*UNIVERSITY OF GRONINGEN*

# *Enhancing the Thermostability of PTDH-CHMO from Rhodococcus sp. HI-31*

## Computationally Designed Libraries Using the FRESCO Method

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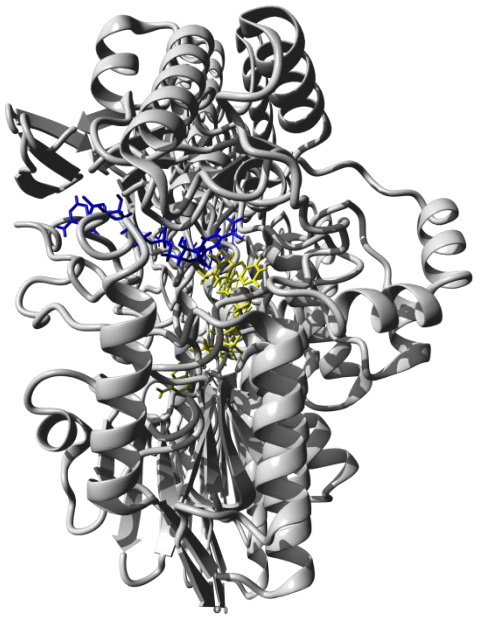

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## Abstract

Cyclohexanone monooxygenase from *Rhodococcus* sp. HI-31 ($CHMO_{Rm}$) is an industrially interesting Baeyer-Villiger monooxygenase which converts ketones to their corresponding esters or lactones. A disadvantage of $CHMO_{Rm}$ is its relatively low thermostability with an apparent melting temperature of 36 °C. For industrial applications it is necessary to increase this $Tm^{app}$. Here the $CHMO_{Rm}$, fused to a phosphite dehydrogenase (PTDH), is engineered using the FRESCO method in order to increase the $Tm^{app}$. 13 mutants had an increase of $\Delta Tm^{app} \geq 1.0$ °C from which a fourfold mutant was created with a $\Delta Tm^{app}$ of 7 °C giving a $Tm^{app}$ of 43 °C.

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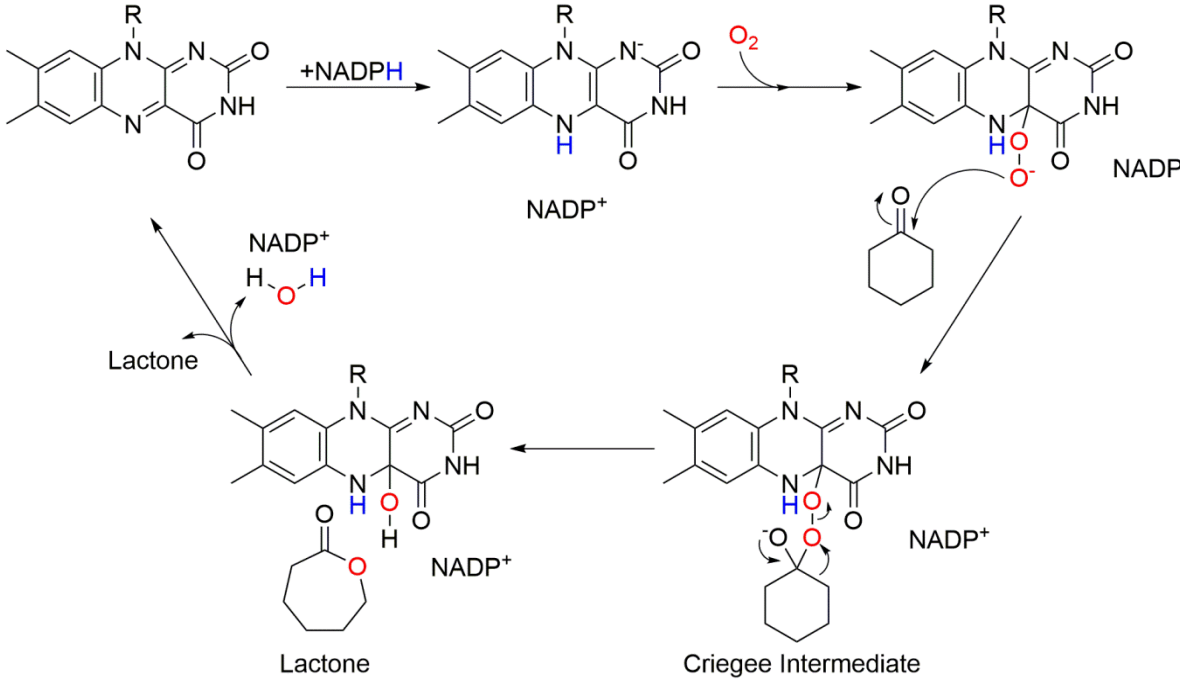
# 1. Introduction

Enzymes are interesting biocatalysts because of their catalytic efficiency and selectivity making them an environmental friendly alternative to metallo- and organocatalysis. A challenge of industrial applications of enzymes is their limited stability during, which was initially overcome by immobilizing the enzyme[1].

Here, the cyclohexanone monooxygenase from *Rhodococcus* sp. HI-31 ($CHMO_{Rm}$) was engineered using the FRESCO method in order to increase the apparent melting temperature ($Tm^{app}$) of 36 °C. Site-directed mutagenesis, expression, purification and determination of the $Tm^{app}$ were performed in high throughput fashion. A selection of stabilizing point mutations were combined to give the fourfold mutant Q409P/N431Y/A115V/A455V. The combined mutant has a $Tm^{app}$ of 43 °C, an increase of 7 °C.

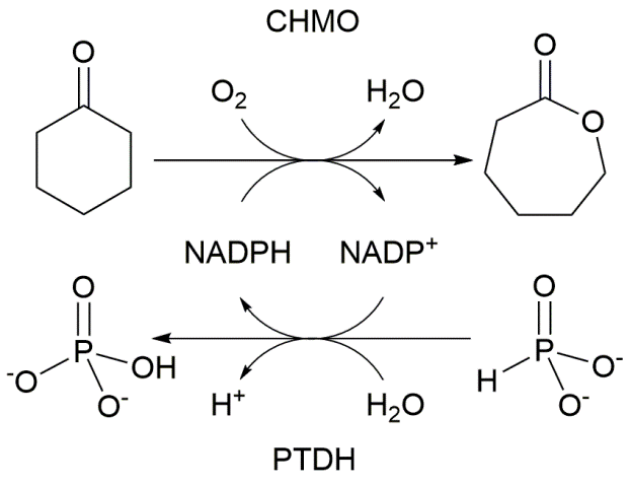
## 1.1. Cyclohexanone Monooxygenase

The Baeyer-Villiger reaction is an industrially important synthetic reaction as it can be used to insert oxygen functionalities into organic compounds thereby yielding –value added compounds useful for the polymer industry as well as the pharmaceutical industry. It was first described by Adolf Baeyer and Victor Villiger in 1899 where menthone was oxidized to the corresponding ε-lactone using Caro's acid (peroxymonosulfuric acid)[2]. The chemical synthesis route uses harsh chemicals such as organic peracids and peroxides which are expensive, explosive and shock sensitive, they are converted to their corresponding carboxylate salts as a waste product[3,4]. Even though the reaction has improved in that more stable organic peroxy acids can be used as oxidants, there is still a need to improve the reaction conditions further. Baeyer-Villiger monooxygenases (BVMOs) such as cyclohexanone monooxygenase (CHMO) (EC 1.14.13.22) can be interesting enzymes to obtain the desired oxidized products via a greener and safer way. It does however require the enzyme to be stable under industrial conditions such as higher temperatures and in organic (co)solvents.




**Scheme 1 Reaction mechanism of a CHMO. Figure adapted from Mirza, I. A. *et al.***

CHMO catalyzes the conversion of cyclohexanone to ε-caprolactone, a precursor for the polymer industry. In the reaction mechanism (Scheme 1) of CHMO, the FAD (flavin adenine dinucleotide) is reduced by NADPH (dihydronicotinamide adenine dinucleotide phosphate) after which the reduced FAD reacts with molecular oxygen to form the C4A-peroxyflavin intermediate. This peroxyflavin acts the same as peracids in the synthetic Baeyer-Villiger reaction. It performs a nucleophilic attack on the carbonyl carbon of a ketone e.g. cyclohexanone, forming the Criegee intermediate. The intermediate rearranges resulting in the ester or lactone e.g. ε-caprolactone and the C4A-hydroxyflavin. In the last stage, water is eliminated from the hydroxyflavin to give the oxidized FAD and $NADP^+$ is released[4,5] thereby completing the cycle.




**Scheme 2 Reaction of CHMO with cyclohexanone to form ε-caprolactone. NADPH is regenerated by phosphite dehydrogenase by oxidizing phosphite to phosphate.**

The CHMO from *Rhodococcus* sp. HI-31 ($CHMO_{Rm}$) is an NADPH dependent flavoprotein which efficiently converts ketones such as cyclohexanone to their corresponding ester or lactones. As NADPH is an expensive cofactor, it is desired to regenerate $NADP^+$ to NADPH. Known examples of enzymes that regenerate NADPH are formate dehydrogenase (FDH), alcohol dehydrogenase (ADH) and phosphite dehydrogenase (PTDH). PTDH has the advantage that the oxidation of phosphite is a cheap sacrificial substrate and the reaction to phosphate is essentially an irreversible process[6]. These enzymes can be added to the reaction mixture to regenerate the reduced cofactor[7] or the PTDH protein can be fused to CHMO to obtain a self-sufficient BVMO[8] (Scheme 2). This makes the protein more attractive for biocatalytic processes, a disadvantage however is that CHMO has a relatively low apparent melting temperature of 36 °C meaning that the thermostability has to be increased.

## 1.2. Strategies For Engineering the Thermostability of Proteins

To achieve prominent increases in thermostability, multiple amino acid substitutions have to be made and combined in a multiple mutant. It has been shown that often the increase in thermostability of single mutations are nearly additive[9]. In order to improve the thermostability of proteins, several approaches are available. The chosen strategy is dependent on the available information of the protein and can be divided in rational and random approaches where the rational approaches can be divided into two groups: **1** amino acid sequence comparison between protein of interest and a homologous, more thermostable protein and **2** Inspection of the 3D

structure of the protein and identifying promising mutations applying concepts of engineering thermostability[10].

Directed evolution is a powerful random approach to generate more thermostable proteins, especially when structural information is absent. Initially, this technique involved iterative cycles of random amino acid changes, resulting in a library of variants which were screened on for example improved enzyme stability. Examples of directed evolution are error prone PCR, degenerate primers and gene shuffling[1]. Directed evolution is notoriously laborious and requires a method for high-throughput screening[11].

Rational approaches can be used when structural information of the protein is available. It is suggested that irreversible thermal denaturation is of a local/partial character as opposed to a global character. By identifying and improving the stability of early unfolding regions, the global stability of the protein also increases[12].

A semirational approach for increasing thermostability can also be used. This consensus approach uses homologous proteins and is based on that even amino acid sequences of homologues mesophilic proteins can be used for improving thermostability. A consensus amino acid sequence is calculated from the homologues based on the hypothesis that amino acids that can be found at a given position of a set of structurally similar proteins (consensus amino acids), have a larger contribution than non-consensus amino acids on the protein stability[9,10,13].

### 1.2.1. FRESCO Method for Generating Stabilizing Mutations

When the crystal structure of a protein is available, the FRESCO[14] (Framework for Rapid Enzyme Engineering by Computational Libraries) protocol (Figure 1) can be used to predict a large number of stabilizing mutations. FRESCO runs three software packages in order to find stabilizing mutations which can be point mutations (using FoldX and Rosettaddg) and disulfide bonds. Stabilizing disulfide bonds are predicted using Dynamic Disulfide Discovery. FoldX and Rosettaddg are used to determine the relative change in folding free energy $\Delta\Delta G^{Fold}$ in the 3D structures of the mutant compared to the wild type protein structure. Mutations with a predicted $\Delta\Delta G^{Fold}$ <-5 kJ mol$^{-1}$ are selected as potentially stabilizing mutations. This cut of can also be less strict (e.g. -2.5 kJ mol$^{-1}$) in order to increase the number of stabilizing mutations. The FRESCO procedure contains in silico screening steps in which chemically unreasonable mutations and mutations which increase flexibility are eliminated thereby reducing in vitro screening[15]. The in silico screening steps are discussed in more detail in section 2.2. Using the FRESCO strategy, increases in thermostability of +35 °C can be reached[14].

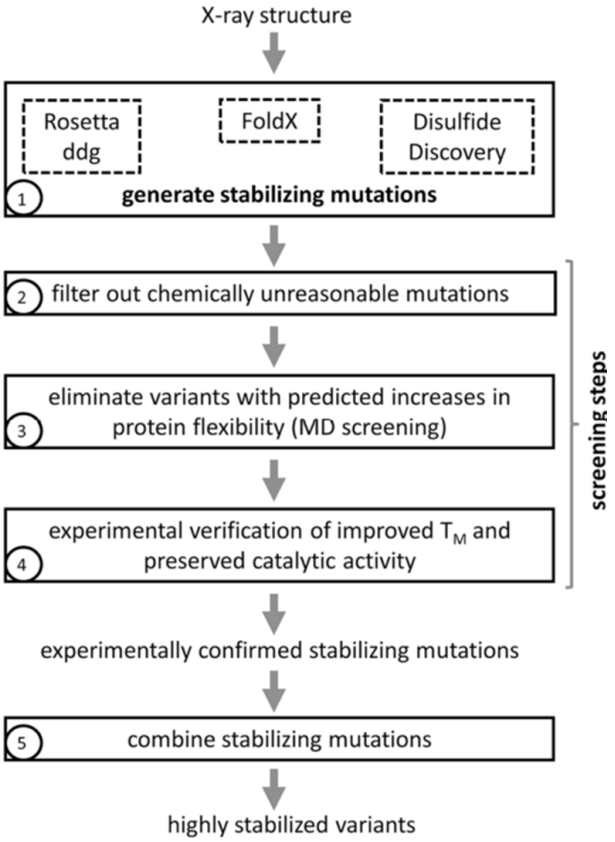



**Figure 1 Representation of the FRESCO protocol[15] showing all steps from generating mutants in step 1 to the highly stabilized variant after step 5.**

## 1.3. ThermoFAD Method for Determining the Apparent Melting Temperature

The unfolding temperature of a protein can be determined using the so called Thermofluor method[16]. This method relies on fluorescent molecular probes such as SYPRO Orange. These dyes have a low quantum yield when present in water, Upon protein unfolding, the dye has a high quantum yield when bound to the hydrophobic surface of the denature protein. FAD containing proteins have the advantage that flavins have the intrinsic property of being fluorescent which lie in the same wavelength area as for typical dyes used for Thermofluor. When a protein is properly folded, the fluorescence of the flavin is usually quenched, however, upon unfolding of an FAD containing protein, the flavin cofactor becomes exposed to the solvent and the fluorescence signal is increased. ThermoFAD measurements require protein sample volumes of only 20 µL, with a concentration ranging from 0.3 to 4.0 mg $mL^{-1}$. The measurement itself takes less than 2 hours and is performed over a temperature range starting from 15-20 °C to 90°C, while fluorescence is measured every 30 s[16].

# 2. Materials and Methods

## 2.1. General Methods

### 2.1.1. SDS-PAGE

Samples from purification experiments were analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) by 24 µL sample to 6 µL 5x SDS loading buffer. For concentrated protein samples such as purified enzyme from large volume expression cultures, 2 µL purified protein was mixed with 22 µL buffer (Tris/HCl 50 mM pH 7.5) and added to 6 µL SDS loading buffer. Samples were incubated at 95 °C for 5 min. 5 µL of PageRuler™ Prestained 5 µL Protein ladder (ThermoFischer) was added to a well of ExpressPlus™ PAGE gel 12% (GenScript). After loading 15 µL of the sample, the gel was run for 20 min at 80 V after which the potential was changed to 140 V for 1 h. Gels were stained using InstantBlue™ Protein Stain (Expedion). Gels were destained using reverse osmosis (R.O.) water.

### 2.1.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to image QuickChange PCR products. 5 µL of PCR product was added to 1 µL 6x Orange Loading Dye (ThermoFisher). 1% agarose gel was made using 1% (w/v) agarose in 1x TAE buffer. 1.5 µL Roti®-GelStain (Carl Roth) was used for gel staining. Samples were run at 90 V for 1 h after which imaging was performed using UV light.

### 2.1.3. Vector and Bacterial Strain

The thermostable 18x PTDH[8,17] protein is fused to the N-terminus of the $CHMO_{Rm}$ protein via a linker (SRSAAG). The His-tag used for purification is fused to the N-terminus of the PTDH protein. Ampicillin was used as a selection marker in the vector pCRE3-$CHMO_{Rm}$. *Escherichia coli (E. coli)* NEB® 10-beta was used for selection and protein expression. $CHMO_{Rm}$ 59.835 kDa, PTDH 36.568 kDa, fused protein including linker and His-TAG 99.078 kDa.

## 2.2. Screening of Potentially Stabilizing Point Mutations Using YASARA

The crystal structure that was used for the FRESCO protocol has the pdb accession code 4RG3 and is one of the five structures available of this protein[5].The by Rosettaddg and FoldX predicted stabilizing mutations were selected and their predicted 3D structures were run in MD simulations. Often the computationally predicted stabilizing mutations contain biophysical errors. Therefore it is necessary to perform a visual inspection of the generated mutants. First the most different MD simulation of both wild type and mutant will be made invisible as often one of the MD simulations behaves as an outlier due to different conformation sampling leading to an exaggerated difference between wild type and mutant. The inspection is performed in several steps and mutations are eliminated when:

1. Mutations lead to an unusual solvent exposure of a hydrophobic side chain, e.g. a surface exposed tryptophan or phenylalanine;
2. The mutations has an increase in unsatisfied H-bond donors/acceptors and cannot be replaced by H-bonds with a water molecule;
3. The mutation violates biophysical criteria, e.g. prolines are not allowed in a helix and methionine not on the surface;
4. The side chain behaves unusually flexible compared to similar wild type residues;

5. The backbone where the mutation is located becomes more flexible or its flanking regions;
6. The overall structure of the mutant has increased in flexibility.

By carrying out these steps, the amount of suggested stabilizing mutations will be decreased to around 25%. In this project, the same procedure was followed for determining stabilizing disulfide bonds, these were however not experimentally tested.

## 2.3. Preparation of Chemically Competent Cells

$CaCl_2$ NEB® 10-beta competent *E. coli* cells were prepared by growing a 5 mL overnight culture at 37°C from a NEB® 10-beta glycerol stock . From this culture, 1 mL was used to inoculate a main culture in a sterile 1L flask containing 200 mL lysogeny broth (LB) and shaken in an INFORS HT Multitron Shaker at 37°C, 135 rpm. Once an $Od_{600}$ between 0.2-0.4 was reached, the culture was transferred to 50 mL Greiner centrifuge tubes and cooled on ice after which they were spun down in a 5810 R Eppendorf centrifuge at 1000 x g, 20 mins, 4°C. The cells were kept on ice in all subsequent steps. The supernatant was removed and the cell pellets were resuspended in 20 mL ice cold sterile 0.1 M $CaCl_2$ and incubated on ice for 15 mins on ice. The resuspended cells were again centrifuged at 1000 x g, 20 mins, 4°C after which the pellet was resuspended in 4 mL ice cold sterile 0.1 M $CaCl_2$ and sterile ice cold glycerol was added to obtain a final glycerol concentration of 10% (V/V). Aliquots were made in sterile 96 deep well plates (50 µL/well), Eppendorf tubes (50 µL, 200 µL, 1000 µL ) and sterile BioRad 96-well PCR plates (20 µL/well). The aliquots were shock frozen in liquid nitrogen and stored at -80°C.

## 2.4. Site-directed Mutagenisis Using QuickChange PCR Method

Creation of the PTDH-$CHMO_{Rm}$ library was performed in a high throughput fashion. Forward and reverse primers were ordered at Sigma-Aldrich in a 96-well plate, 12-Channel with a concentration of 100 µM in water (Scale 0.025 µmole and DST purification). From the forward primers and reverse primers, 5 µL of each was added to a sterile microtiter plate using an electronic multichannel pipette and 240 µL of Milli-Q water was added to obtain the FRESCO I primer mix with a final concentration of 2 µM of both forward and reverse primer. The positions of the mutants in the 96 well plate is shown in Table 1.

**Table 1 Overview of the position of the desired mutants (FRESCO I) in the PCR plate and primer plates. The positions H10, H11 and H12 were left empty such that controls could be added for the PCR reaction and also for later experiments such as expression and ThermoFAD.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | D10H | D10Y | A11M | A11V | H25F | H25L | T35V | S62A | H63F | H63W | H63Y | T82W |
| **B** | E88K | E88Q | E91K | E91Q | A115I | A115L | A115V | D119A | D119K | D119P | D119R | D119S |
| **C** | D119T | D120K | D120T | E121Q | T164I | T164L | T164V | T167V | A169R | S196K | E200Q | E200T |
| **D** | E202R | T205I | T205V | T205W | P216W | I230W | D233K | E252A | E252H | E252K | E252L | E252P |
| **E** | E252R | E252T | E264Q | E264R | E264T | N266W | E271A | D274K | G278A | R280Y | A290W | E293T |
| **F** | E297Q | E309K | E312K | T316I | T316K | D333A | D333M | S334F | Y337W | E338S | E346T | A349F |
| **G** | E357K | H371I | E372T | D383H | D398K | D398N | D398R | D404K | D404N | D407Q | D407R | D407S |
| **H** | Q409P | N431M | N431Y | S440T | E442L | E446A | S449T | D450R | D450T | | | |

**Table 2 Overview of the mutants of the FRESCO II primer mix plate.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | T451M | Y454W | A455I | A455V | E475A | E475K | E475Q | T487K | D490N | R516Q | S534K | S534R |
| **B** | D98K | D129A | T205F | E239A | E263A | E357H | E357R | E446K | E468K | | | |

Template DNA was prepared by making 8 minipreps of wild type (WT) PTDH-CHMO$_{Rm}$, containing the ampicillin resistance gene, using a QIAprep Spin Miniprep Kit according to the supplied protocol. The obtained plasmid stock concentrations are given in Table 3. For the QuickChange PCR reaction, tube 3 was taken and a stock of 1 ng/µL was made by taking 25 µL of the main stock and adding 1245 µL sterile Milli-Q water. Of the 1 ng/µL stock, 5 µL was added to each well of an iQ ™ 96-Well PCR plate using an electronic Sartorius multidispensing pipette. 10 µL of *Pfu*Ultra II Hot Start master mix 2x was added to each well followed by 5 µL of the FRESCO I primer mix using an electronic multichannel pipette. The PCR plate was covered with a Bio-Rad Microseal® 'B' seal and placed in a Bio-Rad CFX96 Touch™ Real-Time PCR machine to perform the PCR reaction using the program as show in Table 4. To each well, 1 µL of DpnI (New England Biolabs) was added and incubated at 37 °C for 4 h or overnight in order to digest methylated parental DNA. The PCR digest product was stored at -20 °C without heat inactivation of DpnI. The same procedure was followed for the FRESCO II mutants (Table 2), these were remaining point mutations which were not included in the first round. For 23 mutants of the FRESCO I plate, (Figure A 1) which were not obtained from the

**Table 3 Concentration of DNA in ng/µL of wild type PTDH-CHMO$_{Rm}$ stocks.**

| Tube | Concentration (ng/µL) |
|---|---|
| **1** | 48.3 |
| **2** | 41.6 |
| **3** | 49.8 |
| **4** | 23.2 |
| **5** | 44.0 |
| **6** | 49.3 |
| **7** | 52.2 |
| **8** | 46.4 |

first QuickChange PCR batch, another QuickChange PCR was performed using the same conditions. For mutant A290W, PCR was performed with the annealing temperature at 52.5 °C as several attempts using 55 °C were unsuccessful. For combining single mutations, also the same procedure was followed with the exception that the PCR product was purified using the Sigma-Aldrich GenElute PCR Clean-Up kit. For a number of samples, Agarose (1%) gel electrophoresis was performed to verify correct DNA size.

**Table 4 PCR Program for generating the PTDH-CHMO$_{Rm}$ library.**

| | PCR step | Temperature (°C) | Time |
|---|---|---|---|
| | **Initial denaturation** | 95 | 4 min |
| | **Denaturation** | 95 | 30 s |
| **24 cycles** | **Annealing** | 55 | 30 s |
| | **Extension** | 72 | 3.5 min |
| | **Final extension** | 72 | 10 min |
| | **Final hold** | 4 | ∞ |

## 2.5. Transformation Into $CaCl_2$ NEB® 10-beta Competent *E. Coli* Cells

### 2.5.1. Transformation in Multiwell Plates

The transformation of PCR digest product was initially performed by adding 5 µL of digest to 50 µL competent cells in a 96-deep well plate and resting on ice for 20 min. Heat shock was performed by placing the plate in a water bath at 42 °C for different amounts of time; 45 s, min, 2 min and by Jorien van der Weerd[18] also 1 min and 1 min 15 s. 500 – 1000 µL of Prewarmed SOC was added to each well after which cells were recovered for 1 h at 37 °C in an MTP shaker (Heidolph Inkubator 1000).

Transformation was also performed in iQ™ 96-Well PCR plates containing 20 or 30 µL competent cells. To these cells, 5 µL digest was added, covered with AeraSeal and incubated on ice for 20 min. Heat shock was performed for 10 s at 45 °C after which the cells were put on ice for 5 min. 150-200 µL prewarmed Super Optimal Broth containing 20 mM glucose (SOC) medium was added to each well and the plate was incubated at 37 °C for 1 h at 1500 rpm in an MTP shaker.

For the FRESCO II mutants, DpnI of the PCR digest was heat inactivated at 80 °C for 20 min. Transformation was performed using 5 µL digest to 40 µL competent cells. An overview of the different conditions for transformation in multiwell plates can be found in Table A 2 including transformation in Eppendorf cups for mutants of the FRESCO I library.

### 2.5.2. Transformation in 1.5 mL Eppendorf Cups

Transformation in 1.5 mL Eppendorf cups was performed by addition of 5 µL PCR digest to 50 µL NEB 10-beta $CaCl_2$ competent cells. This procedure was performed for all FRESCO II mutants, mutant combinations and FRESCO I mutants for which no colonies were obtained yet after 5 transformation rounds using PCR plates and 96-deep well plates. As mentioned in section 2.4, only for the PCR product of mutant combinations, the PCR digest product was purified using a purification kit. When purified the PCR product was eluted with 15 µL elution buffer of which 3 µL were used for transformation of 50 µL $CaCl_2$ competent cells. The cells were incubated on ice for 20 min after which heat shock was performed for 45 s at 42 °C. Prewarmed SOC medium (600 µL) was added and the cells were incubated at 37 °C for 1 h at 700 rpm in an Eppendorf ThermoMixer C. The culture was pelleted in an Eppendorf micro centrifuge after which the pellet was resuspended in ~50 µL remaining liquid. The cells were plated on LB agar plates containing 50 µg $mL^{-1}$ ampicillin and spread using 3 mm glass beads.

## 2.6. Sequencing of Transformants

Sequencing of the FRESCO I plate was performed using the PlateSeq service of Eurofins for which an ampicillin agar plate containing a colony of the mutant is directly send to Eurofins Scientific (after overnight growth), a duplicate was made of this plate using a 96-deep well plate containing LB agar ampicillin 50 µg $mL^{-1}$. For the FRESCO II plate the plasmid isolation was performed in house by using either the QIAprep Spin Miniprep kit from Qiagen or High Pure Plasmid Isolation Kit from Roche. Sequencing of the in house isolated plasmid was performed by GATC Biotech or Eurofins Scientific.

## 2.7. Expression and Purification

### 2.7.1. Preparation of Precultures for Expression of the PTDH-CHMO$_{Rm}$ Library

Of the FRESCO I library, a glycerol stock (Master plate) was made by stabbing into the agar plate with a multichannel pipette and inoculating a 96 well plate 500 µL $LB_{amp}$ (50 µg $mL^{-1}$). The plate was covered with AeraSeal and incubated at 37 °C, 1050 rpm in an MTP shaker. Of the preculture, a glycerol stock (masterplate) was made by adding glycerol to a final concentration of 30% v/v. Inoculation of cultures from the masterplate was done using a cryo-replicator CR1000 from Enzyscreen. Precultures were grown overnight in a 96-deep well plate containing 500 µL $LB_{amp}$ (50 µg $mL^{-1}$).

### 2.7.2. Lysis Test

Two lysis buffers were used to find the best method to lyse the cell pellets from expression cultures in 96-deep well plates. Lysis was performed using lysis buffer 1 (Table 5), BugBuster® 1x protein extraction reagent and FastBreak™ 1x cell lysis reagent containing additional lysozyme (from hen egg white, 0.2 mg $mL^{-1}$) and DNAse (0.3 U $mL^{-1}$). Expression cultures 1 mL were made of the mutants D10H, E88K and of wild type by adding 10 µL preculture to 1 mL Terrific Broth (TB) supplemented with 50 µg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose. All were expressed in triple and the individual mutants and wild type cultures of these triples were combined to ensure homogeneous samples for cell lysis. After homogenization, the cultures were spun down for 20 min, 4 °C, 2250 x g. The supernatant was removed and cell pellets were frozen at -80 °C for 15 min after which they were thawed by placing the 96-deep well plate in room temperature water. To the cell pellet were added 200 µL of Lysis buffer, 300 µL FastBreak and 50 µL BugBuster. Incubation was performed for 1 h at 20 °C in an MTP shaker at 700 rpm. The samples were made to equal volumes (300µL) and spun down for 45 min, 4°C, 2250 x g. 24 µL cell free extract of each sample was taken and added to 6 µL 5x SDS loading buffer. The cell pellets were also resuspended in 300 µL buffer of which also 24 µL sample was added to 6 µL 5x SDS loading buffer. SDS-PAGE was performed using 15 µL sample in order to determine the most efficient lysis method.

**Table 5 Constituents of the two used lysis buffers**

| | Lysis Buffer 1 ($mL^{-1}$) | Lysis Buffer 2 ($mL^{-1}$) |
|---|---|---|
| Lysozyme | 1 mg | 1 mg |
| DNAse | 5 U | 0.5 mg |
| $MgCl_2$ (100 mM) | 5 mM | 10 mM |
| Tris/HCl 50 mM pH 7.5 | Fill to 1 mL | Fill to 1 mL |

### 2.7.3. Expression in 96-deep Well Plates and High Throughput Purification

Initially, expression of the enzyme library was performed by addition of preculture in a 1:100 ratio to 1 mL/well Terrific Broth (TB) containing 50 µg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose. In later experiments the expression of the enzyme library was performed by adding 200 µL preculture to 800 µL TB (20/100 ratio) containing 50 µg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose final concentration. The plate was covered with AeraSeal and incubated at 24 °C for ~36 h in an MTP shaker at 1050 rpm. The cells were harvested by spinning down in

a 5804R Eppendorf centrifuge for 20 min at 2250 x g, 4 °C. The supernatant was removed and the cells were lysed with lysis buffer (Table 5) using the steps shown in Table 6. After lysis, the cells were spun down for 45 min at 2250 x g in a 5904R Eppendorf centrifuge at 4 °C. After centrifugation, the cell free extract was transferred to a UNIFILTER microplate (Whatman) and centrifuged until all cell free extract was run through into a 96-deep well plate. The filtered cell free extract was transferred to a AcroPrep Advance 1 mL 1.2 µM Supor filter plate containing 100 µL Ni Sepharose. After careful resuspension with the resin, the plate was incubated for 1 h at 4 °C. The flowthrough was collected by centrifugation at 200 x g for 10 – 15 s. The resin was washed with 200 µL Tris/HCl 50 mM pH 7.5 followed by 200 µL Tris/HCl 50 mM pH 7.5 + 5 mM imidazole. The protein was eluted with 2 times 100 µL Tris/HCl 50 mM pH 7.5 + 500 mM imidazole after which the second fraction was used for desalting and the first fraction was used for SDS-PAGE. Desalting was performed by adding the eluted fraction to a pre-equilibrated (according to protocol) PD MultiTrap G-25 column, containing 500 µL Sephadex G-25 per well. The protein was eluted by centrifugation at 800 x g for 2 min.

**Table 6 Steps used for the expression cultures using 1/100 and 20/100 ratios of preculture**

| Amount of preculture | 1/100 | 20/100 |
|---|---|---|
| **Step 1** | Freeze at -80 °C, 15 min | Add 200 µL lysis buffer 2 |
| **Step 2** | Thaw plate in RT water | Incubate at 20 °C, 30 min, 700 rpm MTP shaker |
| **Step 3** | Add 200 µL lysis buffer 1 | Freeze at -80 °C, 45 min |
| **Step 4** | Incubate at 20 °C, 1 h, 700 rpm MTP shaker | Thaw plate in RT water |
| **Step 5** | Spin down 45 min 3700 rpm, 4 °C | Spin down 45 min 3700 rpm, 4 °C |

### 2.7.4. Expression in 24-deep Well Plates and High Throughput Purification

Expression of the FRESCO I library was also performed in four 24-deep well plates by adding 50 µL preculture to 2 mL TB/well containing 50 µg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose. The plates were covered with AeraSeal and incubated at 24 °C in a New Brunswick Innova 44 incubator at 135 rpm for ~36 h. The cells were harvested by centrifugation in 2250 x g, 30 min, 4 °C in a 5904R Eppendorf centrifuge. The cell pellets were resuspended in 200 µL lysis buffer 2 per well, transferred to a 96-deep well plate and incubated at 20 °C, 30 min, 700 rpm. The plate was then placed at -80 °C for 45 min after which it was thawed in room temperature water after which 50 µL Tris/HCl 50 mM pH 7.5 was added. The plate was spun down at 2250 x g for 30 min after which 100 µL cell free extract was transferred to a UNIFILTER microplate and spun down until all cell free extract was run through. The filtered cell free extract was transferred to a AcroPrep Advance 1 mL 1.2 µM Supor filter plate containing 100 µL Ni Sepharose. Purification was performed as described in section 2.7.3.

### 2.7.5. Expression and Purification of Large Volume Cultures of Wild Type and Double Mutant (100 mL - 200 mL)

For the expression to obtain larger quantities of protein, an expression culture was made in an Erlenmeyer flask containing either 100 mL (double mutant (DM)) or 200 mL (wild type) TB supplemented with 50 μg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose and addition of 1/100 volume preculture. The flask was shaken at 24 °C 135 rpm in a New Brunswick Innova 44 incubator shaker for ~36 h ($OD_{600}$ WT 7.4, $OD_{600}$ DM 9.2). The cells were harvested by spinning down at 3000 x g for 25 min. The supernatant was removed and the cell pellet was either directly used for further purification or frozen at -20 °C. The pellet was resuspended in 5 x the pellet weight 50 mM Tris/HCl, pH 7.5. For the sonication of smaller volumes (up to 5 mL) a 3 mm stepped microtip was used at 40% amplitude for 10 min, 3 sec on/off while cooling in icewater to avoid degradation of the protein. For larger resuspension volumes (~15 mL) sonication was performed using a 6 mm stepped microtip at 75% amplitude for 15 min, 3 sec on/off while being stirred and cooled in a water-cooled glass holder (1 °C). The cell debris and aggregates were removed by centrifugation (15000 rpm, 45 min, 4 °C, JA-17 Beckmann rotor). The cell free extract was loaded onto a pre-equilibrated Ni Sepharose column via a 0.45 μm filter and incubated for 1 h with the resin at 4 °C on a rocking table. The cell free extract was eluted and the column was washed with 3 column volumes (CV) 50 mM Tris/HCl, pH 7.5, followed by 3 CV 50 mM Tris/HCl, pH 7.5 containing 5 mM imidazole. The protein was eluted with a maximum of 3 CV 50 mM Tris/HCl pH7.5, containing 500 mM imidazole. Excess imidazole was removed by applying the protein to an EconoPac 10-DG desalting column. The protein was divided in two portions of which one was supplemented with glycerol (10% v/v final concentration) after which the protein was flash frozen as beads in liquid nitrogen and stored at -80 °C. Protein concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific) at 440 nm.

## 2.8. Determining the Apparent Melting Temperature Using ThermoFAD

To determine the apparent melting temperatures ($Tm^{app}$) of the mutants, ThermoFAD was performed on mutant enzymes using at least two different rounds of expressions in order to obtain duplicate values. The ThermoFAD measurements were performed by adding 20 μL desalted protein sample in a well of an iCycler iQ PCR plate which was covered with a Bio-Rad Microseal® 'B' seal. The samples were subjected to a temperature program running from 20 °C – 99 °C and measuring the fluorescence every 30 s using a CFX96 Real-Time System with C1000 Touch Thermal Cycler (BioRad), using the SYBR Green fluorescence filter for detection. ThermoFAD was also performed on the cell free extract before purification in order to see if the same $Tm^{app}$ is obtained as for purified protein.

## 2.9. Combining Stabilizing Mutants and Determining the Apparent Melting Temperature

Mutants resulting in a $\Delta Tm^{app} \geq 1.0$ °C were selected to be combined to obtain a highly stable enzyme variant. Selection of mutants was performed by first checking for possible sequence overlap of primers and by viewing all stabilizing mutations in YASARA to identify possible structural clashes of the mutations. Mutations with the highest increase in $\Delta Tm^{app}$ were chosen to be combined first with the additional criterion that they do not induce a change in charge to the protein. To ensure that the mutant with multiple mutations was still expressed and had an increase in $\Delta Tm^{app}$, the mutants were expressed and ThermoFAD was performed. This was

done for the first 6 combined mutants by expressing them all at the same time in duplo in a 96-deep well plate, purification was performed as described in section 2.7.3.

# 3. Results

## 3.1. Screening of Potentially Stabilizing Point Mutations Using YASARA

Using YASARA, 852 different point mutations were screened *in silico* according to the criteria as mentioned in section 2.2. From these, 114 (~13%) mutants were selected as potentially stabilizing. Of these 114 mutant 113 were created using QuickChange PCR and the mutant enzymes were expressed and purified in order to determine the $Tm^{app}$.

## 3.2. Site-directed Mutagenesis Using QuickChange PCR Method

Most mutants of the first FRESCO I QuickChange PCR round were obtained. Due to unsuccessful transformation attempts in 96-well plates for 23 mutants of FRESCO I, a new QuickChange PCR was performed. Analysis of the PCR product by (1%) agarose gel electrophoresis showed the expected band size for most mutants.
For mutant A290W only a faint band present of expected size was present (Figure 2 and Figure A 4),. Upon sequencing, the correct mutants were obtained with the exception of the mutant A290W, therefore a QuickChange PCR was performed according to the protocol as shown in Table 4 and once using a lower annealing temperature of 52.5 °C. The correct mutant was however never obtained and no further attempts were made to obtain it.

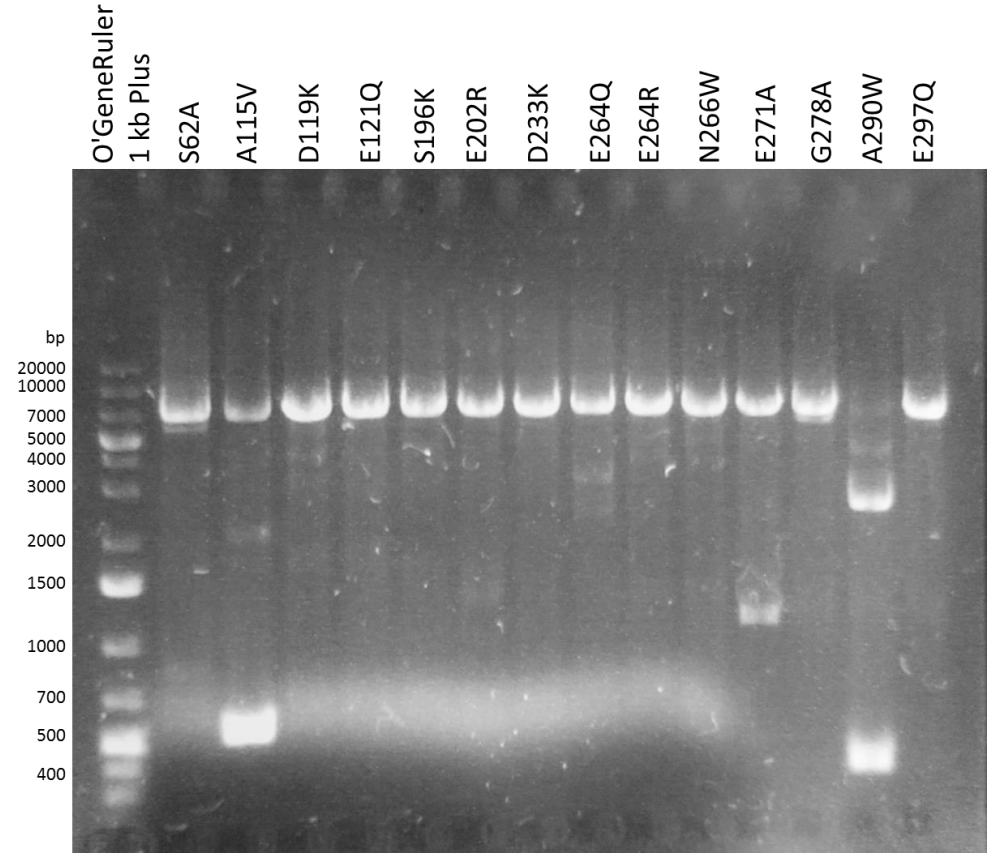



**Figure 2 Agarose (1%) gel of a selection of mutants from the FRESCO I library for which a second PCR reaction was performed.**

## 3.3. Transformation in Multiwell Plates and 1.5 mL Eppendorf Cups

Transformation of PCR product was performed in 96-deep well plates and 96 well PCR plates as described in section 2.5.1. It was found that 45 s heat shock at 42 °C and concentrating cells by centrifugation before plating works most efficient for 96-deep well plates. Transformation led however to inconsistent results in the amount of obtained colonies. An overview of the performed transformations and their conditions can be found in Table A 2. Possible causes for the inconsistency are **1** competent cells which were not sufficiently competent, **2** DpnI was not heat inactivated after digestion, **3** the amount of time used for heat shock at 42 °C was not optimal. Initially, transformation in 96-deep well plates was performed using heat shock for 1 min or longer which appeared to be too long. The use of unpurified PCR product also negatively affects the transformation efficiency. This was seen when performing transformations in Eppendorf cups with purified and unpurified PCR products. Purified PCR product gave 20 – 100 colonies, while unpurified, DpnI inactivated, PCR product would give 20 colonies at most. Transformation in Eppendorf cups showed that the used cells are sufficiently competent. It would be advised to purify the PCR product using a 96 PCR clean-up kit, which however also requires a vacuum system of € 459.50 - € 668 (Sigma-Aldrich, QIAGEN). As an alternative, a different protocol for competent cells could be used to increase transformation efficiency. An example are electro competent cells, however this will require a 96 well electroporation plate. Another alternative is a different protocol for chemically competent cells such as the use of rubidium chloride which has been shown to work when performing transformation in 96-deep well plates.

## 3.4. Expression and Purification

### 3.4.1. Lysis Test

The lysis test using FastBreak, BugBuster and lysozyme buffer as described in 2.7.1 showed that BugBuster was inefficient in lysing the cell pellet as can be seen by SDS-PAGE in Figure 3. The protein was not present in the cell free extract and only in the pellet for the two mutants and wild type. FastBreak and lysozyme buffer showed equal results. As FastBreak contains non-ionic detergents which could interact with the enzyme and interfere with later ThermoFAD measurements, it was chosen to perform cell lysis using the lysozyme buffer.

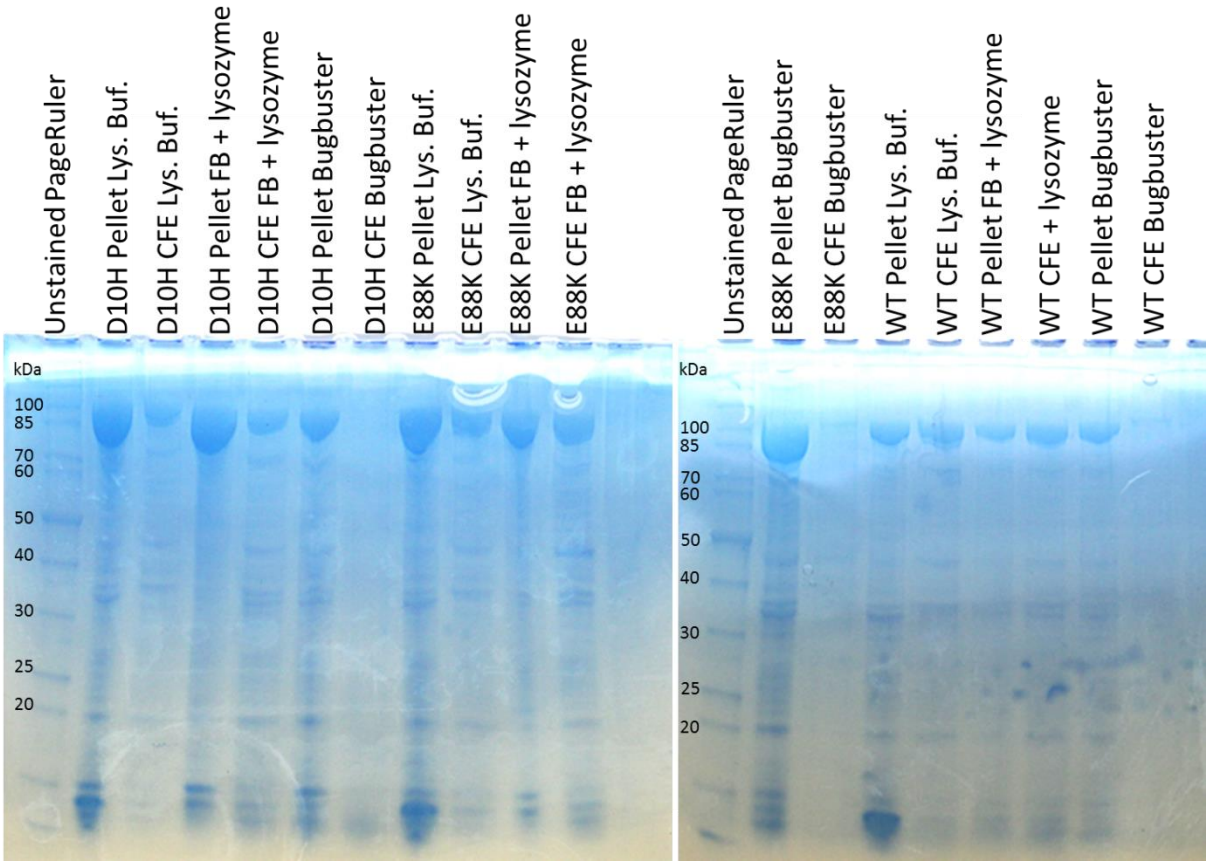



**Figure 3 SDS-PAGE gel showing the pellet and cell free extract (CFE) fractions after lysis of two different mutants and wild type using three different methods for lysis.**

### 3.4.2. Expression in 96-deep Well Plates

Expression using 10 µL preculture to 1 mL TB containing 50 µg $mL^{-1}$ ampicillin and 0.02% ($v.v^{-1}$) L-arabinose led to insufficient signal in ThermoFAD measurements as can be seen in Figure 5. When using 200 µL preculture, the fluorescence intensity drastically improved (Figure 8), meaning that the amount of protein was higher than for the expression culture using a smaller amount of preculture. This could also be noticed after cell lysis and during purification as the cell free extract now had an intense yellow color while this was not noticed for the other expression cultures. For desalting the second elution fraction was taken as this appeared by eye to be the most concentrated fraction as the color was more yellow than for the first elution fraction. The fractions were not combined as the desalting plate can only handle a maximum of 100 µL of protein sample. It has to be noted that for the expression cultures starting from 200 µL preculture, also the lysis method was slightly changed as mentioned in section 2.7.3 which

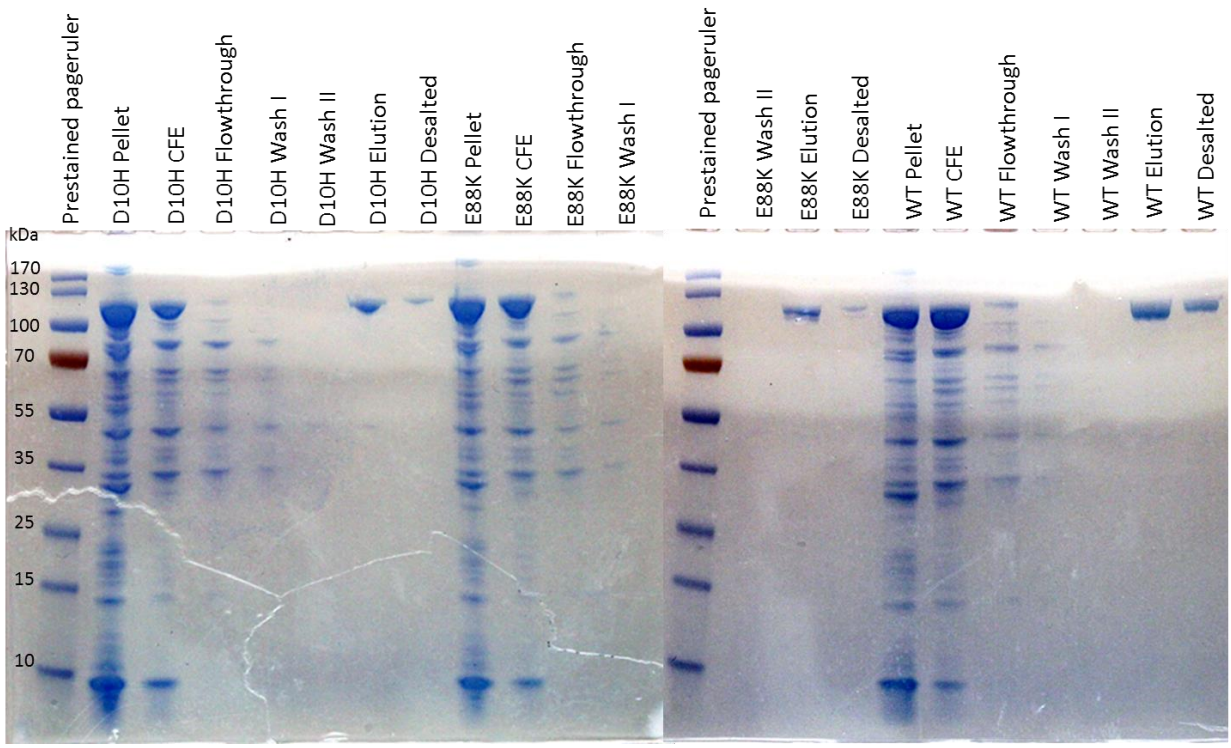



**Figure 4 SDS-PAGE gel of purification process of a 96-deep well plate expression using 10 µL preculture.**

could have contributed to the improvement of the amount of enzyme in the CFE. No comparison was made between the two lysis methods. The concentration of the enzyme was measured one time for all mutants expressed using 200 µL preculture.

For the expression cultures using 200 µL preculture, there were still some mutants that gave no fluorescence signal in ThermoFad measurements. In order to find if the problem lies in expression or insoluble protein, SDS-PAGE was performed on the pellet and cell free extract after lysis. Figure 7 and figure A 3 show the protein content in the pellet and CFE. It can be seen that in almost all cases, the protein is mainly present in the pellet faction, meaning that the protein is no longer soluble. In the case of D119A, there is also no protein visible in the pellet suggesting that the protein was not expressed.

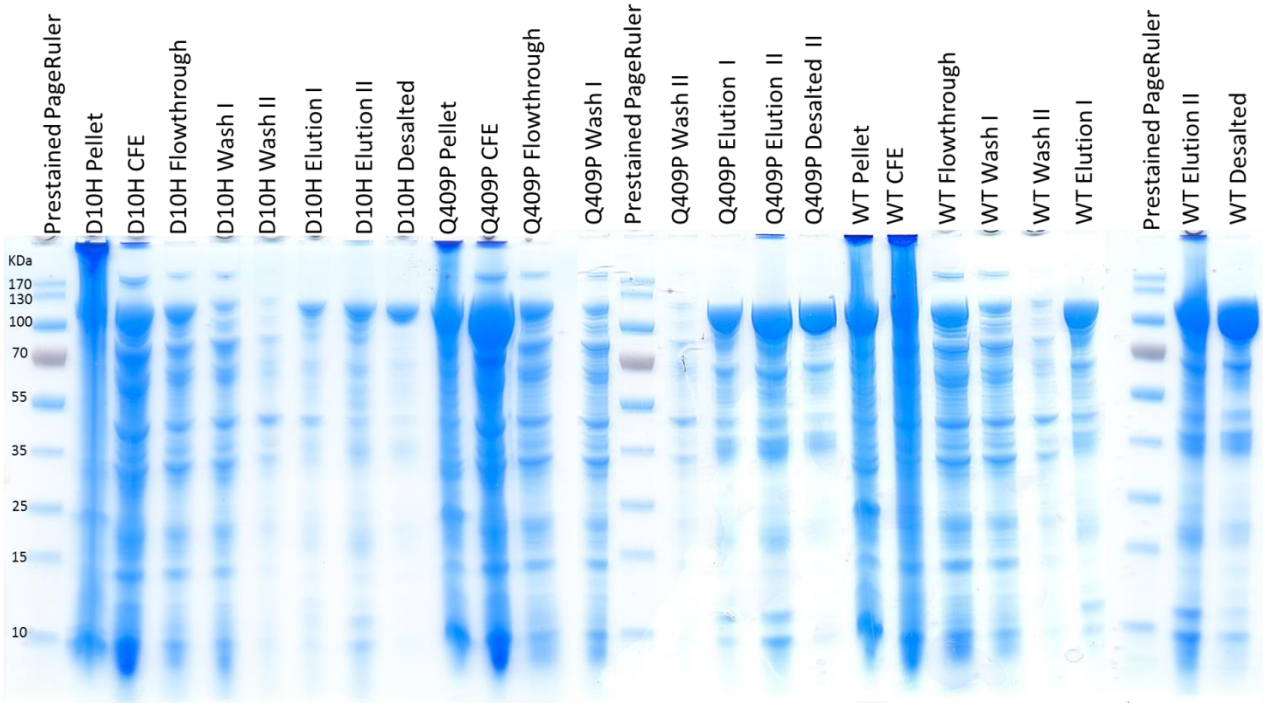



**Figure 6 SDS-PAGE gel of the purification process of a 96-deep well plate expression using 200 µL preculture.**

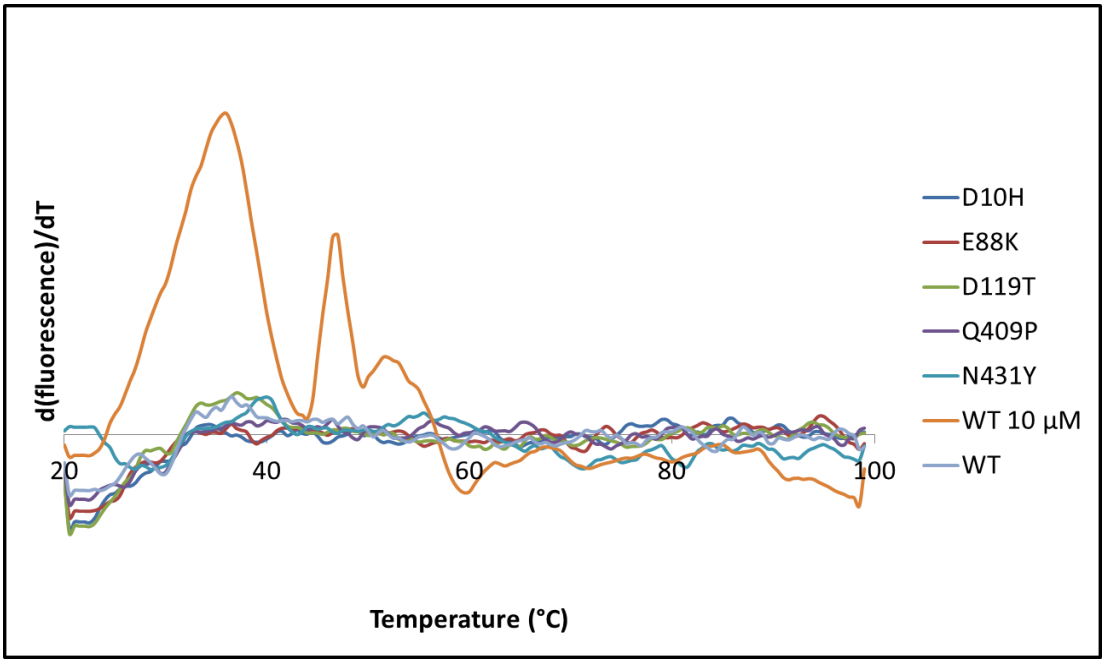



**Figure 5 ThermoFAD melt curve derivative of 5 different mutant proteins and wild type expressed in a 96-deep well plate using 10 µL preculture. As a reference, wild type with a concentration of 10 µM was added obtained from a large culture purification**

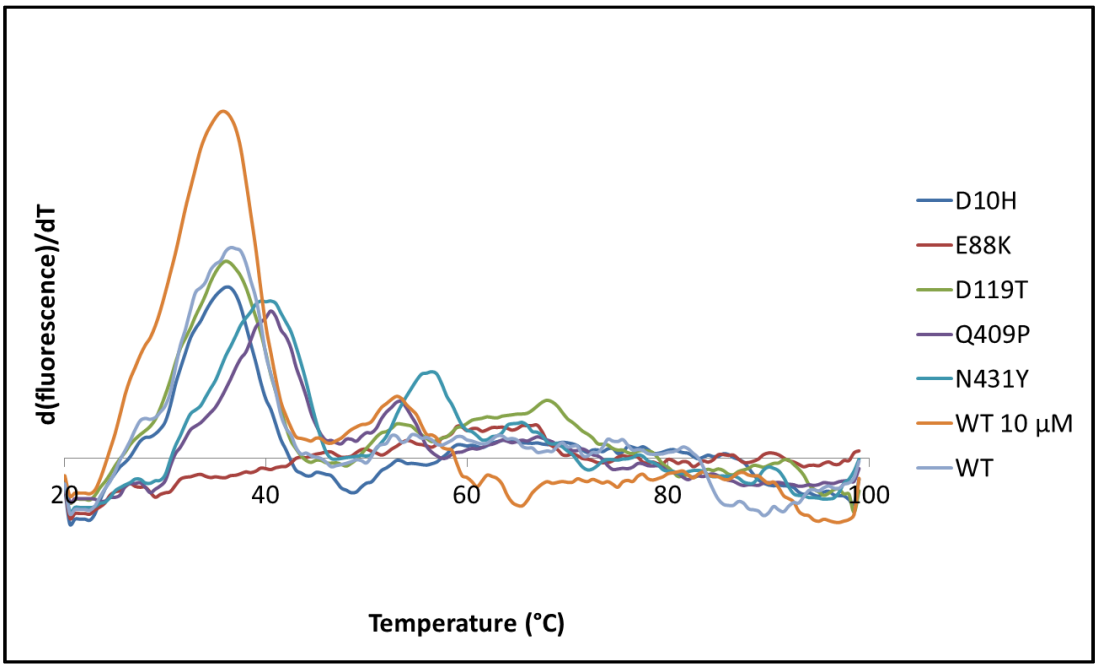



**Figure 8 ThermoFAD melt curve derivative of mutants and wild type purified from 96-well expression using 200 µL preculture. As a reference, wild type with a concentration of 10 µM was added obtained from a large culture purification**

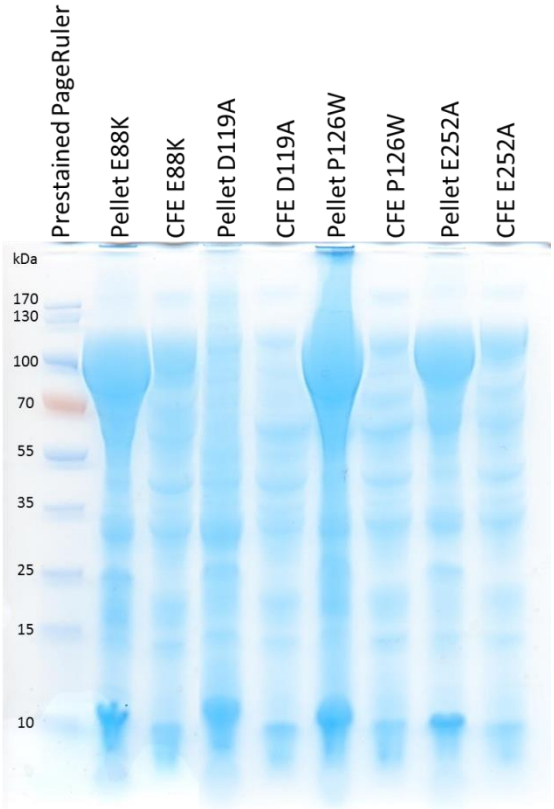



**Figure 7 SDS-PAGE of pellet and CFE after lysis of mutants where no signal was present in ThermoFAD experiments.**

### 3.4.3. Expression in 24-deep Well Plates

Purified mutant enzymes produced from growing in 24 well plates also gave sufficient intensity in fluorescence as can be seen in Figure 10. Figure 9 shows the SDS-PAGE of two mutants and wild type. Samples of the pellet and cell free extract after lysis are not shown. As expression in 96-deep well plates gave sufficient results for ThermoFad measurements, 24-deep well plates were not used for further expressions. The use of 24-deep well plates can be an option for proteins which have lower levels of expression.

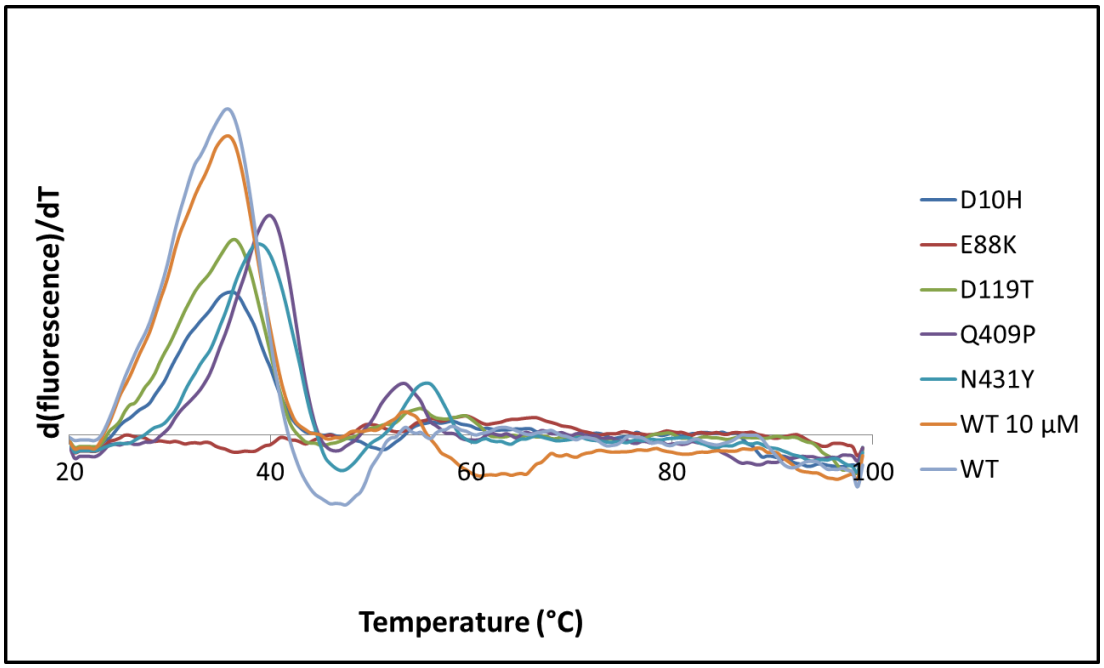



**Figure 10 ThermoFAD melt curve derivative of mutants and wild type purified from a 2 mL culture in 24 well plates**

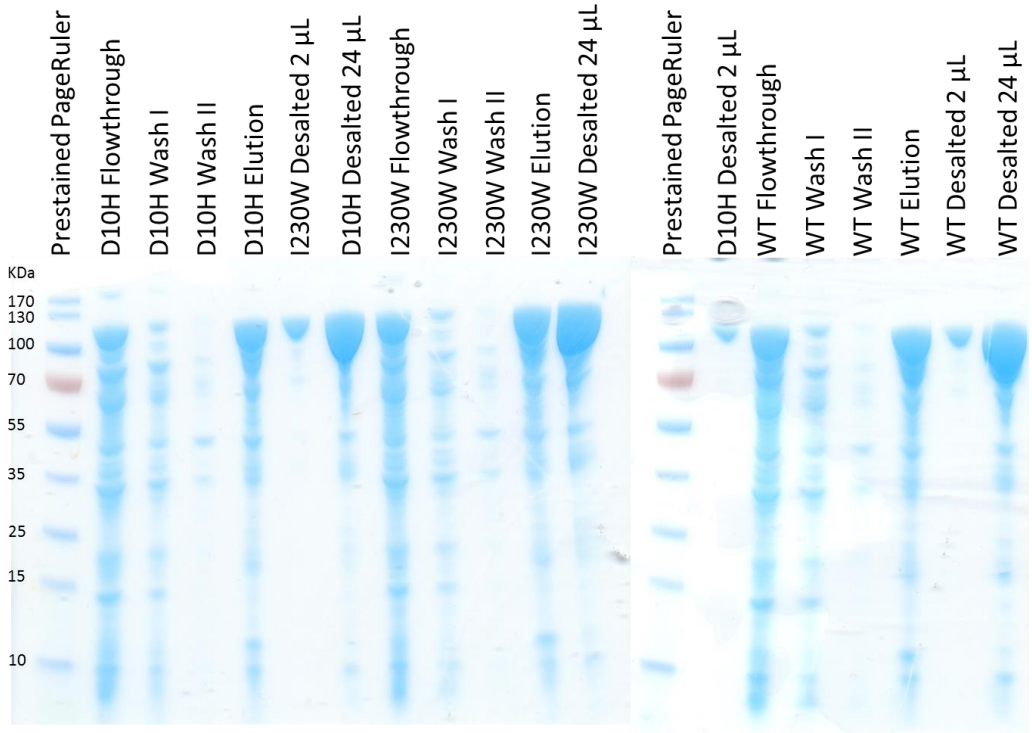



**Figure 9 SDS-PAGE of selection of mutants from expression using 24 deep well plates. For the desalted protein samples of 2 µL and 24 µL were taken as the protein concentration was not known.**

### 3.4.4. Expression and Purification of Large Volume Cultures of Wild Type and Double Mutant (100 mL – 200 mL)

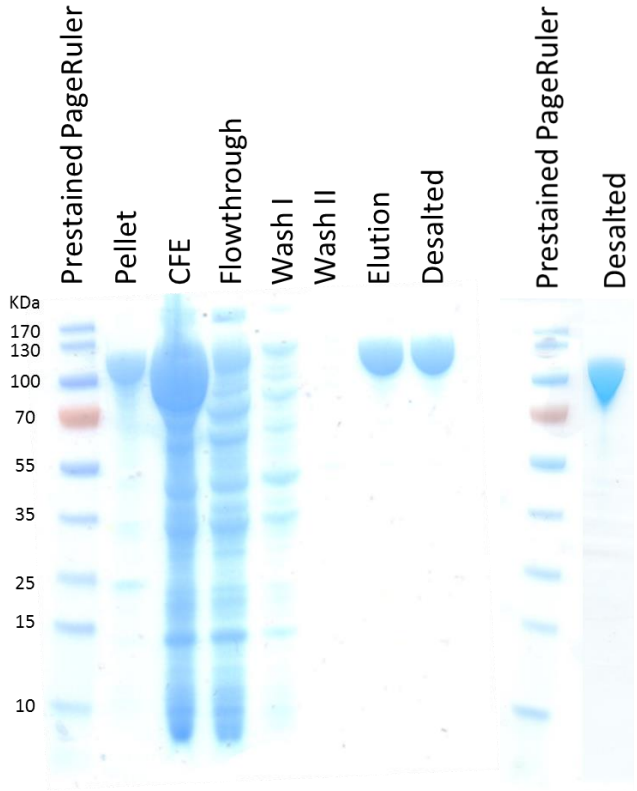



**Figure 11 SDS-PAGE of the double mutant (Q409P/N431Y) (left) and of the WT (right) enzyme after desalting.**

Expression and purification of WT (200 mL culture) and double mutant Q409P/N431Y was performed successfully. For the WT a previous experiment failed as the enzyme was not sufficiently cooled during sonication, leading to degradation of the enzyme. For the WT protein, the elution fraction was collected in three batches. After desalting these had concentrations of 93, 38 and 35 µM. For the double mutant one fraction was collected and desalted and had a concentration of 40 µM. Figure 1 shows the SDS-PAGE of the double mutant and wild type indicating that the protein is pure. For the wild type only a gel was made of the protein after desalting.

## 3.5. Determining the Apparent Melting Temperature Using ThermoFAD

The screening of 113 mutants using ThermoFAD resulted in 34 mutants with a $\Delta Tm^{app} \geq 0.5$ °C of which 13 had a $\Delta Tm^{app} \geq 1.0$ °C which are shown in A 5 39 mutations led to a decrease of $\Delta Tm^{app} \geq -0.5$ °C and 22 with $\Delta Tm^{app} \leq -0.5$ °C. For 16 mutants, no ThermoFAD data was obtained. An overview of all point mutations and their corresponding a $\Delta Tm^{app}$ can be found in Table A 5 of Appendix A. Mutations resulting in a $\Delta Tm^{app} \geq 1.0$ °C (Table 7 and Figure 12) were selected to be combined. In the case of stabilizing mutants at the same position, the mutant with no change in charge was chosen to be added. For all mutations the proximity to each other in the structure was checked in order to avoid possible clashes.

The cell free extract was also used for ThermoFAD in order to see if the protein gave the same a $\Delta Tm^{app}$ as for the purified enzyme Table 8. It was found that measurements on CFE and purified protein in almost all cases does not show the same result and also not the same shift in

**Table 7 Overview of the mutations which had an increase in the $\Delta Tm^{app} \geq 1.0$ °C.**

| Mutation | Average $Tm^{app}$ ( °C) | $\Delta Tm^{app}$ ( °C) | st. dev. |
|---|---|---|---|
| **WT** | 36.00 | 0.00 | 0.39 |
| **D10Y** | 37.00 | 1.00 | 0.00 |
| **A11M** | 37.00 | 1.00 | 0.00 |
| **E91K** | 37.17 | 1.17 | 0.24 |
| **E91Q** | 37.50 | 1.50 | 0.41 |
| **A115V** | 38.00 | 2.00 | 0.00 |
| **D119R** | 37.00 | 1.00 | 0.50 |
| **T164L** | 37.17 | 1.17 | 0.24 |
| **R280Y** | 37.25 | 1.25 | 0.25 |
| **Q409P** | 40.20 | 4.33 | 0.24 |
| **N431Y** | 39.80 | 3.67 | 0.60 |
| **A455V** | 37.75 | 1.75 | 0.25 |
| **S534R** | 37.00 | 1.00 | 0.00 |

temperature difference. This suggests that for this protein, determining the apparent melting temperature using ThermoFAD on the CFE is not suitable. It has to be noted that the values for CFE were measured only once. In the melt curve derivative graphs, a second peak can be seen around 55 °C. This peak can be found in purified enzyme and is even more pronounced for ThermoFAD measurements on the CFE (Figure 13). It was unknown if this second peak is an artifact or if it comes from the enzyme itself and is shifted to a higher temperature because of interactions with proteins in the CFE, as molecular and protein crowding can tune protein stability[19]. WT protein was analyzed by ThermoFAD supplementing it with BSA or CFE of a non-induced WT culture[a]. Analysis of CFE of a non-induced WT culture shows a fluorescence signal at 57 °C and 71 °C. When performing ThermoFAD on a protein which should not give a fluorescence signal such as BSA, it was found that also BSA shows fluorescence at 64 °C.

[a] The used CHMO concentration in all samples was 10 µM. BSA concentrations were 1, 5, 10, 75, 150, 300 mg $mL^{-1}$. CFE concentrations 1, 5, 10, 20, 25, 30, 35 mg $mL^{-1}$.

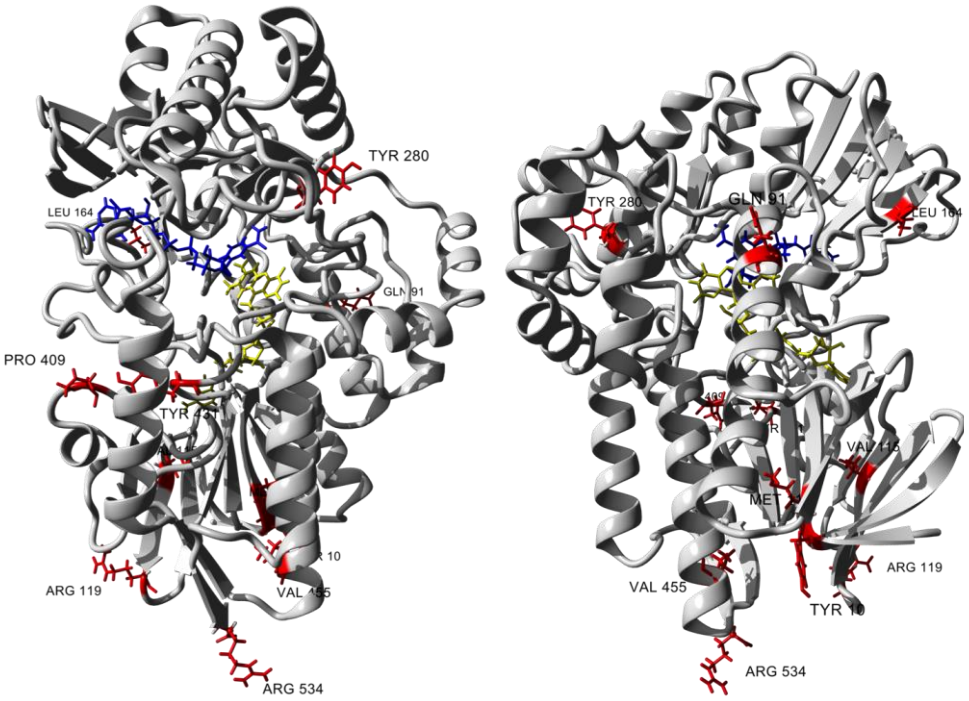



**Figure 12 Enzyme structure of CHMO showing in red the mutations which were selected for combination. In blue and yellow are shown the NADPH and FAD cofactors.**

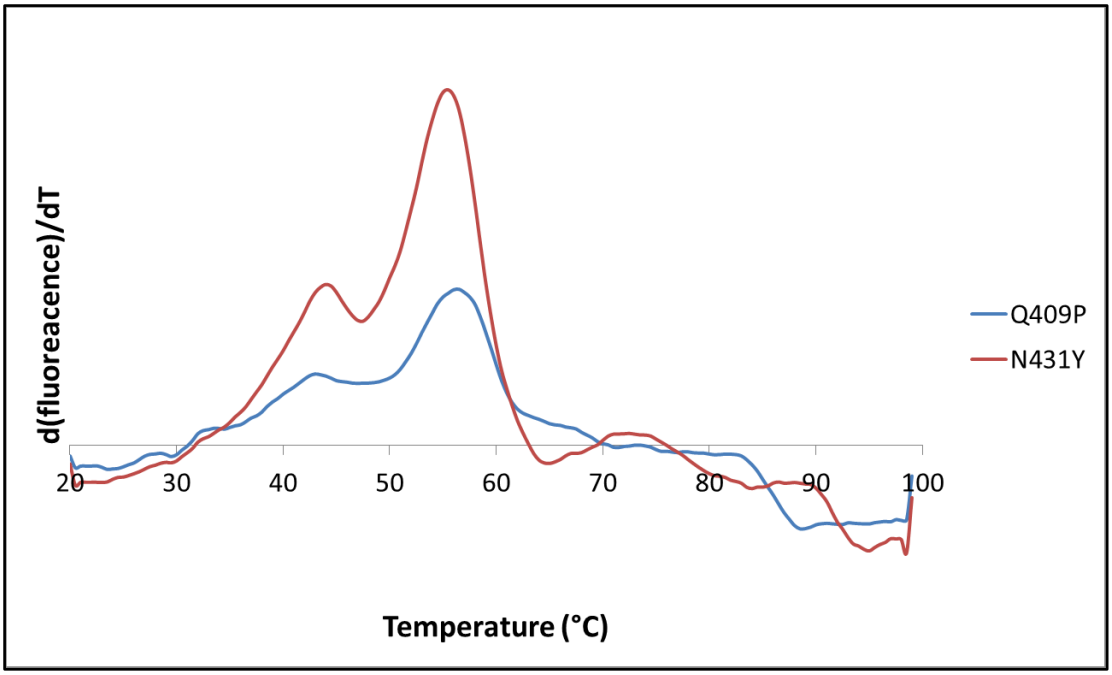



**Figure 13 ThermoFAD melt curve derivative from CFE measurement of mutants Q409P and N431Y showing a second peak around 55 °C.**

**Table 8 Mutants for which the $Tm^{app}$ could be determined using CFE and compared to purified enzyme. ND not determined.**

| Mutation | Average $Tm^{app}$ (°C) purified | $Tm^{app}$ CFE (°C) | ΔTm (°C) purified/CFE | Mutation | Average $Tm^{app}$ (°C) purified | $Tm^{app}$ CFE (°C) | ΔTm (°C) purified/CFE |
|---|---|---|---|---|---|---|---|
| **D10H** | 36.00 | 37.50 | 1.50 | **I230W** | ND | 37.00 | |
| **A11M** | 37.00 | 39.50 | 2.50 | **E264Q** | 36.00 | 36.00 | 0.00 |
| **A11V** | 35.25 | 37.00 | 1.75 | **E264R** | 35.75 | 38.00 | 2.25 |
| **H25F** | 35.25 | 37.50 | 2.25 | **E264T** | 36.25 | 37.00 | 0.75 |
| **H25L** | 34.75 | 37.50 | 2.75 | **D274K** | 35.83 | 37.00 | 1.17 |
| **T35V** | 36.17 | 37.50 | 1.33 | **G278A** | 31.17 | 35.00 | 3.83 |
| **S62A** | 32.50 | 36.50 | 4.00 | **R280Y** | 37.50 | 38.50 | 1.00 |
| **H63F** | 32.25 | 35.50 | 3.25 | **E293T** | 35.50 | 38.00 | 2.50 |
| **H63W** | ND | 34.00 | | **E309K** | 36.17 | 39.50 | 3.33 |
| **H63Y** | 35.50 | 38.00 | 2.50 | **Y337W** | 36.00 | 37.50 | 1.50 |
| **E91K** | 37.17 | 39.00 | 1.83 | **D398K** | 36.50 | 38.00 | 1.50 |
| **E91Q** | 37.50 | 39.50 | 2.00 | **D398N** | 36.00 | 37.50 | 1.50 |
| **A115I** | 35.17 | 36.50 | 1.33 | **D398R** | 36.33 | 39.00 | 2.67 |
| **D119R** | 37.00 | 39.50 | 2.50 | **D404K** | 36.00 | 38.50 | 2.50 |
| **D119S** | 36.50 | 40.00 | 3.50 | **D404N** | 36.33 | 38.50 | 2.17 |
| **D120T** | 36.00 | 37.50 | 1.50 | **D407Q** | 34.25 | 37.00 | 2.75 |
| **E121Q** | 35.83 | 40.00 | 4.17 | **D407R** | 34.33 | 37.50 | 3.17 |
| **T164I** | 36.83 | 39.50 | 2.67 | **Q409P** | 40.33 | 43.00 | 2.67 |
| **T164L** | 37.17 | 39.50 | 2.33 | **N431M** | 35.50 | 42.00 | 6.50 |
| **T164V** | 36.17 | 39.00 | 2.83 | **N431Y** | 39.67 | 44.00 | 4.33 |
| **A169R** | 36.50 | 39.50 | 3.00 | **S440T** | 35.00 | 29.00 | 6.00 |
| **E200Q** | 35.00 | 37.50 | 2.50 | **D450R** | 36.25 | 38.00 | 1.75 |
| **T205V** | 36.33 | 47.50 | 11.17 | **D450T** | 36.50 | 38.50 | 2.00 |

**Table 9 Initial order in which stabilizing mutations were planned to be added**

| Mutation | Combined Mutant nr. |
|---|---|
| **D10Y** | 10x |
| **A11M** | 5x |
| **E91Q** | 7x |
| **A115V** | 3x |
| **D119R** | 11x |
| **T164L** | 6x |
| **R280Y** | 8x |
| **Q409P** | 2x |
| **N431Y** | 2x |
| **A455V** | 4x |
| **S534R** | 9x |

## 3.6. Combining Stabilizing Mutations

Stabilizing mutation were combined as shown in Table 9 up till the 7x combined mutant. The double mutant was made choosing the two mutation giving the largest increase in $Tm^{app}$. From MD simulations these mutations appear to be stabilizing the same part in the enzyme as can be seen in Figure 14. Here the mutations Q409P and N431Y show increased rigidity in the same part of the enzyme. The combined mutants were expressed up till the 6x mutant. The double mutant has a $Tm^{app}$ of 41.50 °C which is lower than expected since mutations are generally additive As mentioned, the double mutant consists of two point mutations which are stabilizing the same part of the protein. Nevertheless, the $Tm^{app}$ of the double mutant is higher than for the individual mutations.

**Table 10 Overview of combined mutants for which ThermoFAD measurements were performed. * Double mutant purified from 100 mL culture, the other double mutant was expressed simultaneously in a 96-deep well plate together with the other combined mutants.**

| | Mutation | Average $Tm^{app}$ ( °C) | $\Delta Tm^{app}$ ( °C) | st. dev. |
|---|---|---|---|---|
| **Double Mutant (DM1)*** | Q409P/N431Y | 41.50 | 5.50 | 0.00 |
| **Double Mutant (DM1)** | Q409P/N431Y | 42.75 | 6.75 | 0.25 |
| **Triple Mutant (TM1)** | DM1 + A115V | 42.50 | 6.50 | - |
| **Quadruple Mutant (QM1)** | TM1 + A455V | 43.00 | 7.00 | 0.00 |
| **Pentadruple Mutant (PM1)** | QM1 + A11M | Insoluble | | |
| **Hexatruple Mutant (HM1)** | PM1 + T164L | Insoluble | | |
| **Double Mutant (DM2)** | A115V/A455V | 38.33 | 2.33 | 0.24 |
| **Triple Mutant (TM2)** | DM1 + T164L | 41.58 | 5.58 | 0.19 |
| **Quadruple Mutant (QM2)** | TM1 + T164L | 41.50 | 5.50 | 0.00 |
| **Pentadruple Mutant (PM2)** | QM1 + T164L | 42.00 | 6.00 | 0.00 |

It was also found that the 5x mutant and 6x mutant gave no fluorescence signal in ThermoFAD measurements. Analysis of the pellet and CFE after lysis using SDS-PAGE (Figure 15) showed that for these mutants the protein was insoluble as the protein was only present in the pellet after lysis. It was decided to create the new combinations excluding the mutation A11M which made the protein insoluble: A115V/ A455V, Q409P/ N431Y/ T164L, Q409P/ N431Y/ A115V/ T164L, Q409P/ N431Y/ A115V/ A455V/ T164L. A115V/ A455 was created to see if A115V had an effect on the melting temperature when combined with A455V as the triple mutant (TM1, Table 10), did not show a significant increase compared to the double mutant (DM1) which was simultaneously expressed in the 96-deep well plate. As the pentadruple mutant was insoluble, the subsequent mutation T164L was added to the previously obtained double mutant (DM1), triple mutant (TM1) and quadruple mutant (QM1). These newly combined multiple mutants were expressed and purified after which their $Tm^{app}$ was determined by ThermoFAD. It was found that new multiple mutants did not show significant increase on the $Tm^{app}$, the addition of T164L to QM1 even dropped the $\Delta Tm^{app}$ when combined with TM1 and QM1 while expecting an increase of 1 °C. It appears that a plateau has been reached.

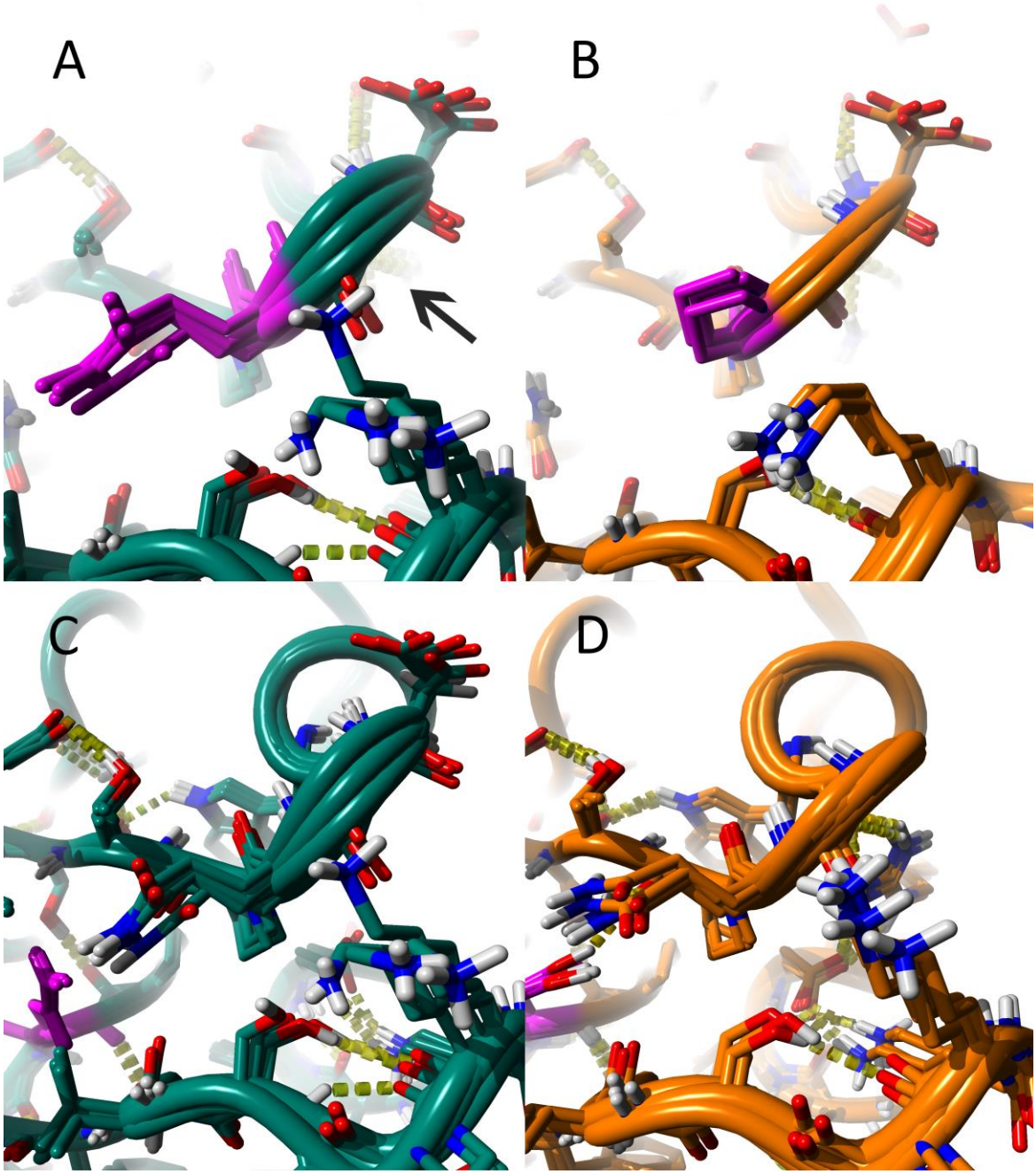



**Figure 14 Comparison of wild type and mutant structures from MD simulations visualized using YASARA. In green the wild type and in orange the mutant, the residue of interest is show in magenta. A) Wild type highlighting residue Q409 B) Mutant structure highlighting the mutation Q409P C) Wild type structure highlighting residue N431 D) Mutant structure highlighting the mutation N431Y. The arrow in image A shows the area in the structure where there is an increase in rigidity. Both mutations are stabilizing the same area in the protein.**

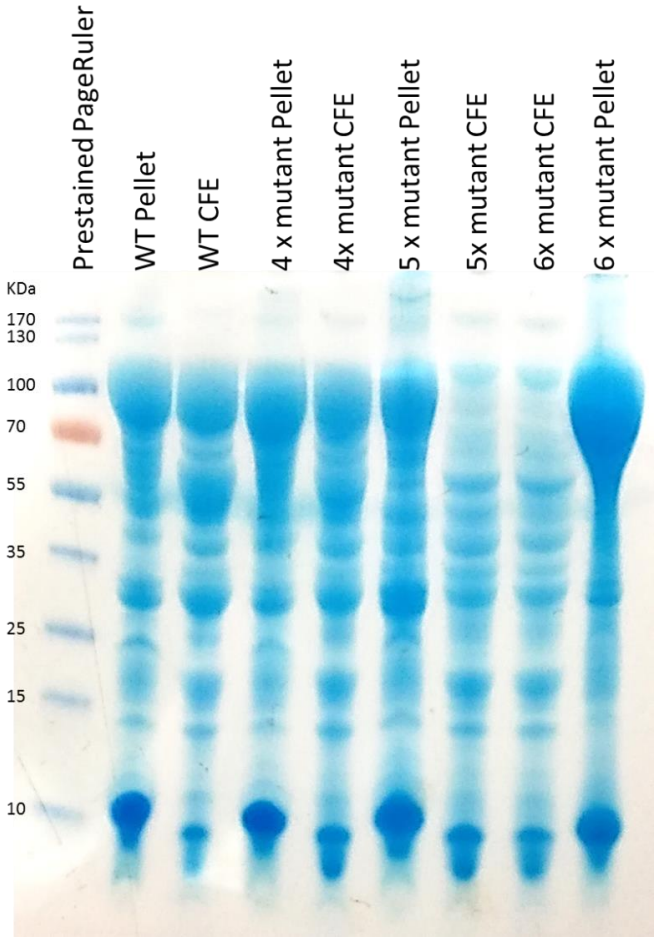



**Figure 15 SDS-PAGE of the pellet and cell free extract after lysis of combined mutants.**

## 4. Discussion

In this project, the aim was to improve the thermostability of the CHMO from *Rhodococcus sp. HI-31*. Potentially stabilizing mutations were generated using the FRESCO protocol after which they were manually inspected using YASARA. Using selection criteria described in section 2.2, 114 (~13%) of the 852 suggested mutations were selected to be created and expressed to be analyzed by ThermoFAD. This is a lower percentage than the expected ~25%, however this is a manual screening step and therefore selection strictness can vary per person.

Using QuickChange PCR, 113 mutants were produced and 1 mutant was not obtained. Lowering the melting temperature by 2.5 °C to 52.5 °C. Using the OligoCalc[20] tool it was found that the reverse primer for this mutation has the potential of forming hairpins and contains 3 self-annealing sites. In order to obtain this mutant, a new reverse primer should be designed.

Transformation of the PCR product into *E. Coli* NEB® 10-beta, using 96-deep well plates and PCR plates, lead to low and inconsistent amounts of colonies. Possible causes were that the DpnI in the PCR product was not heat inactivated, cells were not sufficiently competent enough to be used with 96 well plates and/or the time for heat shock was not optimal. It was found later on that indeed using PCR digest is less efficient than using purified PCR product. The reduced efficiency of using unpurified PCR product could potentially be compensated by deactivating

the DpnI and using competent cells which have higher competence such as electro competent cells or using a different protocol for chemically competent cells.

Lysis of cell pellets obtained from expression in 96-deep well plates was shown to be equally efficient for lysis with FastBreak™ 1x cell lysis reagent (supplemented with lysozyme from hen egg white, 0.2 mg $mL^{-1}$ and DNAse 0.3 U $mL^{-1}$) and lysis buffer 1 (Table 5). Lysis with BugBuster® 1x protein extraction reagent did not work as the protein was only present in the insoluble fraction after lysis. The protocol accompanying BugBuster® states that the extraction efficiency is somewhat strain-dependent and works particularly efficient for BL21 and derivatives thereof. As the used strain for expression for CHMO was NEB® 10-beta, it might be that this lysis reagent is not suitable for this strain.

Expression of the CHMO mutants worked best when using a 1:5 ratio of preculture to TB medium instead of the conventional 1:100 ratio. This was visible in color (more intense yellow color) of the CFE and in ThermoFAD measurements. The two different methods for preparing the expression however cannot be fully compared to each other as the lysis methods was also adjusted when changing from the 1:100 to the 1:5 ratio. The freezing time at -80 °C was 3 times longer with 45 min and performed after incubation with lysis buffer for 30 min at 20 °C. As the main object was to produce enough protein for ThermoFAD measurements, no attempts were made to identify if lysis method or expression conditions were the main factor in obtaining a larger amount of protein.

Larger volume expression (100 – 200 mL) showed to work well using the described protocol. As the WT $CHMO_{Rm}$ has low thermostability, it is important to sufficiently cool the cell suspension during sonication. This was apparent when the wild type was expressed as sonication using ice water to cool appeared to be insufficient and the protein was lost in the flowthrough due to degradation. Cooling the cell suspension using a water cooled glass holder (1 °C) worked well and the wild type enzyme was purified successfully.

Determination of the apparent melting temperature of mutant enzymes should be performed on purified enzyme. ThermoFAD on the cell free extract does show a peak for the CHMO enzyme, however this $Tm^{app}$ is not equal to the $Tm^{app}$ of purified enzyme and is also not shifted with the same amount of degrees for different mutants therefore the actual $Tm^{app}$ cannot be determined using CFE. For ThermoFAD on CFE also a second peak is visible around 55 °C with high intensity. This peak is also sometimes visible in samples of purified enzyme but with much lower intensity. A problem when this peak has high intensity is that the foot of the curve can overlap with the signal of the mutant CHMO especially when the mutant has a $Tm^{app}$ above 40 °C. Also the CFE can contain compounds that fluoresce, as it contains the complete cell including free FAD. FAD in its stacked (closed) form is reported to be non-fluorescent as there is an intramolecular interaction possible between the adenine and isoalloxazine ring of FAD which results in quenching of fluorescence[21]. This could be verified by performing ThermoFAD on free FAD and also by adding free FAD to the CFE of a non-induced culture. Fluorescence in BSA samples can be caused by compounds such as carotenoids as it is a protein which serves in transporting a wide range of metabolites, nutrients and other molecules[22] which can still be present in the protein after purification.

## 5. Outlook

As it appears that a plateau has been reached for the $Tm^{app}$ of the enzyme it would be helpful to identify the possible cause. This could be done by looking at the B-factors (B-FIT method) in the proteins X-ray structure which indicates the flexibility of certain regions[23]. Another option is to use constrained network analysis (CNA) where thermal unfolding of a protein is simulated making it possible to identify early unfolding regions[24].

Activity assays should be performed on the generated thermostable multiple mutant as loss of activity can occur even when the changed amino acids are not near the active site. Mutations can also have an effect on the incorporation of the NADPH and FAD cofactors. The thermostable mutant can also obtain a new optimal temperature for activity which should be determined.

Next to determining the $Tm^{app}$, the half-time should be determined of the thermostable multiple mutant as this is an important parameter for biocatalysis

Potentially, disulfide bonds could be created in order to increase the $Tm^{app}$. This however does have the disadvantage that disulfide bonds can be cleaved by reduction leading to loss of the stabilizing function of these bonds.

## 6. Conclusion

Using the FRESCO method, 114 potentially stabilizing mutations were selected and 113 were created using QuickChange PCR. Transformation of PCR product into chemically competents NEB® 10-beta *E. coli* showed to be a bottleneck in the process of obtaining mutants. Optimization of this process is therefore required.

Expression and purification in high-throughput worked efficient. Some mutants showed to be insoluble under the used conditions. Using ThermoFAD, 34 mutants with a $\Delta Tm^{app} \geq 0.5$ °C were identified. Of these 34, 13 had a $\Delta Tm^{app} \geq 1.0$ °C. 39 mutations led to a decrease of $\Delta Tm^{app} \geq -0.5$ °C and 22 with $\Delta Tm^{app} \leq -0.5$ °C. For combined mutations, a fourfold mutant was created with an increased $\Delta Tm^{app}$ of 7

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# 7. Appendix A

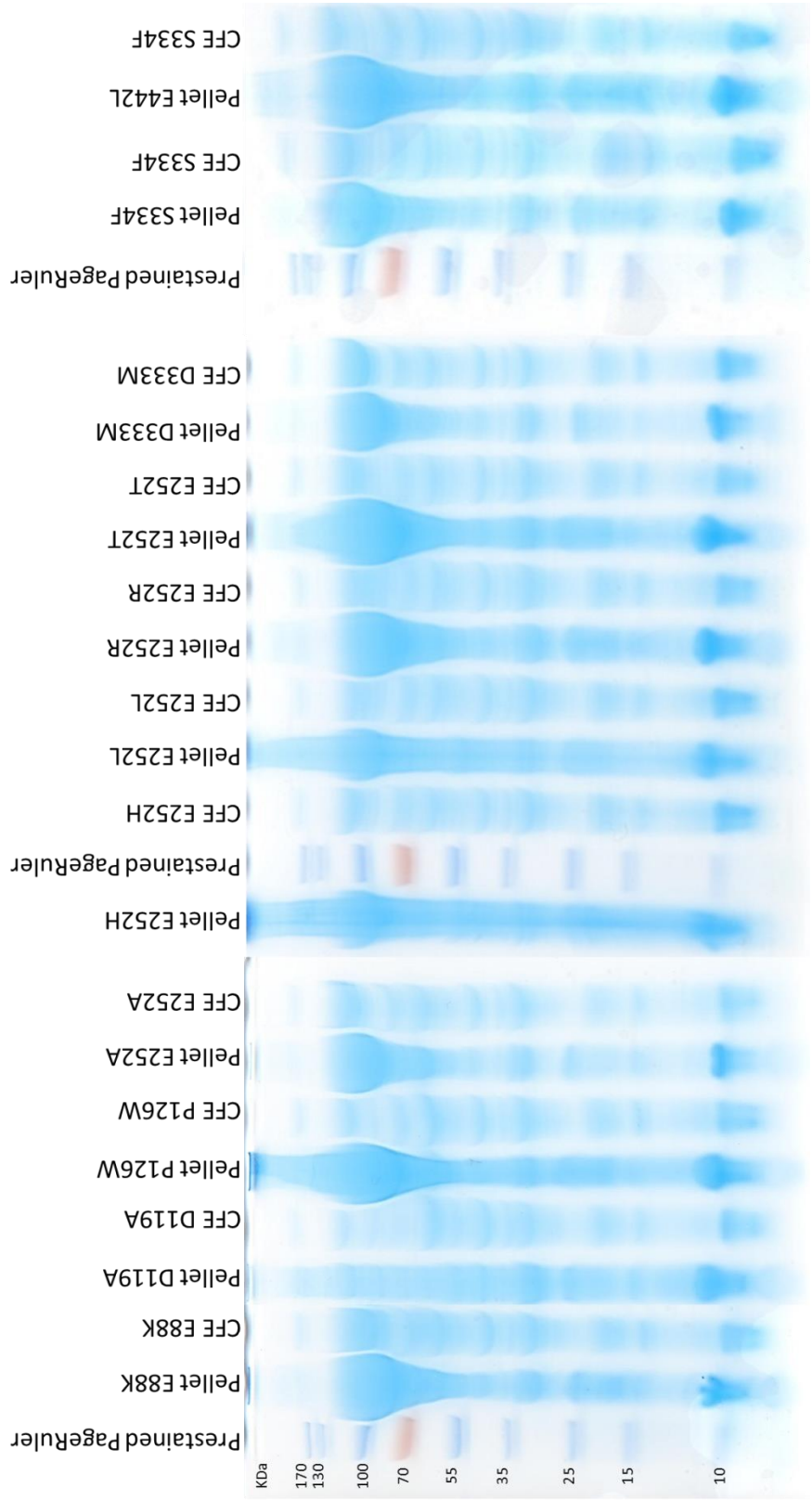
| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | S62A | A115V | D119K | E121Q | S196K | E202R |
| **B** | D233K | E264Q | E264R | N266W | E271A | G278A |
| **C** | A290W | E297Q | E312k | E338S | E346T | D404K |
| **D** | D407S | Q409P | E446A | S449T | D450T | |

**A 1 Overview of mutants for which a new QuickChange PCR was performed**

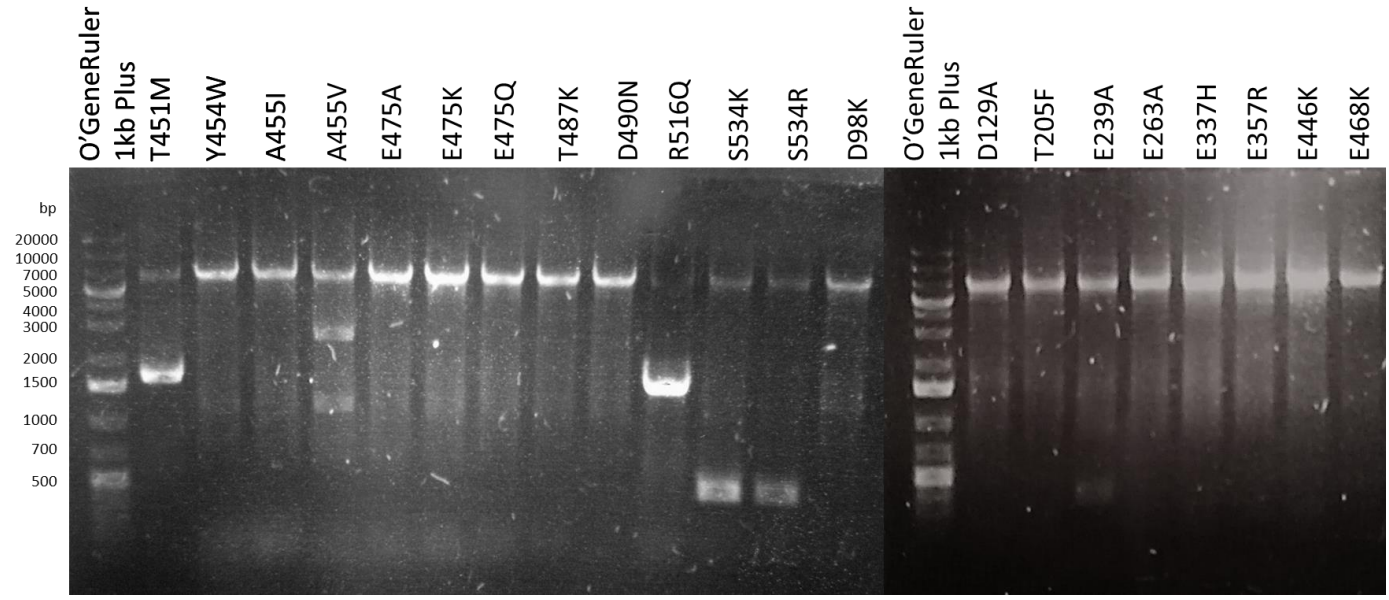
**A 2 Overview of different transformation rounds and their conditions. [1] As the wells of the PCR plate were empty 5 µL of sterile MiliQ was added to each well. [2] DpnI was inactivated for the PCR digest. [3] The recovered cells (1000 µL) were divided over two Eppendorf cups of which one was spun down after which the pellet was resuspended and 20 µL was plated and. Of the other sample 20 µL was directly plated onto 24 well agar plate. [4] The cells should have been spun down for only 30 s - 2 min, too long**

| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 | Round 6 | Test DWP | FRESCO II |
|---|---|---|---|---|---|---|---|---|
| **Container** | 96 PCR plate | 96 DWP | 96 PCR plate | 96 PCR plate | Eppendorf cups | Eppendorf cups | 96 DWP | 96 DWP |
| **Amount comp. cells (µL)** | 20 | 50 | 20 | 20 | 50 | 50 | 50 | 40 |
| **Amount PCR digest (µL)** | 5 | 5 | 0.5 | 1 | 5 | 5[1] | 5 | 5[2] |
| **Heat shock time (s)** | 10 | 120 | 10 | 10 | 45 | 45 | 45 | 45 |
| **Heat hock method at 42°C** | Water bath | Water bath | Water bath | Water bath | Heat block | Heat block | Water bath | Water bath |
| **SOC (µL)** | 150 | 500 | 200 | 200 | 500 | 500 | 1000 | 1000 |
| **Spun down (y/n)** | n | y | y | y | y | y | y/n[3] | y |
| **Time and speed** | | 10 min, 3600 rpm | 20 min, 3600 rpm | 20 min, 3600 rpm | 20 min, 13500 rpm | 20 min, 13500 rpm | 20 min, 3700 rpm[4] | 20 min, 3700 rpm[4] |
| **Supernatant removed (µL)** | | 300 | 180 | 180 | 400 | 400 | 480 | 970 |
| **Amount plated (µL)** | 50 | 50 | 40 | 40 | 50 | 50 | 20 | 20 |
| **Spreading/drying method** | Small glass beads | Small glass beads | Small glass beads | Small glass beads | Air dried | Air dried | Air dried | Small glass beads |
| **Percentage of mutants for which colonies were obtained** | 6 | 0 | 53 | 6 | 91 | 15 | Concentrated: 80<br>Not concentrated: 40 | 10 |
| **Amount of mutants for transformation** | 93 | 93 | 93 | 93 | 23 | 27 | 5 | 21 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | D10H | D10Y | A11M | A11V | H25F | H25L | T35V | S62A | H63F | H63W | H63Y | T82W |
| **[Protein] µM** | 10.07 | 6.71 | 5.37 | 12.08 | 8.05 | 4.70 | 12.08 | 9.40 | 10.07 | 12.08 | 12.08 | 8.05 |
| **B** | E88K | E88Q | E91K | E91Q | A115I | A115L | A115V | D119A | D119K | D119P | D119R | D119S |
| **[Protein] µM** | 4.70 | 30.20 | 13.42 | 16.78 | 10.07 | 12.08 | 14.09 | 2.68 | 13.42 | 8.72 | 8.72 | 8.05 |
| **C** | D119T | D120K | D120T | E121Q | T164I | T164L | T164V | T167V | A169R | S196K | E200Q | E200T |
| **[Protein] µM** | 11.41 | 10.74 | 10.07 | 6.04 | 2.01 | 10.74 | 14.77 | 3.36 | 2.68 | 2.68 | 1.34 | 3.36 |
| **D** | E202R | T205I | T205V | T205W | P216W | I230W | D233K | E252A | E252H | E252K | E252L | E252P |
| **[Protein] µM** | 11.41 | 10.74 | 10.07 | 6.04 | 2.01 | 10.74 | 14.77 | 3.36 | 2.68 | 2.68 | 1.34 | 3.36 |
| **E** | E252R | E252T | E264Q | E264R | E264T | N266W | E271A | D274K | G278A | R280Y | A290W | E293T |
| **[Protein] µM** | 0.67 | 1.34 | 7.38 | 5.37 | 5.37 | 6.71 | 6.71 | 6.71 | 8.72 | 18.79 | ND | 4.70 |
| **F** | E297Q | E309K | E312K | T316I | T316K | D333A | D333M | S334F | Y337W | E338S | E346T | A349F |
| **[Protein] µM** | 5.37 | 5.37 | 3.36 | 6.04 | 3.36 | 9.40 | 4.70 | 4.03 | 4.03 | 8.72 | 5.37 | 3.36 |
| **G** | E357K | H371I | E372T | D383H | D398K | D398N | D398R | D404K | D404N | D407Q | D407R | D407S |
| **[Protein] µM** | 6.71 | 3.36 | 4.70 | 6.71 | 8.05 | 10.07 | 4.03 | 3.36 | 2.01 | 6.71 | 8.72 | 6.04 |
| **H** | Q409P | N431M | N431Y | S440T | E442L | E446A | S449T | D450R | D450T | | WT | WT |
| **[Protein] µM** | 4.70 | 4.70 | 6.04 | 8.72 | 1.34 | 6.04 | 3.36 | 1.34 | 5.37 | | 4.03 | 8.72 |




**A 3 SDS-PAGE of the pellet and cell free extract after lysis of mutant which gave no fluorescence signal in ThermoFAD measurements**




**A 4 Agarose (1%) gel of mutants from the FRESCO II library**

**A 5 Overview of single point mutations with their corresponding melting temperatures and standard deviation. Mutant A290W was not obtained from QuickChange PCR. ND: not determined.**

| Mutation | Average $Tm^{app}$ (°C) | ΔTm (°C) | st. dev. | Mutation | Average $Tm^{app}$ (°C) | ΔTm (°C) | st. dev. | Mutation | Average $Tm^{app}$ (°C) | ΔTm (°C) | st. dev. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **D10H** | 36.00 | 0.00 | 0.00 | **E202R** | 36.00 | 0.00 | 0.00 | **A349F** | 36.50 | 0.50 | |
| **D10Y** | 37.00 | 1.00 | 0.00 | **T205F** | 36.50 | 0.50 | 0.00 | **E357H** | 33.50 | -2.50 | 0.00 |
| **A11M** | 37.00 | 1.00 | 0.00 | **T205I** | 36.25 | 0.25 | 0.25 | **E357K** | 36.67 | 0.67 | 0.24 |
| **A11V** | 35.25 | -0.75 | 0.25 | **T205V** | 36.33 | 0.33 | 0.47 | **E357R** | 36.00 | 0.00 | 0.00 |
| **H25F** | 35.25 | -0.75 | 0.25 | **T205W** | 36.75 | 0.75 | 0.25 | **H371I** | 36.50 | 0.50 | 0.50 |
| **H25L** | 34.75 | -1.25 | 0.25 | **P216W** | | ND | | **E372T** | 36.25 | 0.25 | 0.25 |
| **T35V** | 36.17 | 0.17 | 0.24 | **I230W** | | ND | | **D383H** | 32.50 | -3.50 | 0.50 |
| **S62A** | 32.50 | -3.50 | 0.00 | **D233K** | 33.75 | -2.25 | 0.25 | **D398K** | 36.50 | 0.50 | 0.41 |
| **H63F** | 32.25 | -3.75 | 0.25 | **E239A** | 37.25 | 1.25 | 4.25 | **D398N** | 36.00 | 0.00 | 0.00 |
| **H63W** | | ND | | **E252A** | | ND | | **D398R** | 36.33 | 0.33 | 0.24 |
| **H63Y** | 35.50 | -0.50 | 0.50 | **E252H** | | ND | | **D404K** | 36.00 | 0.00 | 0.00 |
| **T82W** | 31.00 | -5.00 | 0.50 | **E252K** | | ND | | **D404N** | 36.33 | 0.33 | 0.24 |
| **E88K** | | ND | | **E252L** | | ND | | **D407Q** | 34.25 | -1.75 | 0.25 |
| **E88Q** | | ND | | **E252P** | | ND | | **D407R** | 34.33 | -1.67 | 0.62 |
| **E91K** | 37.17 | 1.17 | 0.24 | **E252R** | | ND | | **D407S** | 36.00 | 0.00 | 0.50 |
| **E91Q** | 37.50 | 1.50 | 0.41 | **E252T** | | ND | | **Q409P** | 40.33 | 4.33 | 0.24 |
| **D98K** | 36.75 | 0.75 | 0.25 | **E263A** | 36.50 | 0.50 | 0.50 | **N431M** | 35.50 | -0.50 | 0.00 |
| **A115I** | 35.17 | -0.83 | 0.47 | **E264Q** | 36.00 | 0.00 | 0.00 | **N431Y** | 39.67 | 3.67 | 0.62 |
| **A115L** | 35.75 | -0.25 | 0.25 | **E264R** | 35.75 | -0.25 | 0.25 | **S440T** | 35.00 | -1.00 | 0.00 |
| **A115V** | 38.00 | 2.00 | 0.00 | **E264T** | 36.25 | 0.25 | 0.25 | **E442L** | | ND | |
| **D119A** | | ND | | **N266W** | 36.00 | 0.00 | 0.00 | **E446A** | 33.00 | -3.00 | |
| **D119K** | 36.83 | 0.83 | 0.24 | **E271A** | 36.67 | 0.67 | 0.47 | **E446K** | 32.00 | -4.00 | 0.79 |
| **D119P** | 36.67 | 0.67 | 0.24 | **D274K** | 35.83 | -0.17 | 0.24 | **S449T** | 36.83 | 0.83 | 0.62 |
| **D119R** | 37.00 | 1.00 | 0.50 | **G278A** | 31.17 | -4.83 | 0.62 | **D450R** | 36.25 | 0.25 | 0.75 |
| **D119S** | 36.50 | 0.50 | 0.41 | **R280Y** | 37.50 | 1.50 | | **D450T** | 36.50 | 0.50 | 0.41 |
| **D119T** | 36.33 | 0.33 | 0.24 | ~~**A290W**~~ | | | | **T451M** | 36.50 | 0.50 | 0.00 |
| **D120K** | 36.00 | 0.00 | | **E293T** | 35.50 | -0.50 | 0.00 | **Y454I** | 36.00 | 0.00 | 0.50 |
| **D120T** | 36.00 | 0.00 | 0.00 | **E297Q** | 35.75 | -0.25 | 0.25 | **A455I** | 36.75 | 0.75 | 0.25 |
| **E121Q** | 35.83 | -0.17 | 0.24 | **E309K** | 36.17 | 0.17 | 0.47 | **A455V** | 37.67 | 1.67 | 0.24 |
| **D129A** | 36.50 | 0.50 | 0.00 | **E312K** | 35.75 | -0.25 | 0.25 | **E457A** | 36.50 | 0.50 | 0.50 |
| **T164I** | 36.83 | 0.83 | 0.24 | **T316I** | 36.33 | 0.33 | 0.47 | **E468K** | 36.13 | 0.13 | 0.41 |
| **T164L** | 37.17 | 1.17 | 0.24 | **T316K** | 35.75 | -0.25 | 0.25 | **E475K** | 36.25 | 0.25 | 0.25 |
| **T164V** | 36.17 | 0.17 | 0.24 | **D333A** | 33.00 | -3.00 | | **E475Q** | 36.25 | 0.25 | 0.25 |
| **T167V** | 35.50 | -0.50 | 0.00 | **D333M** | | ND | | **T487K** | 34.50 | -1.50 | 0.00 |
| **A169R** | 36.50 | 0.50 | 0.41 | **S334F** | | ND | | **D490N** | 32.75 | -3.25 | 0.25 |
| **S196K** | 33.50 | -2.50 | 0.00 | **Y337W** | 36.00 | 0.00 | 0.41 | **R516Q** | 27.50 | -8.50 | 0.00 |
| **E200Q** | 35.00 | -1.00 | | **E338S** | 36.00 | 0.00 | 0.00 | **S534K** | 36.75 | 0.75 | 0.20 |
| **E200T** | 35.50 | -0.50 | 0.41 | **E346T** | 36.50 | 0.50 | 0.00 | **S534R** | 37.00 | 1.00 | 0.00 |