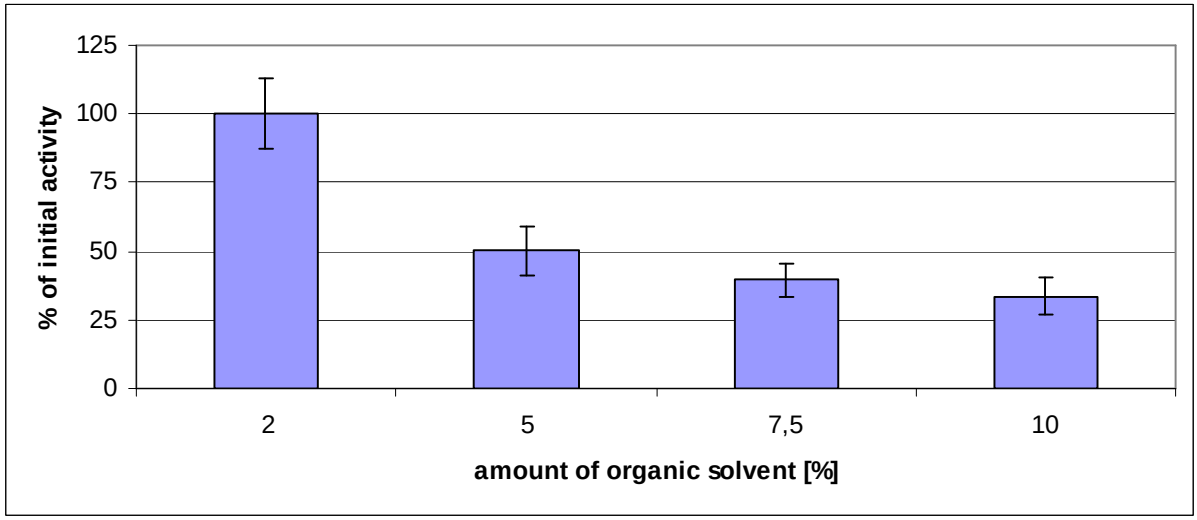

Figure 43: Determination of the C_{50} in NMP: the obtained activity with 2% co-solvent is set to 100% and all other values are related to this worth. The standard deviation is indicated by error bars.

11.2 Characterisation of the combined mutant 11_G9/13_G6

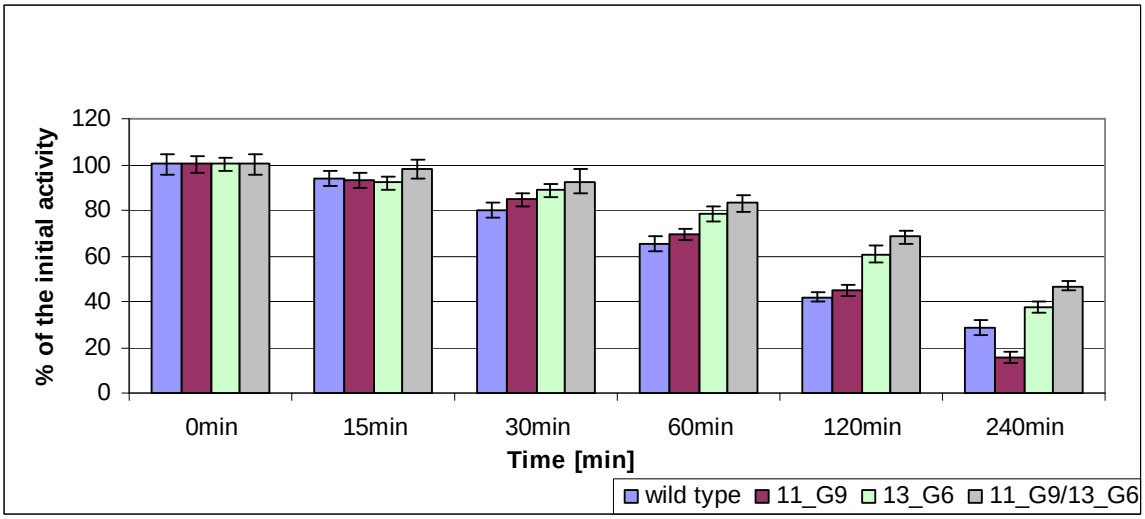

Figure 44: Characterisation of 11_G9/13_G6 with 5% DMF as organic co-solvent: The average activities of the wild type (blue), 11_G9 (violet), 13_G6 (bright green) and their combination 11_G9/13_G6 (grey) are shown. The standard deviation is indicated by error bars.

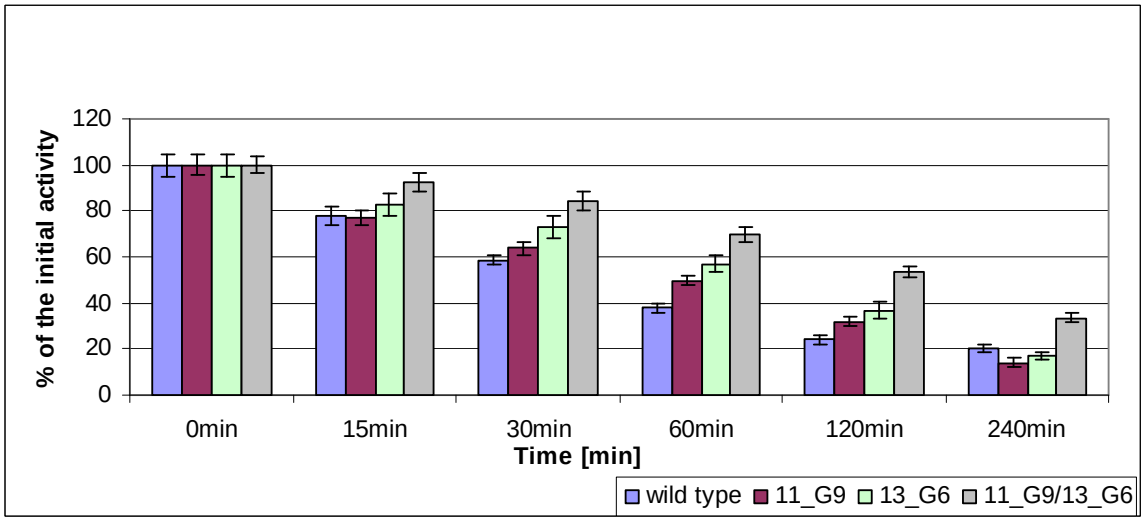

Figure 45: Characterisation of 11_G9/13_G6 with NMP as organic co-solvent: The average activities of the wild type (blue), 11_G9 (violet), 13_G6 (bright green) and their combination 11_G9/13_G6 (grey) are shown. The standard deviation is indicated by error bars.

11.3 Strain deposited in the culture collection

Before the strain was deposited in the culture collection it was sequenced.

Table 16: Strain deposited in the culture collection

Culture collection number	Designation	Plasmid	Host strain	Resistance marker
3094	11_G9/13_G6	pMS470 11_G9/13_G6	*E. coli* Top10F'	Ampicillin

The Vector NTI file of 11_G9/13_G6 was named pMS470 11_G9_13_G6. This file is part of the CD.

11.4 DNA sequences

11.4.1 P1syn

atgactgcgaccaacaagcaggtgatcctgaaagattatgtatctggcttccctaccgaatctgatttcgattttaccaccactacggtagaactgcgcgtgccggaag
gcactaactccgttctggtgaagaacctgtacctgtcttgtgatccgtatatgcgtatccgtatgggcaagccggacccgtctaccgcagcgctggcgcaggcttata
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11.4.2 11_G9

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11.4.3 13_G6

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ctggttggtctgttccatggcaagaatgtgggcaaacaagtcgtagttgtcgcgcgtgaataa

11.4.4 11_G9/13_G6 (stab)

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11.4.5 3_L6/10_K5/stab

atgactgcgaccaacaagcaggtgatcctgaaagattatgtatctggcttccctaccgaatctgatttcgattttaccaccactacggtagaactgcgcgtgccggaag
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11.5 Protein sequences

11.5.1 P1syn

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SNIIYKRIRIQGFVVSDFYDKYSKFLEFVLPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVV
VARE

11.5.2 11_G9

MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGKPDPSTAALA
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AFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDAVLVNMNMHGRIAVCGMISQYNLENQEGVHNL
SNIIYKRIRIQGFVVSDFYDKYSKFLEFVLPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVV
VARE

11.5.3 13_G6

MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGKPDPSTAALA
QAFTPGQPIQGYGVSRIIESGHPDYKKGDLLWGIVAWEEYSVITPMTHAHFKIQHTDVPLSYYTGLLGM
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SNIIYKRIRIQGFVVSDFYDKYSKFLEFVLPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVV
VARE

11.5.4 11_G9/13_G6

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AFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDAVLVNMNMHGRIAVCGMISQYNLENQEGVHNL
SNIIYKRIRIQGFVVSDFYDKYSKFLEFVLPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVV
VARE

11.5.5 3_L6/10_K5/stab

MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRICMGKPDPSTAALA
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PGMTAYTGFYEVCSPKEGETVYVSAASGAVGQLVGQLAKMMGCYVVGSAGSKEKVDLLKTKFGFDD
AFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDAVLVNMNVHGRIAVCGMVSQYNLENQEGVHNL
SNIIYKRIRIQGFVVSDFYDKYSKFLEFVLPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVV
VARE

11.5.6 Alignment of the protein sequences

```
P1syn          MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGK     60
13_G6          MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGK     60
11_G9          MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGK     60
11_G9/13_G6    MTATNKQVILKDYVSGFPTESDFDFTTTTVELRVPEGTNSVLVKNLYLSCDPYMRIRMGK     60

P1syn          PDPSTAALAQAYTPGQPIQGYGVSRIIESGHPDYKKGDLLWGIVAWEEYSVITPMTHAHF    120
13_G6          PDPSTAALAQAFTPGQPIQGYGVSRIIESGHPDYKKGDLLWGIVAWEEYSVITPMTHAHF    120
11_G9          PDPSTAALAQAYTPGQPIQGYGVSRIIESGHPDYKKGDLLWGIVAWEEYSVITPMTHAHF    120
11_G9/13_G6    PDPSTAALAQAFTPGQPIQGYGVSRIIESGHPDYKKGDLLWGIVAWEEYSVITPMTHAHF    120

P1syn          KIQHTDVPLSYYTGLLGMPGMTAYAGFYEVCSPKEGETVYVSAASGAVGQLVGQLAKMMG    180
13_G6          KIQHTDVPLSYYTGLLGMPGMTAYAGFYEVCSPKEGETVYVSAASGAVGQLVGQLAKMMG    180
11_G9          KIQHTDVPLSYYTGLLGMPGMTAYTGFYEVCSPKEGETVYVSAASGAVGQLVGQLAKMMG    180
11_G9/13_G6    KIQHTDVPLSYYTGLLGMPGMTAYTGFYEVCSPKEGETVYVSAASGAVGQLVGQLAKMMG    180

P1syn          CYVVGSAGSKEKVDLLKTKFGFDDAFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDA    240
13_G6          CYVVGSAGSKEKVDLLKTKFGFDDAFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDA    240
11_G9          CYVVGSAGSKEKVDLLKTKFGFDDAFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDA    240
11_G9/13_G6    CYVVGSAGSKEKVDLLKTKFGFDDAFNYKEESDLTAALKRCFPNGIDIYFENVGGKMLDA    240

P1syn          VLVNMNMHGRIAVCGMISQYNLENQEGVHNLSNIIYKRIRIQGFVVSDFYDKYSKFLEFV    300
13_G6          VLVNMNMHGRIAVCGMISQYNLENQEGVHNLSNIIYKRIRIQGFVVSDFYDKYSKFLEFV    300
11_G9          VLVNMNMHGRIAVCGMISQYNLENQEGVHNLSNIIYKRIRIQGFVVSDFYDKYSKFLEFV    300
11_G9/13_G6    VLVNMNMHGRIAVCGMISQYNLENQEGVHNLSNIIYKRIRIQGFVVSDFYDKYSKFLEFV    300

P1syn          LPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVVVARE                   345
13_G6          LPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVVVARE                   345
11_G9          LPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVVVARE                   345
11_G9/13_G6    LPHIREGKITYVEDVADGLEKAPEALVGLFHGKNVGKQVVVVARE                   345
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11.6 Tables

TABLE 7-1. VAN DER WAALS DISTANCES FOR INTERATOMIC CONTACTS

Contact Type	Normally Allowed (Å)	Outer Limit (Å)
H · · · H	2.0	1.9
H · · · O	2.4	2.2
H · · · N	2.4	2.2
H · · · C	2.4	2.2
O · · · O	2.7	2.6
O · · · N	2.7	2.6
O · · · C	2.8	2.7
N · · · N	2.7	2.6
N · · · C	2.9	2.8
C · · · C	3.0	2.9
C · · · CH_2	3.2	3.0
CH_2 · · · CH_2	3.2	3.0

Figure 46: Van der Waals distances for interatomic contacts [61]

Properties of amino acids

Amino acid residue	pK_a of ionizing side chain[a]	Average residue mass[b] (daltons)	Monoisotopic mass (daltons)[b]	Occurrence in proteins[c] (%)	Percent buried residues[d] (%)	V_i[e] (Å³)	van der Waals volume[f] (Å³)	Accessible surface area[g] (Å²)	Ranking of amino acid polarities[h]
Alanine	–	71.0788	71.03711	7.5	38 (12)	92	67	67	9 (7)
Arginine	12.5 (>12)	156.1876	156.10111	5.2	0	225	148	196	15 (19)
Asparagine	–	114.1039	114.04293	4.6	10 (2)	135	96	113	16 (16)
Aspartic acid	3.9 (4.4–4.6)	115.0886	115.02694	5.2	14.5 (3)	125	91	106	19 (18)
Cysteine	8.3 (8.5–8.8)	103.1448	103.00919	1.8	47 (3)	106	86	104	7 (8)
Glutamine	–	128.1308	128.05858	4.1	6.3 (2.2)	161	114	144	17 (14)
Glutamic acid	4.3 (4.4–4.6)	129.1155	129.04259	6.3	20 (2)	155	109	138	18 (17)
Glycine	–	57.0520	57.02146	7.1	37 (10)	66	48		11 (9)
Histidine	6.0 (6.5–7.0)	137.1412	137.05891	2.2	19 (1.2)	167	118	151	10 (13)
Isoleucine	–	113.1595	113.08406	5.5	65 (12)	169	124	140	1 (2)
Leucine	–	113.1595	113.08406	9.1	41 (10)	168	124	137	3 (1)
Lysine	10.8 (10.0–10.2)	128.1742	128.09496	5.8	4.2 (0.1)	171	135	167	20 (15)
Methionine	–	131.1986	131.04049	2.8	50 (2)	171	124	160	5 (5)
Phenylalanine	–	147.1766	147.06841	3.9	48 (5)	203	135	175	2 (4)
Proline	–	97.1167	97.05276	5.1	24 (3)	129	90	105	13 (–)
Serine	–	87.0782	87.03203	7.4	24 (8)	99	73	80	14 (12)
Threonine	–	101.1051	101.04768	6.0	25 (5.5)	122	93	102	12 (11)
Tryptophan	–	186.2133	186.07931	1.3	23 (1.5)	240	163	217	6 (6)
Tyrosine	10.9 (9.6–10.0)	163.1760	163.06333	3.3	13 (2.2)	203	141	187	8 (10)
Valine	–	99.1326	99.06841	6.5	56 (15)	142	105	117	4 (3)

Figure 47: Properties of amino acids [59, 60]

11.7 Chemicals

Table 17: Used chemicals

Chemicals	Producer
α-methyl-trans-cinnamaldehyde	SAFC /Sigma-Aldrich Handels GmbH, Vienna, Austria
Ampicillin	Sigma-Aldrich Handels GmbH, Vienna, Austria
Aqua bidest. "Fresenius"	Fresenius Kabi Austria GmbH., Graz, Austria
Bacto™ Peptone	Becton Dickinson and Company, Sparks, MD, USA
Bacto™ Yeast Exctract	Becton Dickinson and Company, Sparks, MD, USA
DMF	Karl Roth GmbH & Co., Karlsruhe, Germany
DNAseI	Sigma-Aldrich Handels GmbH, Vienna, Austria
Glycerol	Karl Roth GmbH & Co., Karlsruhe, Germany
IPTG	Karl Roth GmbH & Co., Karlsruhe, Germany
K_2HPO	Karl Roth GmbH & Co., Karlsruhe, Germany
KH_2PO_4	Karl Roth GmbH & Co., Karlsruhe, Germany
LB-agar (Lennox)	Karl Roth GmbH & Co., Karlsruhe, Germany
LB-medium (Lennox)	Karl Roth GmbH & Co., Karlsruhe, Germany
Lysozyme	Karl Roth GmbH & Co., Karlsruhe, Germany
MES	Karl Roth GmbH & Co., Karlsruhe, Germany
$MgCl_2$	Karl Roth GmbH & Co., Karlsruhe, Germany
NaCl	Karl Roth GmbH & Co., Karlsruhe, Germany
NADPH	Codexis, Inc, Redwood City, USA
NaOH	Karl Roth GmbH & Co., Karlsruhe, Germany
NMP	Karl Roth GmbH & Co., Karlsruhe, Germany
Toluene	Karl Roth GmbH & Co., Karlsruhe, Germany
Trypton/Pepton aus Casein	Karl Roth GmbH & Co., Karlsruhe, Germany
Tween20	Sigma-Aldrich Handels GmbH, Vienna, Austria
Yeast Extract	Karl Roth GmbH & Co., Karlsruhe, Germany

11.8 CD-Content

The CD contains all data and calculations done during this diploma thesis. Beside the diploma thesis also the precedent lap course is present on the CD.

Table 18: CD-content

CD -Content	
Lab course	Data
	Analysis
	Sequencing
	Presentations
	Conclusion
Diploma thesis	Data
	Analysis
	Sequencing
	Protein gels
	Culture collection
	Vector NTI files of the created mutants
	Presentations
	References
	Diploma thesis