Universität für Bodenkultur Wien
University of Natural Resources
and Life Sciences, Vienna

Master Thesis

Molecular Engineering of a Biorecognition Element for Glucose Biosensors

Submitted by

Andreas SCHULLER, BSc

in the framework of the Master program

Biotechnology

in partial fulfilment of the requirements for the academic degree

Diplom-Ingenieur

Vienna, October 2021

Supervisor:

Ass. Prof. Dipl.-Ing. Dr. Roland Ludwig
Institute of Food Technology
Department of Food Science and Technology

Affidavit

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

VIENNA, October 2021 Andreas SCHULLER *(manu propria)*

Preface

The research presented in this master thesis was conducted in the framework of the Austrian Science Fund (FWF) project “Routing the flow of electrons from oxidoreductases to electrodes” with the project number P 31019-N28.

Acknowledgement

I would like to thank my supervisor **Ass.Prof. Dipl.-Ing. Dr. Roland Ludwig** for his scientific support and the chance to pursue this thesis in his research group.

A special thank you goes to **Franziska Schachinger, MSc** for her guidance during my work, her constant support and all the lively scientific discussions. I also greatly appreciate the trust she paid me when complex tasks had to be fulfilled.

Furthermore, I want to thank all other members of the **LudwigLab – Biocatalysis & Biosensing Research Group** for warmly welcoming me in the group and their help and support in the laboratory whenever I needed it.

Content

Abstract

Over the last years, the interest in fungal FAD-dependent glucose dehydrogenases (GDH) for the application in biosensors has grown immensely. These fungal GDHs exhibit superior properties, e.g. a high substrate specificity and turnover number for glucose, which is needed for the application in blood glucose biosensors. For this study, GDH of the ascomycete *Glomerella cingulata* (*Gc*GDH) was linked enzymatically with cytochrome *cz* with sortase A to achieve direct electron transfer (DET) from the catalytic dehydrogenase subunit (DH) to the electrode without the need of a mediator, as the GDH itself cannot directly transfer electrons to the electrode. To facilitate intraprotein electron transfer, the interface of *Gc*GDH was mutated to clear the interface both sterically (removal of one glycosylation site, A53P_N54D) and electrostatically (exchange of lysine, K480G). Both modified *Gc*GDH subunits as well as the wildtype enzyme were expressed in *Pichia pastoris* X33 and produced in a 5-L fermenter. Cytochrome *cz* was expressed by *Escherichia coli* BL21 in shaking flasks as well as in a high-density fermentation in a 5-L fermenter. A chimeric fusion enzyme was created by linking *Gc*GDH_K480G to the cytochrome *cz* by transpeptidation (sortagging). The chimeric enzyme Cyt*cz*-*Gc*GDH_K480G was then successfully produced and a purification strategy was developed. In pH dependent activity profiles, the parental enzyme *Gc*GDH_K480G showed a similar specific activity (235 U/mg) as the chimeric enzyme Cyt*cz*-*Gc*GDH_K480G (217 U/mg). Both enzymes showed a lower activity than the wild-type *Gc*GDH (314 U/mg) at the pH optimum of pH 6. The spectroscopic investigation of the cytochrome showed the successful reduction of the cytochrome upon glucose addition when *Gc*GDH subunits and cytochrome *cz* subunits were combined unlinked and for the chimeric fusion enzyme as well. In final amperometric experiments, the subunits were tested unlinked but also the chimeric enzyme. The unlinked *Gc*GDH_K480G (35.0 nA) achieved higher currents than the chimeric enzyme (5.1 nA) upon glucose addition. Nevertheless, the concept of chimeric fusion enzymes exhibiting DET for the application on biosensors could be proven.

Abstrakt

In den letzten Jahren wuchs das Interesse an Glucose-Dehydrogenasen (GDH) von Pilzen, die FAD als Cofaktor beherbergen, immens. Diese GDHs besitzen sehr vorteilhafte Eigenschaften, wie hohe Substratspezifität und Stoffumsatz, die eine Anwendung auf Biosensoren ermöglichen. Für diese Untersuchung wurde die GDH des Ascomyceten *Glomerella cingulata* enzymatisch mit Cytochrom *cz* mithilfe von Sortase A verbunden um direkten Elektronentransfer (DET) von der katalytischen Dehydrogenase-Untereinheit (DH) ohne Mediator zu ermöglichen, denn GDHs allein sind dazu nicht fähig. Um den Elektronentransfer innerhalb des Proteins zu erleichtern, wurden über dem aktiven Zentrum etwaige sterische (Glykosylierungsstellen, A53P_N54D) und elektrostatische (Lysinrest, K480G) Behinderungen entfernt. Die beiden modifizierten *Gc*GDH-Untereinheiten, sowie das Wildtyp-Enzym, wurden in *Pichia pastoris* X33 exprimiert und in einem 5 L-Fermenter hergestellt. Cytochrom *cz* wurde in *Escherichia coli* BL21 exprimiert und in Schüttelkolben hergestellt. Zusätzlich wurde eine Hochdichte-Fermentation in einem 5 L-Fermenter versucht. Mithilfe von Transpeptidierung (Sortagging) wurde *Gc*GDH_K480G mit Cytochrom *cz* verbunden, um ein chimäres Protein herzustellen. Für dieses wurde im Anschluss eine Aufreinigungsstrategie entwickelt. In pH-abhängigen Aktivitätsprofilen zeigten *Gc*GDH_K480G (235 U/mg) und die chimäre Cyt*cz*-*Gc*GDH_K480G (217 U/mg) ähnliche spezifische Aktivitäten. Beide Spezies lagen aber deutlich unter der spezifischen Aktivität der Wildtyp *Gc*GDH (314 U/mg) am pH-Optimum von pH 6. Spektroskopische Experimente zeigten die erfolgreiche Reduktion des Cytochrom cz bei Zugabe von Glucose, sowohl bei den unverbundenen Spezies in Lösung als auch beim chimären Protein. In amperometrischen Experimenten wurden die Untereinheiten unverbunden, sowie als chimäres Protein getestet. Mit der unverbundenen *Gc*GDH_K480G (35.0 nA) konnten höhere Stromstärken als mit dem chimären Protein (5.1 nA) erreicht werden. Nichtsdestotrotz konnte das Konzept der chimären Enzyme für DET auf Biosensoren bewiesen werden.

1 Introduction

The interest in the FAD-dependent glucose dehydrogenase of the ascomycete *Glomerella cingulata* (*Gc*GDH) has increased over the last years for application in glucose biosensor for blood glucose monitoring in diabetic patients as well as for biofuel cells [1], [2]. *G. cingulata* (anamorph: *Colletotrichum gloeosporioides*) is known to be a plant pathogen which causes anthracnose in a variety of different cultivated and non-cultivated plants. Extracellular GDH is believed to play a role in the mechanism of pathogenicity via the reduction of phenoxy radicals and quinones produced by the plant to defend itself against fungal invasion [3]. Nevertheless, fungal FAD-dependent GDHs exhibit very beneficial properties for the application in biosensors because of their thermostability, high turnover rate, substrate specificity and oxygen independence [4], [5]. Oxygen independence of GDH is a major advantage over glucose oxidases (GOx) used in biosensors which is affected by the presence of oxygen leading to errors when determining blood glucose [6], [7]. FAD-dependent GDHs generally catalyze the reaction:

D-glucose + quinone → D-glucono-1,5-lactone + quinol

Biosensors can be categorized in different generations (Figure 1). First generation sensors sense the consumption of O_2 or the formation of H_2O_2 and therefore usually employ oxidases as recognition elements. Second generation biosensors, where an enzyme is combined with a mediator to achieve mediated electron transfer to the electrode (MET), are investigated since 1984 [8]. The concept of third generation biosensors eliminates the need of sometimes toxic redox mediators with also high redox potentials that are prone for interfering signals as the enzyme itself is able to transfer electrons generated from substrate conversion directly to the electrode (DET). Because DET properties of fungal glucose dehydrogenase are not commonly observed due to their structure with the FAD cofactor deeply buried inside the enzyme [9], there is the need for an electron-transferring domain which must be connected to the enzymatic unit [10], [11]. For this study, cytochrome *cz* from *Chlorobaculum tepidum* was linked to the N-terminus of *Gc*GDH by transpeptidation using sortase A from *Staphylococcus aureus* to create a chimeric enzyme for the application on

electrodes. Such fused enzymes occur also in nature and are the template for this artificial fusion enzymes (e.g. cellobiose dehydrogenase) [12].

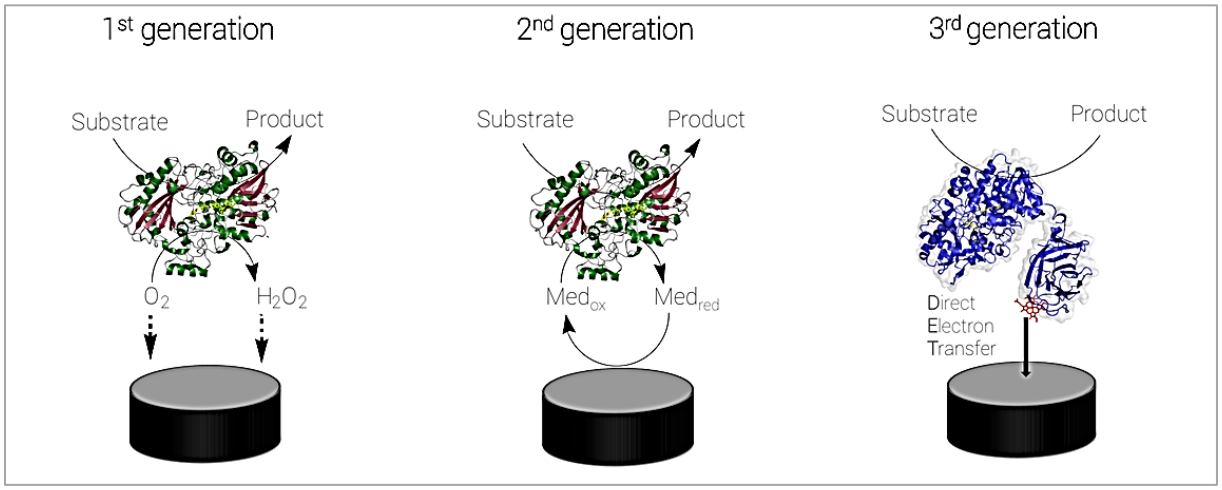

Figure 1: Generations of biosensors (taken from: https://www.directsens.com/technology-biosensors/).

Production of *Gc*GDH and cytochrome *cz* poses many challenges as both subunits cannot be expressed by the same expression organism. *Gc*GDH can only be functionally expressed by eukaryotic systems (*P. pastoris*) where it is controlled by a methanol-inducible AOX promoter and secreted into the supernatant upon induction [13]. Also, the extremely inefficient expression of *Gc*GDH in *E. coli* is reported [13]. Cytochrome *cz* on the other hand needs to be expressed in *E. coli* recombinantly, where 8 additional proteins (ccmA-H) must be cloned into the host beside the gene coding for cytochrome *cz*. This protein cassette is needed to efficiently incorporated heme into cytochrome *cz* and release it into the periplasmic space [14], [15]. *P. pastoris* however is not able to produce cytochrome *c* because a different cytochrome maturation is used [16]. Once produced and purified, both subunits are linked enzymatically with the transpeptidase sortase A of *Staphylococcus aureus* known as “sortagging” [17].

In this study, different mutations are introduced to the enzymatic *Gc*GDH subunit to alter the interface of the enzyme. The main goal of these mutations is the modification of the enzyme interface to facilitate access of cytochrome *cz* to withdraw electrons

taken up by FAD in the active site after the enzymatic reaction. The interface is cleared both sterically by altering a glycosylation site (Figure 2, C) as well as electrostatically by replacing a lysine residue with glycine (K480G, Figure 2, B). All variants carry a N-terminal linker for sortagging. The *Gc*GDH and cytochrome *cz* subunits are produced by fermentation and then, the biochemical and electrochemical performance of the sole *Gc*GDH subunits and the linked fusion enzyme are investigated.

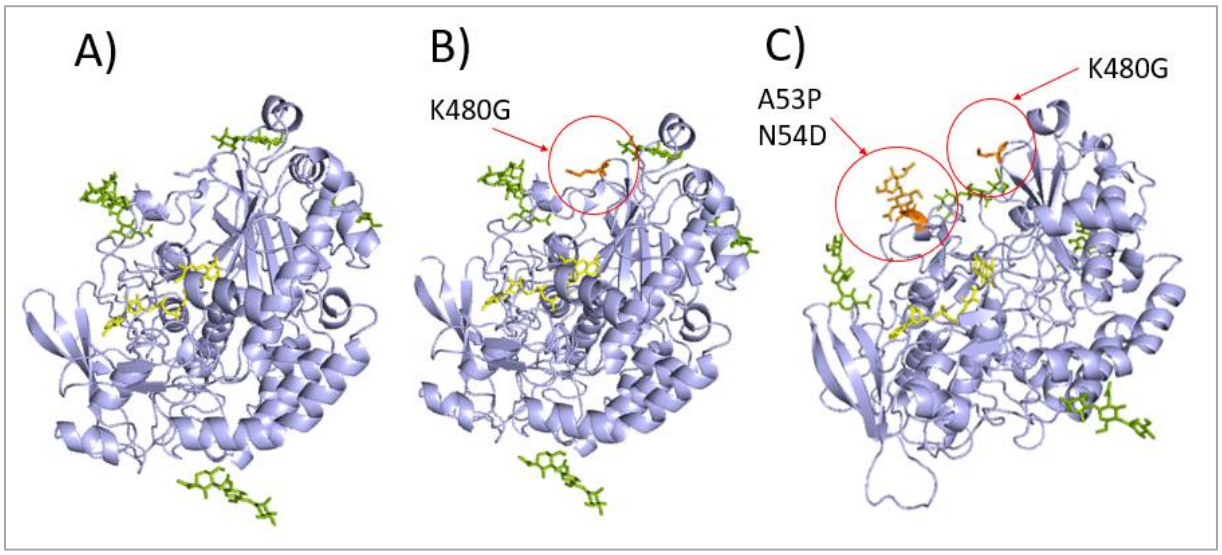

Figure 2: Comparison of the GcGDH variants. A, GcGDH_WT, B, GcGDH_K480G, C, GcGDH_A53P_N54D_K480G. FAD cofactor is shown in yellow, glycosylations are shown in green and mutations are shown in orange.

2 Material and methods

2.1 Used standard media

The following compositions for the standard media were used for the preparation of liquid media. In case plates had to be casted, 15 g/L agar agar was added. After preparation, the media were sterilized by autoclaving for 15 min at 121 °C. In case the standard media were supplemented with antibiotics, the readily prepared stock solutions in the laboratory were used. Usually, one thousandth of the culture volume of antibiotic stock was added after autoclaving to end up with optimal cultivation conditions.

Table 1: Used antibiotic concentrations in standard media

Antibiotic	Stock concentration	Concentration in culture
Ampicillin	100 mg/mL	100 mg/L
Chloramphenicol	35 mg/mL	35 mg/L
Zeocin	100 mg/mL	Yeast: 100 mg/L
		Bacteria: 25 mg/L

For induction, one thousandth of the culture volume of 100 mM IPTG stock solution was used.

2.1.1 Luria Broth and low-salt Luria Broth

Table 2: Composition of LB medium.

LB medium	
Peptone from casein	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

Luria broth (LB medium) was used for the cultivation of bacteria. When it was used in combination with zeocin, the concentration of NaCl was reduced to 5 g/L in the low-salt LB medium (LS-LB medium).

2.1.2 Yeast extract peptone dextrose medium

Table 3: Composition of YPD medium.

YPD medium	
Peptone from casein	20 g/L
Yeast extract	10 g/L
D-glucose	4 g/L

The Yeast extract peptone dextrose (YPD) medium was used for the cultivation of yeast cultures.

2.1.3 Terrific Broth medium

Table 4: Composition of TB medium.

TB medium	
Peptone from casein	10 g/L
Yeast extract	24 g/L
Glycerol	4 mL/L
1 M potassium phosphate buffer pH 7.5	100 mL/L

The terrific broth (TB) medium was used for the cultivation of *E. coli* NEB5α for the preparation of electrocompetent cells.

2.1.4 Super Optimal broth with Catabolite repression

When a super optimal broth with catabolite repression (SOC) medium was needed, the readily prepared SOC Outgrowth Medium of New England Biolabs (NEB, Catalog number: B9020 S) was used.

2.1.5 Buffered Glycerol Complex medium

Table 5: Composition of BMGY medium.

BMGY medium	
Peptone from casein	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L
1 M potassium phosphate buffer pH 6.0	100 mL/L

The buffered glycerol complex (BMGY) medium was used for the cultivation of yeast cultures when protein was expressed. The buffer in the medium is required to keep the pH constant while protein expression.

2.2 Preparation of electrocompetent *E. coli* cells

Six hundred mL of TB medium were inoculated with a fresh overnight culture of *Escherichia coli* NEB5α and incubated with 160 rpm of shaking at 37°C. The culture was harvested at around 1 OD_{600} and centrifuged. From here on cell have to be kept cold by working over ice. Afterwards, the pellet was resuspended in 250 mL ice-cold glycerol (10 % in water) and centrifuged, resuspended in 150 mL glycerol (10 %) and again centrifuged. The remaining pellet was again resuspended in 20 mL glycerol (10 %) and centrifuged before being resuspended in 1 mL glycerol (10%). All centrifugation steps were carried out with 4000 rpm at 4°C for 15 min. The aliquots of 50 µL were stored at -80 °C. All other electrocompetent cells used such

as *E. coli* BL21 for protein expression have already been prepared following this protocol and have therefore been available.

2.3 Transformation of cells

The different *Glomerella cingulata* glucose dehydrogenase (*Gc*GDH) domains were all expressed in *Pichia pastoris* X33, whereas the cytochrome *cz* domain was expressed in *E. coli* BL21. Additionally, all plasmids were transformed into *E. coli* NEB5α.

2.3.1 Transformation of cells by electroporation

For electroporation of *E. coli* NEB5α, around 200 ng of the plasmid (either pET-22b(+) or pPICZA) were used. The pET-22b(+) plasmid contains the gene for the cytochrome *cz* and an ampicillin resistance cassette, the pPICZA plasmid contains the gene for the *Gc*GDH domain and a zeocin resistance. Generally, the pET-22b(+) vector was used for expression in *E. coli*, whereas the pPICZA vector was used for expression in *P. pastoris*. pPICZA also carries an ORI (Origin of Replication) suitable for *E. coli* cells. The plasmid was mixed with 50 µL of bacterial suspension and transferred to a chilled electroporation cuvette. After electroporation with voltage of 1.8 kV and pulse length of 1.8 msec, 500 µL of SOC medium were added and the cells were incubated on a shaker with 450 rpm at 37°C for one hour in to develop the antibiotic resistance. When carrying the pET-22b(+) plasmid, cells were plated on LB medium plates containing ampicillin. If the pPICZA plasmid was introduced, LS-LB medium plates containing zeocin were used. Positive colonies were visible on the following day. Although *E. coli* NEB5α was not used for protein production, all constructs were transformed into these cells to facilitate plasmid preparation, sequencing and long-term storage of the plasmid.

Prior to the electroporation of *P. pastoris* X33, the pPICZA plasmid was linearized by a SacI-HF (New England Biolabs) restriction. Other than for *E. coli*, *P. pastoris* cells require the linearized plasmid in order to insert the gene of interest into their genome. For the restriction, a DNA concentration of around 50 ng/µL in 50 µL is optimal. For the restriction digest, an incubation phase of 10 min at 37°C was followed by a deactivation phase of the restriction enzyme of 20 min at 65°C. All components of the SacI

restriction can be seen in Table 6. The added volumes of nuclease free water and plasmid DNA depended on the concentration of the original plasmid DNA.

Table 6: Components of the SacI restriction.

SacI restriction	
SacI restriction enzyme	2 µL
CutSmart 10x buffer	5 µL
Plasmid DNA (185 ng/µL)	15 µL
Nuclease free water	28 µL
	50 µL

For electroporation of *P. pastoris* X33, around 100-200 ng of the linearized pPICZA plasmid containing a zeocin resistance and the gene for the *Gc*GDH domain and a homologous region for integration under the AOX (alcohol oxidase) promoter were mixed with 50 µL suspension of electrocompetent cells in a chilled electroporation cuvette. Immediately after electroporation with voltage of 2.0 kV and pulse length of 3.0 msec, 500 µL of ice cold 1 M sorbitol were added. Afterwards, 500 µL of YPD medium were added and the cells were horizontally incubated on a shaker with 100 rpm at 30°C for 3-4 h to give the *P. pastoris* cells time to restore their cell wall and to develop the antibiotic resistance. The cells were then plated on YPD medium plates containing zeocin. Positive colonies were visible after around two days.

2.3.2 Transformation of cells by heat shock

For heat shock transformation, around 400 ng of pET-22b(+) plasmid (containing the gene for the cytochrome *cz* and an ampicillin resistance) and 400 ng of the pEC86 plasmid (containing the cytochrome *c* maturation cassette and a chloramphenicol resistance) [15] were first mixed and then transferred to 50 µL of heat shock competent *E. coli* BL21 cells and incubated for 30 min on ice. Usually, lower concentrations such as 200 ng plasmid are sufficient for the transformation but because of the double transformation, the transformation efficiency with both plasmids would be to low when low amounts of DNA are added.

Afterwards, the cells were heated to 42°C for 60 sec and were subsequently placed back to ice for 5 min, then 500 µL of SOC medium were added. After 10 min on ice, the cells were incubated on a shaker with 450 rpm at 37°C for one hour. One hundred µL of the cell suspension, as well as the complete pellet after centrifugation, were platted on two separate LB medium plates containing both, ampicillin and chloramphenicol for selection of double transformed clones. The plates were incubated at 37°C. Positive colonies were visible on the following day.

2.4 Plasmid preparation

To verify that a gene was incorporated correctly and has no unwanted mutations, a plasmid preparation followed by sequencing of the plasmid was performed. For this purpose, all plasmids were transformed into *E. coli* NEB5α as well (without the pEC86 plasmid).

For extraction of the plasmid from the cells, 2 mL of either LB medium or LS-LB medium (in case zeocin was used as an antibiotic) were inoculated with a single positive *E. coli* NEB5α colony from the plate. After 16 to 24 h, a plasmid preparation of the culture was performed with the Monarch Plasmid Miniprep Kit (NEB). The DNA was eluted in autoclaved milliQ water instead of the elution buffer because the elution buffer can cause interferences with follow up experiments such as the sequencing. The plasmid DNA was then prepared in a dilution suitable for the requirements of the sequencing company and were sent to an external company for Sanger sequencing using standard primers for pET or pPIC vector, respectively.

2.5 PCR and mutagenesis of the *Gc*GDH gene

The pPICZA plasmid containing the *Gc*GDH_WT gene or *Gc*GDH_K480G variant gene were obtained from BioCat. The *Gc*GDH_K480G variant then served as a PCR template to introduce further mutations to achieve the *Gc*GDH_A53P_N54D_K480G variant. Mutagenesis was performed with the Q5 Site directed Mutagenesis Kit (NEB). The PCR reaction was prepared according to Table 7. The template DNA was obtained

by a plasmid preparation of *E. coli* NEB5α. During this reaction, a PCR using primers that carry the mutated codons were designed and the whole plasmid was copied by PCR. Because the original non-mutated plasmid templated derived from *E. coli* had the mutation, but the mutated products generated PCR is not methylated, the restriction enzyme (DpnI) in the KLD (Kinase, Ligase and DnpI) only digest the template. Therefore, only mutated DNA will remain. For optimal digest of nonmutated plasmid it is important to keep the amount of template added to the PCR reaction at a minimum.

Table 7: PCR reaction for the mutagenesis.

PCR reaction mixture	
Q5 Hot Start High-Fidelity 2x Master Mix	12.5 µL
10 µM Forward Primer	1.25 µL
10 µM Reverse Primer	1.25 µL
Template DNA (1-25 ng/µL)	1 µL
Nuclease-free water	9 µL
	25 µL

Suitable forward and reverse primers to anneal to the desired DNA segments were designed and ordered from Microsynth. To ensure perfect primer annealing, PCR was performed with an annealing temperature gradient between 60°C and 65°C. The outcome of the PCR reaction was controlled by agarose gel electrophoresis and the bands were afterwards extracted again. Afterwards, a KLD digest according to Table 8 was performed to demethylate the DNA. The mixture was incubated for 5 min.

Table 8: KLD digest of the PCR product.

KLD digest	
PCR product	1 µL
2x KLD Reaction Buffer	5 µL
10x KLD Enzyme Mix	1 µL
Nuclease-free water	3 µL
	10 µL

The reaction product was then transformed into *E. coli* NEB5α and after the appearance of positive colonies on plates, a plasmid preparation and Sanger sequencing was done to ensure correct incorporation of the mutation and that the plasmids were correctly ligated without errors in the reading frame.

2.6 Agarose gel electrophoresis

To control the outcome of a PCR and purify genetic material agarose gel electrophoresis was performed. A gel was casted with 30 mL agarose (0.8 %) containing 2 µL of SYBR safe. The PCR samples were mixed with DNA gel loading dye containing glycerol to ensure proper loading of the sample into the wells of the gel and was then loaded to the wells. Additionally, 5 µL of DNA marker (1 kb plus, NEB) were injected into one well to estimate the size of the DNA in the samples. A voltage of 10 V per cm of gel length was applied. As a buffer, TAE buffer (TRIS-Acetate-EDTA buffer) was used. After electrophoresis, a picture of the gel was taken in the GelDoc (BioRad). The bands on the gel were cut out under UV light and the genetic material was recovered with the Monarch DNA Gel Extraction Kit (NEB), if required.

2.7 Preparation of glycerol stocks

Glycerol stocks of all transformed organisms were prepared to create a culture collection for long-term storage of plasmids. For this purpose, a sterile glass tube with 2 mL of medium was inoculated with a single positive colony of the organism and was put on a shaker overnight at 37°C and shaking. YPD medium containing zeocin was used for *P*. pastoris cultures and LB medium (or LS-LB medium in case zeocin was used as an antibiotic) containing ampicillin and chloramphenicol was used for *E. coli* cultures. On the next day, 1 mL of this overnight culture was transferred into a cryotube and mixed with 1 mL of glycerol (30 %). The glycerol stocks were stored at -80 °C.

2.8 Determination of cell density and wet biomass

Cell density was determined by measuring the optical density at 600 nm (OD_{600}). If needed, the cell suspension was diluted to stay in a linear range of the curve between 0.1 and 0.8 OD_{600}. Wet biomass was determined by pipetting a defined sample volume into a pre-weighed sample tube. After the cells were centrifuged at maximum speed, the supernatant was pipetted off and the tube was weighed. Afterwards, the sample tube weight was subtracted.

2.9 Production of *Gc*GDH in *P. pastoris*

The different *Gc*GDH variants of the chimeric enzymes were expressed in *P. pastoris* X33 and fermentation was done in a 5-L Eppendorf BioFlo 120 bioreactor. The cells secrete the *Gc*GDH into the culture supernatant because a secretion tag was added at the beginning of the gene construct which facilitated purification after the fermentation. Because the protein background in the medium is much lower compared to crude extract, the purification of a target protein is much simpler. This is one of the advantages of the *P. pastoris* expression system.

For the pre-preculture, 20-40 mL of BMGY or YPD medium containing zeocin were inoculated with a single positive culture of *P. pastoris* X33 carrying the pPICZA plasmid with the *Gc*GDH gene. This culture was incubated on a shaker at 30°C overnight. The

complete preculture was then used for the inoculation of the preculture of 200-400 mL BMGY or YPD medium containing zeocin. The preculture was cultivated on a shaker at 30 °C until a suitable cell density was reached.

Table 9: Composition of the Pichia Basal Salts Medium.

Pichia Basal Salts Medium	
Chemical	Concentration
Phosphoric acid (85 %)	26.7 mL/L
$CaSO_4 \cdot 2\ H_2O$	1.18 g/L
K_2SO_4	18.2 g/L
$MgSO_4 \cdot 7\ H_2O$	14.9 g/L
KOH	4.13 g/L
Glycerol	40 g/L
Pichia Trace Metal solution (PTM)	4.35 mL/L

Table 10: Composition of the Pichia trace metal solution (PTM).

Pichia Trace Metal solution (PTM)	
Chemical	Concentration
$CuSO_4 \cdot 5\ H_2O$	6 g/L
NaI	0.08 g/L
$MnSO_4 \cdot H_2O$	3 g/L
$Na_2MoO_4 \cdot 2\ H_2O$	0.2 g/L
H_3BO_3	0.02 g/L
$CoCl_2$	0.5 g/L
$ZnCl_2$	20 g/L
$FeSO_4 \cdot 7\ H_2O$	65 g/L
Biotin	0.2 g/L
Sulfuric acid	5 mL/L

After assembly of the 5-L Eppendorf BioFlo 120 fermenter according to the Eppendorf BioFlo120 manual, 3.2 L of Pichia basal salt medium (Table 9) were poured into the vessel, the pH sensor (Mettler Toledo) was calibrated between pH 4 and pH 7, and the complete fermenter was autoclaved. Afterwards, the temperature was adjusted to 30 °C and the DO (dissolved oxygen) sensor was calibrated while stirring and airflow were already switched on. The DO sensor was calibrated with N_2 (0 % DO) and air (100 % DO). The pH was adjusted to 5.0 with ammonia. 14 mL sterile filtered PTM solution (which was already prepared and sterilized by filtration) (Table 10) were added through the septum to complete the medium. Afterwards, the fermenter was inoculated with the preculture. pH and temperature were kept constant during the fermentation. Ammonia was pumped into the vessel when the pH exceeded 5.0.

Base: 25 % ammonia solution

Sparging: compressed air, flow rate: 3.5 vvm (volume air per volume culture medium)

Stirrer speed: 700–800 rpm

After the glycerol in the initial media was used as carbon source during the batch phase, the glycerol feed was started. Ammonia is used as the nitrogen source.

Glycerol feed medium: 400 mL 50 % glycerol with 4.8 mL PTM solution

The batch phase is monitored based on the oxygen consumption. Because the cell density increases and more oxygen is needed, the DO level will decrease to almost zero in the first batch phase. At the end of the batch phase, when all the glycerol is used up and the cells cannot further grow and stop consuming oxygen, the DO increases immediately. When the DO in the fermenter rose - which is the case after approximately 20 h - above a certain set limit (20-40 %), glycerol feed medium was pumped automatically by the control unit into the fermenter and cells use oxygen again, resulting in DO spikes. Feeding with glycerol feed medium was done for around 8 h. During this fed batch phase, the biomass is increasing without induction of protein expression. After the glycerol fed batch phase, the feed medium was changed to methanol supplemented with PTM. Protein expression is induced by activating the AOX promoter, leaving methanol as the carbon source. In this phase, the cells also require ammonia base as nitrogen source which is added automatically by the control unit based on the pH level, which was maintained at pH 5.0.

Methanol feed: 1 L methanol with 12 mL PTM solution

During the expression phase, production of the target protein was monitored based on the activity of the *Gc*GDH with a DCIP assay (Section 2.15). As soon as the activity did not further increase anymore, the fermentation process was stopped and the culture was harvested. The expression phase lasted between 24 and 48 h. The culture was then centrifuged with 4000 rpm at 4 °C for 20 min. The cell pellet was discarded and the supernatant was taken for further purification.

2.10 Purification of *Gc*GDH

After fermentation, cells were removed by centrifugation (SLC6000 rotor, 4000 rpm, 20 min, 4°C) and the culture supernatant was vacuum filtered with a filter pore size of 3–5 µm. Subsequently, a diafiltration with a cellulose membrane (Sartorius Vivaflow

50 cassette for molecular weight cut-off: 10 kDa) was performed to decrease the volume of the supernatant and to equilibrate the sample to the start buffer for the following ion exchange chromatography (IEX).

2.10.1 Ion exchange chromatography (IEX)

Start buffer: 50 mM Tris, pH 5.5 – 6.5

Elution buffer: 50 mM Tris, 500 mM NaCl, pH 5.5 – 6.5

Theoretical pI (*Gc*GDH): 4.59 (Expasy, ProtParam tool)

Column: DEAE Sepharose, anion exchange, XK 26/20, 56 mL, GE Healthcare

System: GE Healthcare purification system (Äkta)

First, the self-packed column was washed from the 20 % ethanol storage solution to water, then to elution buffer and was then equilibrated with the start buffer until the pH in the column was adjusted. The sample was then loaded onto the column, proteins bound to the column due to their charge. This happened in case the pH applied in the column was at least one scale higher than the pI of the protein. To elute the bound enzyme again, the ionic strength in the column was increased by setting a linear gradient of high conductive elution buffer over around 5 column volumes (CV). The eluting fractions containing the *Gc*GDH were collected and concentrated by centrifuging with Amicon centrifugal tubes (MWCO: 10 kDa) with 4000 rpm at 4 °C.

2.11 Production of cytochrome *cz* in *E. coli*

The cytochrome *cz* domain of the later chimeric enzyme was obtained by fermentation. For this purpose, two different methods were performed and compared. The fermentation in all cases was carried out with *E. coli* BL21 carrying the pET-22b(+) plasmid (carrying the cytochrome *cz* gene) and the pEC86 plasmid (carrying the genes for the cytochrome *c* maturation cassette). The cytochrome *cz* is secreted into the periplasmic space because of the N-terminal *pelB* secretion sequence that was added to the protein by using the pET-22b(+) plasmid.

2.11.1 Fermentation in 2-L shaking flasks

For the pre-preculture, a non-baffled shaking flasks with 50 mL of LB medium containing ampicillin and chloramphenicol was inoculated with a single of the *E. coli* BL21 cells in the morning and was put a shaker shaking at 160 rpm at 37 °C. In the late afternoon, two non-baffled shaking flasks with 250 mL LB medium containing ampicillin and chloramphenicol were inoculated with equal amounts – 10 % of the preculture – of the pre-preculture and were put on a shaker with 160 rpm at 37 °C for around 20 h. On the next day, eight 2-L non-baffled shaking flasks with 600 mL LB medium containing ampicillin and chloramphenicol were inoculated with equal amounts of the preculture. After inoculation, the cells were kept growing at 160 rpm and at 37 °C until OD_{600} of 0.4 was reached. Afterwards, the flasks were placed immediately on a shaker at 25 °C for 30 min to equilibrate the temperature. The protein expression was then induced by the addition of 600 µL of 100 µM IPTG to each flask and lasted for around 20 h at 160 rpm shaking at 25 °C. After expression, the cells were harvested by centrifugation at 4000 rpm and 4 °C for 15 min.

2.11.2 High cell-density fed batch fermentation in a 5-L bioreactor

A different approach was the fermentation of cytochrome *cz* in a 5-L Eppendorf BioFlo 120 fermenter [18]. For the preculture, a non-baffled shaking flask with 200 mL LB medium containing ampicillin and chloramphenicol was inoculated with a single colony of *E. coli* BL21 cells and was put on a shaker at 37 °C for about 24 h.

Table 11: Stock solutions for the cytochrome cz fermentation in the fermenter.

Stock solution	Chemical	Concentration
10x Phosphate/ citric acid buffer pH 6.3	KH_2PO_4	133 g/L
	$(NH_4)_2HPO_4$	40 g/L
	Citric acid	17 g/L
$MgSO_4$ solution	$MgSO_4 \cdot 7\ H_2O$	500 g/L
Thiamine solution	Thiamine -HCl	20 g/L
70 % glucose	Glucose	700 g/L
32 % yeast extract	Yeast extract	320 g/L
100x Trace element solution	Fe (III) citrate	10.0 g/L
	$CoCl_2 \cdot 6\ H_2O$	0.25 g/L
	$MnCl_2 \cdot 4\ H_2O$	1.50 g/L
	$CuCl_2$	0.12 g/L
	H_3BO_3	0.30 g/L
	$Na_2MoO_4 \cdot 2\ H_2O$	0.25 g/L
	Zn acetate $\cdot 2\ H_2O$	1.30 g/L
	EDTA	0.84 g/L

The stock solutions in Table 11 were used to prepare the medium for the fermentation. The 10x phosphate/citric acid buffer pH 6.3, the 70 % glucose solution and the 32 % yeast extract solution were autoclaved. The $MgSO_4$ solution, thiamine solution and the 100x trace metal element solution were sterilized by filtration. For the preculture, 200 mL LB medium containing ampicillin and chloramphenicol were inoculated with a single positive colony of *E. coli* BL21 carrying both the pET-22b(+) and the pEC86 plasmid. The culture was put on a shaker and incubated at 30 °C overnight.

After assembly of the fermenter, the vessel was filled with 231 mL 10x phosphate/citric acid buffer, 1974 mL water and 98 mL 70 % glucose solution and autoclaved. The

medium was afterwards completed with the addition of 5.53 mL $MgSO_4$ solution, 23.1 mL 100x trace element solution, 0.525 mL thiamine solution and 3.5 mL ampicillin as well as 3.5 mL chloramphenicol. After completion, pH was adjusted to 6.8 and a temperature was equilibrated to 30 °C. The DO sensor was calibrated with N_2 (0 % DO) and air (100 % DO) and a dissolved oxygen concentration of 30 % was set.

Base: 25 % ammonia solution

Acid: 10x phosphate/citric acid buffer, pH 6.3

Sparging: Compressed air

Stirrer speed and sparging were controlled by a cascade program preinstalled on the Eppendorf BioFlo 120 to keep the microbial growth in an exponential phase [19].

After inoculation with the preculture, the batch phase lasted until the initially added glucose was metabolized, then the glucose feed was started.

Feed medium: 70 % glucose solution supplemented with 40 mL/L $MgSO_4$ solution, 12.5 mL/L 100x trace element solution and 2.3 mL/L thiamine solution

The fed batch was done for around 8 h with a constant growth rate. Afterwards, the temperature was set to 20 °C and the DO limit was reduced to 20 %. Induction was then done with 100 µM IPTG and 3 mL of 32 % yeast extract solution. The expression phase lasted for around 12 h. After expression, the cells were harvested by centrifugation at 4000 rpm and 4 °C for 15 min. It is important to immediately continue with the periplasmic extraction as freezing of the cell pellet would result in a partial breakage of the cells.

2.12 Purification of cytochrome *cz*

2.12.1 Periplasmic extraction by osmotic shock

After centrifugation, the culture supernatant was discarded because the cytochrome *cz* is matured in the periplasmic space of the cell and not further secreted. Periplasmic extraction facilitates the recovery of the protein because it breaks the periplasmic

space open by osmotic shock without releasing cytoplasmic contents. This already represents the first purification step.

Resuspension buffer: 20 % sucrose, 30 mM Tris, pH 8

Osmotic shock solution: chilled 5 mM $MgSO_4$

First, the cell pellet was properly resuspended but with avoiding unnecessary mechanical stress for the cells in resuspension buffer and transferred to a glass beaker. The suspension was stirred on ice at 160 rpm for 10 min. Afterwards, the cells were centrifuged with 4000 rpm at 4 °C and the supernatant was fully removed and collected to check if it contains cytochrome, too. The supernatant however should not contain protein. The cell pellet was now resuspended in the osmotic shock buffer and incubated on ice for 20 min with additional stirring at 160 rpm. The cells were afterwards pelleted by centrifuging with 10000 g at 4 °C for 25 min. The now pink supernatant was filtered to remove particles.

2.12.2 Nickel affinity chromatography (HisTrap)

The cytochrome *cz* domain also carries a polyhistidine-tag to facilitate purification. From previous studies it is known that imidazole in the elution buffer does not interfere with the histidine ligands that are coordinated to the heme. The six histidine residues of the affinity tag interact with the nickel ions which are bound to nitrilotriacetic acid groups of the column material. Thus, the target protein will selectively bind to the column with low amounts of unspecifically bound protein.

Start buffer: 50 mM Tris, 0–150 mM NaCl, pH 7.5 – 8

Elution buffer: 50 mM Tris, 0–150 mM NaCl, 500 mM imidazole, pH 7.5–8

Column: IMAC, XK 16/20

Before loading, the self-packed column was flushed with water and afterwards flushed with the elution buffer to elute all possible protein contaminants on the column. After equilibration to the start buffer, the sample was loaded onto the column. To elute the cytochrome *cz* again, the concentration of elution buffer containing imidazole in the column was increased by setting a linear gradient of elution buffer concentration over

around 3 CV over the course of one h. The eluting fractions containing the cytochrome *cz* were collected and concentrated in by centrifuging with Amicon centrifugal tubes (MWCO: 3 kDa) with 4000 rpm at 4 °C.

2.12.3 Size exclusion chromatography (SEC)

During size exclusion chromatography, the proteins in the sample have different residence times in the column. A separation according to size of the proteins happens inside the column, as bigger proteins are eluted faster than smaller proteins since small protein are retained longer in the porous structures of the resin. The main purpose of SEC in cytochrome *cz* purification is the separate the holo-form with the incorporated heme cofactor from the apo-form of the protein which does not contain the cofactor. As the running buffer is not critical for this SEC, the buffer choice depended on practical reasons.

Column: Superdex 75, XK16/100, 120 mL bed size

The highly concentrated sample was injected through the sample loop and pumped onto the column with a slow flow rate (0.3 mL/min) of running buffer. When the UV_{280} signal exceeded a certain set limit, the fraction collection started automatically, and the peaks were separately collected in fraction tubes. Beside the red color, the correct fraction which contained the cytochrome *cz* could then be determined by performing an SDS PAGE with samples of all eluted peaks.

2.13 SDS-PAGE

With a SDS-PAGE, proteins in a sample are separated according to their molecular weight. The smaller the molecular weight of the protein is, the faster it can migrate through the gel. The proteins are uniformly negatively charged by adding sodium dodecyl sulfate (SDS). Estimation of the molecular weight is done by the comparison to a simultaneously applied protein marker solution.

Prior to being pipetted into the wells of the gel, the samples protein concentration was estimated to not overload the lanes. Afterwards, the samples were mixed with 2x Laemmli buffer and boiled at 95 °C for 5-10 min to completely denature the proteins.

The samples were then pipetted into the wells in the vertically positioned gel in the electrophoresis buffer which is filled with running buffer. The total sample volume should not exceed 20 µL. It was also made sure that the gel was completely covered with running buffer. A constant voltage of 290 V was applied, and the process was stopped when the dye reached the bottom of the gel.

Gel: Mini-PROTEAN TGX Stain-Free (4-20 %), BioRad

Marker: Precision Plus Protein Standard, BioRad

Running buffer: 100 mM Tris /Glycine /SDS buffer

Stain-free gels contain trihalo agents that stain proteins – mostly tryptophans – with luminescence. The detection was done in the GelDoc (BioRad) and the image was saved for documentation.

2.14 Determination of protein concentration

2.14.1 Bradford protein assay

For measuring the total protein concentration of a sample, a protein assay by Bradford was performed. 600 µL of premixed Bradford reagent (containing Coomassie Blue) were mixed with 15 µL sample in a cuvette and incubated for 15 min. After incubation, absorbance at 595 nm was measured and protein concentration was calculated using a BSA standard curve with concentrations between 0.1 and 1 mg/mL. The Bradford assay is suitable for impure protein samples, such as crude extract, but must be taken with care. The assay is based on protein interaction with Coomassie Blue, interacting mostly with tryptophan residues. Cytochromes are small proteins and sometimes have no or only one tryptophan residue which results in underestimation of protein concentration if this assay would be used for pure protein samples.

2.14.2 Spectroscopic measurement with diode array spectrophotometer

Protein concentration of very pure samples was measured spectroscopically. Cytochrome *cz* concentration was measured via absorbance at the reduced-heme-specific peak at 551 nm after the addition of disodiumdithionite to fully reduce the

cytochrome, where an absorbance coefficient of 30 mM^{-1} cm^{-1} was used for calculation. Cytochrome *cz* has almost no peak at 280 nm which is usually used to calculate protein concentrations. Protein concentrations of very pure GDH samples were measured via absorbance at 280 nm, where an absorbance coefficient of 101.19 mM^{-1} cm^{-1} was used for calculation. A suitable dilution was chosen to not exceed absorbance values higher than 1. For all spectroscopic measurements, a quartz cuvette with a pathlength of 1 cm and a sample volume of 200 µL was used.

2.15 DCIP assay

With the DCIP assay, the activity of the produced *Gc*GDH domains was measured spectrophotometrically. DCIP (2,6-dichlorophenolindophenol) is a blue electron acceptor substance. If *Gc*GDH converts its substrate glucose, the electrons that are generated will reduce the DCIP, resulting in decolorization. The decline of absorbance at 520 nm was measured over the course of 180 sec. Afterwards, the slope of the linear part of the curve was determined and the U/mL were calculated with the UV WinLab by PerkinElmer. An enzyme activity coefficient of 434.78 was used in combination with the law of Lambert-Beer.

Table 12: DCIP assay.

Substance	Volume
0.1 M sodium acetate buffer pH 5.5	780 µL
3 mM DCIP solution	100 µL
1 M glucose solution	100 µL
Sample containing *Gc*GDH	20 µL
	1000 µL

After preparing a mastermix of the buffer, the DCIP solution and the glucose solution (Table 12), the cuvette was incubated for 15 min at 30 °C to equilibrate optimal reaction temperature. The photometer can measure at six slots simultaneously. The enzyme solution was put onto a spatula device that helps to apply the sample in each

of the six cuvettes simultaneously and was quickly stirred into the mixture. The sample containing the *Gc*GDH needed to be diluted, if the decline of absorbance happened to rapidly and did not allow quantification. The sodium acetate buffer was used for all DCIP assays except for the pH profile of the *Gc*GDH domains, where different buffers were tested.

2.16 Sortagging of the *Gc*GDH domain and the cytochrome *cz* domain

For the sortagging reaction, *Staphylococcus aureus* sortase A pentamutant [20] was used for the fusion of the two linker domains. The protein was produced by other laboratory members and was already available. The sortase A naturally occurs in gram-postive bacteria which secrete the protein. The secreted protein recognizes the motif LPXTG and adds predominantly glycopeptides to the cell wall of the bacteria. In our case, the cytochrome *cz* carried a C-terminal linker with the LPETG motif. Sortase cuts the sequence between T and G and links the sequence to any free G residue. This free G residue is provided by the *Gc*GDH. The cut-off tag from the LPETG motive also contains a C-terminal G and must be removed from the reaction mix by constant rebuffering of the sample with diafiltration using an Amicon tube. As also the product of the reaction - the linked chimeric protein - has the LPETG motif again, the sortase can also cut the product which is the reason why this reaction has a theoretical maximum efficiency of 50 %.

In order to push the reaction towards a higher product yield in preliminary experiments done by colleagues, reaction conditions regarding temperature, ratio of proteins, incubation time and effect of rebuffering were tested. We came to the conclusion that the temperature should be between 4 and 30 °C and the reaction should run overnight or 6 h, respectively. Cytochrome and GDH should be mixed equimolarly and on tenth of sortase should be added.

The reaction was carried out in a sortase buffer containing 50 mM HEPES, 5 mM $CaCl_2$, 100 mM NaCl at pH 7.0. The *Gc*GDH domain and the cytochrome *cz* domain were mixed equimolarly in an Amicon centrifugal tube (MWCO: 3 kDa). The reaction was performed in the centrifuge at 30 °C for 6 h with constant rebuffering. The centrifuge was set to 4000 rpm and was stopped every 30 min to refill the buffer.

Because the sortagging reaction resulted in a mix of the chimeric enzyme, *Gc*GDH domain, cytochrome *cz* domain, sortase A and C-terminal ends of the cytochrome *cz* domain (these should mostly be eliminated due to constant rebuffering) and therefore needed to be further purified.

2.17 Purification of the chimeric enzyme

Purification of the chimeric enzyme was carried out in two steps. Firstly, size exclusion chromatography (SEC) was carried out to remove the remaining cytochrome *cz* (12 kDa) and sortase (25 kDa). Immediate removal of the sortase is also important in to stop the reaction. Additionally, free C-terminal ends of cytochrome *cz*, which were not successfully removed by centrifugation during the reaction, could also be removed by SEC.

Column: Superdex 75, XK16/100, 120 mL bed volume

Because the remaining *Gc*GDH domain and the chimeric enzyme have very similar sizes and properties – also considering that *Gc*GDH is glycosylated and is therefore not a homogenous protein as not each *Gc*GDH has the sample glycosylation pattern - those two species had to be separated by IEX chromatography after SEC. The overall calculated pI of the chimeric protein and the free *Gc*GDH is similar. However, as the cytochrome *cz* has a very high pI above 9, we assume that the chimera binds stronger to the column because of the attached cytochrome domain.

Running buffer: 50 mM sodium acetate buffer, pH 4

Elution buffer: 50 mM sodium acetate buffer, 750 mM NaCl, pH 4

Column: Source 15 S, cation exchange, 10 mL

Before loading of the sample, the column was properly equilibrated to pH 4. The remaining *Gc*GDH domain went through without binding while the chimeric enzyme bound to the column. For the elution, the ionic strength in the column was increased and a linear gradient was set for 10 CV. After elution, the chimeric enzyme was concentrated and stored at -20 °C.

2.18 Photometer assays for cytochrome reduction by *Gc*GDH

To test whether the *Gc*GDH can reduce the cytochrome *cz* upon substrate conversion, (linked and unlinked) an assay in the diode array spectrometer was performed. The absorbance of the reduced heme peak at 551 nm was tracked in a time curve to observe cytochrome *cz* reduction. Every 5 sec, a complete spectrum between 190 and 1100 nm was recorded.

The cytochrome *cz* and *Gc*GDH domain were mixed equimolarly to a final concentration of 5 µM of each in 200 µL sample volume. The reaction was carried out in a sodium phosphate-borate-citrate buffer [21] at pH 6 and at 30°C. For this purpose, the quartz cuvette (pathlength: 1 cm) and all solutions were prewarmed to reaction temperature. After starting the measurement, glucose was added to reach a concentration of 50 mM in the cuvette as soon as the absorbance signal at 551 nm was stable. The measurement was carried out for at least 2500 sec. At the end, disodiumdithionite was added to reach the maximum reduction of cytochrome *cz*.

2.19 Cyclic voltammetry

The electrochemical characterization of the cytochrome *cz* domain was done with cyclic voltammetry at different pH values and different scan rates to determine the midpoint redox potentials in dependence of the pH. In cyclic voltammetry the sample is applied to an electrode which is then scanned in cycles in a defined potential range, as readout the current is measured. A suitable potential range was chosen where the redox peak couple of the cytochrome *cz* could be clearly observed. Scan rates from 20 mV/s to 1000 mV/s were used, three cycles of each scan rate were measured. Different pH values between pH 9 and pH 4 of a phosphate-borate-citrate buffer system [21] were used.

As electrodes, gold electrodes (BASinc) with a self-assembled thiol monolayer (SAM) with a diameter of 1.6 mm were used. 1-Thioglycerole was used for the SAM modification. Firstly, electrochemical cleaning of the electrodes for 10 cycles at a scan rate of 50 mV/s in a potential range between -205 mV and -1200 mV was done mostly to electrochemically remove thiols from prior measurements. To activate the gold

surface, the electrodes were incubated in basic H_2O_2/ NaOH for 10 min. Afterwards, one cycle with the previously mentioned electrochemical conditions was performed and the electrodes are incubated overnight in a mixture of fresh 1-thioglycerol and ethanol (9 µL 1-thioglycerol in 10 mL ethanol). To prepare the electrodes, 20 µL of 100 µM cytochrome *cz* were pipetted onto the gold surface. Afterwards, a dialysis membrane (Thermo fisher snakeskin, MWCO: 3.5 kDa) which was soaked in assay buffer, was put over the electrode (without the introduction of air bubbles) and secured with a rubber band and parafilm.

For the measurement in a three-electrode setup, the cell was filled with 20 mL buffer, where 100 mM NaCl as a supporting electrolyte was added. As a reference electrode, a Ag|AgCl electrode was used. A platinum electrode was used as a counter electrode. The potential was then applied, starting from the higher potential. During all measurements, the cell was sparged with N_2 reduce the O_2 background in the buffer. The applied potential was later converted to potential versus standard hydrogen electrode (SHE) for easy comparison.

2.20 Amperometry

Amperometric measurements were done with the unlinked species as well as with the chimeric enzyme. In amperometry, a defined potential above the redox potential of a sample is applied to the sample, the current is measured over time and current increase after addition of substrate is monitored. After the addition of glucose to cell, the resulting current was documented over time. Thiol-modified gold electrodes (SAM) were used for the measurements. Cleaning and preparation were done as mentioned in 2.19. Prior to the measurements, a home-crafted Teflon holder with a cavity to entrap a defined volume over the electrode surface was put over the electrode which only allowed a specified amount of enzyme to be put on the electrode. After application of 20 µL containing 200 µM *Gc*GDH domain and 200 µM cytochrome *cz* (200 µM chimeric enzyme respectively) onto the gold surface, a dialysis membrane was put over the electrode and was secured by rubber bands and parafilm.

For the measurement, the cell was filled with 20 mL McIlvaine buffer (pH 5) supplemented with 100 mM NaCl as a supporting electrolyte was used. As a reference

electrode, a Ag|AgCl electrode was used, platinum electrode was used as a counter electrode. A surrounding circulating water bath was used to keep the cell at 30 °C. A constant potential of 1 mV (vs. Ag|AgCl) was applied. After the current was constant, 50 mM glucose was added to the cell.

3 Results and discussion

3.1 Production of the *Gc*GDH domains

3.1.1 Cloning

Production of the different *Gc*GDH domains was done in *P. pastoris* X33. The *Gc*GHD_K480G gene was ordered and arrived already cloned into a pPICZA plasmid also carrying a zeocin resistance gene. The *Gc*GDH gene expression is under regulation of a AOX promoter inducible by methanol. To obtain the *Gc*GDH_WT gene and the *Gc*GDH_A53P_N54D_K480G gene, the plasmid with *Gc*GDH_K480G was mutated by site directed mutagenesis with PCR. After the reaction, agarose gel electrophoresis of the reaction products was performed.

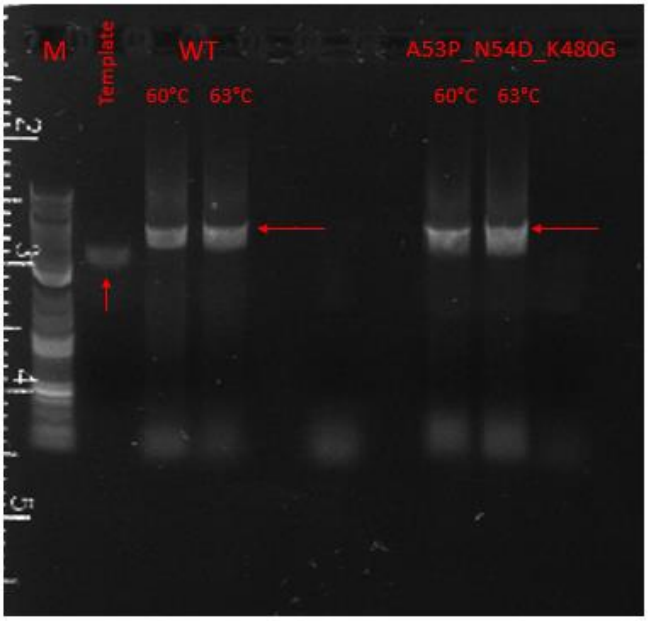

Figure 3: Agarose gel after temperature gradient PCR.

Figure 3 shows the agarose gel after PCR. Distinct bands can be seen at 60 °C and 63 °C primer annealing temperature for both variants (red arrows). This observation indicates a successful amplification of both new plasmids. The bands were cut out and

gel extraction was performed. Afterwards, the plasmids were transformed into *E. coli* NEB5α by electroporation and spread out on agar plates.

For each variant, five colonies were picked, and a plasmid preparation was performed. The plasmids were then sent for Sanger sequencing (Microsynth) to control correct incorporation of the aimed mutations. For *Gc*GDH_A53P_N54D_K480G one colony carried the wanted mutation, this plasmid was then transformed into *P. pastoris* X33 by electroporation. For the *Gc*GDH_WT plasmid however, no colony incorporated the desired mutation. Even newly designed primers and changed PCR conditions (also with added GC enhancers) did not lead to a successful PCR reaction, so the plasmid was readily ordered from BioCat and transformed into *P. pastoris* X33 by electroporation.

3.1.2 *Gc*GDH_WT

Because the gene of interest is integrated into the genome which can also occur at random loci or in copies, not all colonies are necessarily equal and can have different expression levels of the protein of interest. Therefore, colonies are first screened to select the one with the highest protein yield. In a small-scale experiment, four positive colonies of *P. pastoris* X33 containing the *Gc*GDH_WT gene were picked and with each colony, 20 mL of BMGY medium containing zeocin in a 100 mL baffled shaking flask were inoculated. In this medium, the culture was grown to generate biomass. When glucose in the medium was used up, protein expression was induced by the addition of methanol (0.5 % of the culture medium). Methanol induction must not happen if glucose is still present in the culture medium. After cultivation, a DCIP assay of the culture supernatants was performed. The culture supernatant of colony 2 showed the highest GDH activity and was therefore chosen for the inoculation of the preculture for fermentation. Expression of *Gc*GDH_WT was done in baffled shaking flasks containing BMGY medium.

For the preculture, 200 mL BMGY medium containing zeocin were inoculated with the colony chosen from the small-scale experiment. Cultivation was done for 28 h at 120 rpm shaking and 30 °C. The 10 baffled 1 L shaking flask containing 200 mL BMGY medium each were then inoculated with equal volumes of the preculture. The batch phase was carried out at 130 rpm shaking and 30 °C for 22.5 h. The last hour of the

batch phase was used to equilibrate the flasks to 25 °C prior to induction. *Gc*GDH_WT expression was then carried out at 130 rpm shaking and 25 °C for 24 h. 1 mL methanol was added to each the culture twice (0.5 % of the culture volume), once to start induction and once on the following day to further provide the carbon source and keep induction of expression ongoing, approximately 20 h apart. A total wet biomass of 57.3 g was measured.

After centrifugation and subsequent sterile filtration, the supernatant was concentrated and rebuffered to the start buffer for IEX chromatography (DEAE Sepharose) by cross flow filtration (MWCO: 10 kDa) yielding 400 mL. As running buffer for the IEX chromatography, 50 mM sodium acetate with pH 6.5 was used. For the elution buffer was the same as the running buffer but supplemented with 500 mM NaCl.

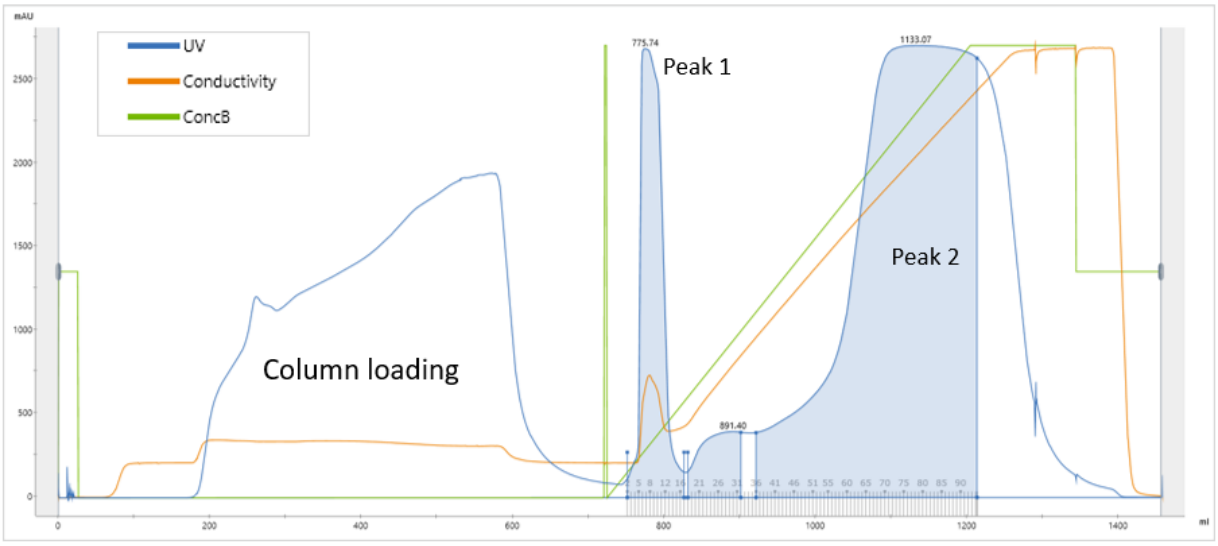

Figure 4: IEX chromatogram of GcGDH_WT, Run 1.

In Figure 4, the blue line represents the absorbance at 280 nm. Elution happened in two peaks, but only Peak 1 showed GDH activity. Because there was still activity left in the flow through, the flow through was loaded onto the column again after the column was properly cleaned and equilibrated to the running buffer for a second run.

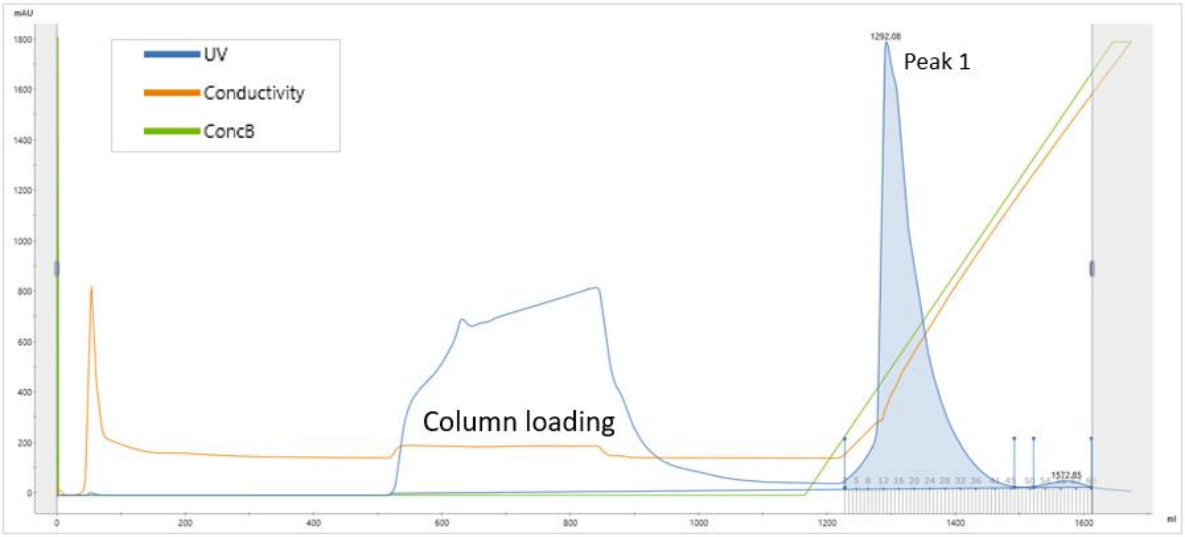

Figure 5: IEX chromatogram of GcGDH_WT, Run 2.

As shown in Figure 5, elution of the second run happened in one peak which showed GDH activity. The chromatograms of the two runs (Figure 4, Figure 5) looked different because protein expression was carried out in a medium containing yeast extract. Run 1 resulted in an overload of the column, leaving the *Gc*GDH partly in the flow through. Before Run 2, the sample was rebuffered to get rid of interfering substances. The resulting peak also showed a shoulder, which might be because of the different glycosylation patterns of the *Gc*GDH. Because Peak 1 of Run 1 and Peak 1 of Run 2 did not have the yellow color expected of GcGDH originating from the FAD cofactor, a SDS-PAGE was done with selected fractions of the different peaks obtained.

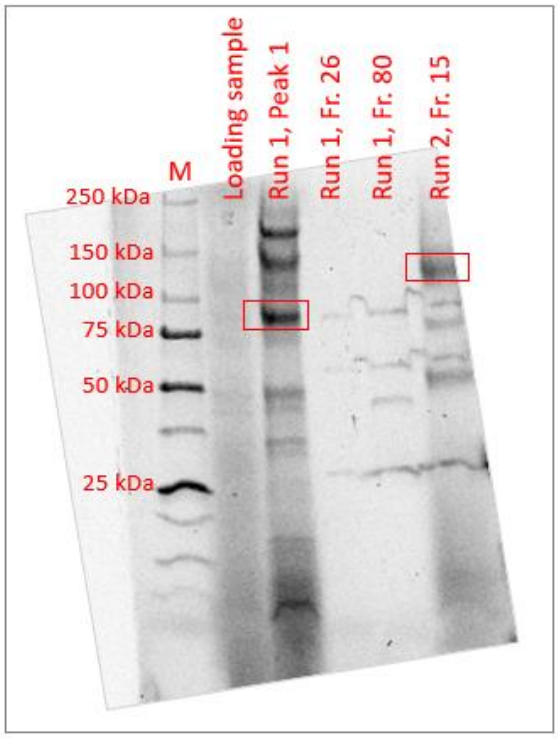

Figure 6: SDS PAGE after IEX chromatography of GcGDH_WT.

On the SDS-PAGE gel shown in Figure 6 we can observe that fractions of Peak 1 of Run 1 and Peak 1 of Run 2 contained the *Gc*GDH (red boxes). The difference in size of the *Gc*GDH in the two peaks might be because of different glycosylation patterns. Those fractions were pooled, concentrated and rebuffered to running buffer to remove containing salts. Fractions of Peak 2 of Run 1 did not contain the *Gc*GDH and were discarded. Nevertheless, the peaks of interest still contained large amounts of impurities and were later further purified by SEC as shown in Figure 7.

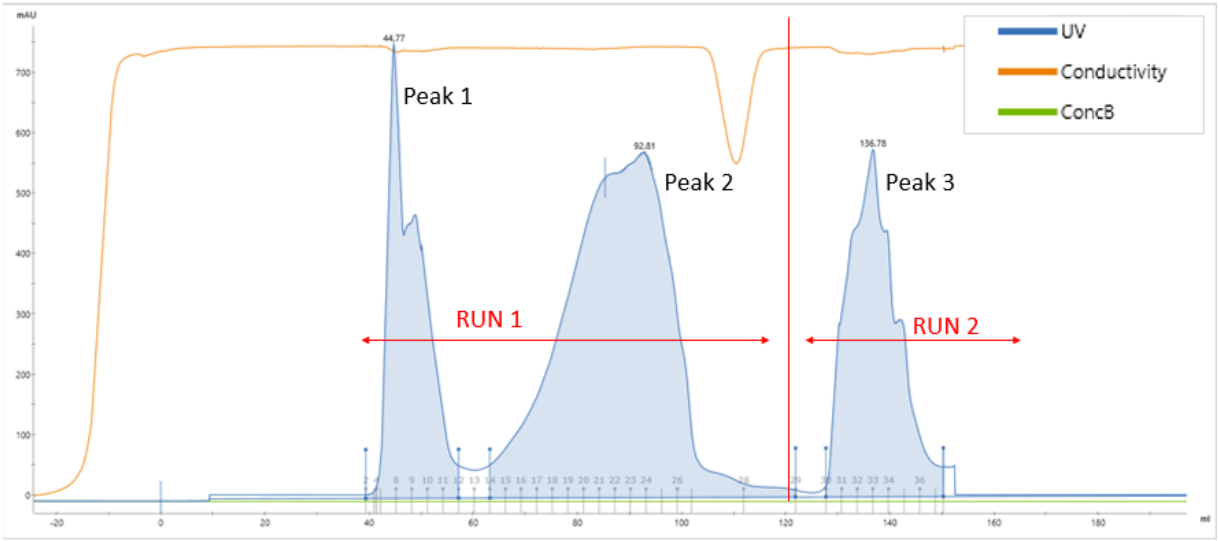

Figure 7: SEC of GcGDH_WT.

In Figure 7, elution of the SEC (Superdex 75) is shown. Loading of the column was done in two runs because only limited amount of sample can be loaded to this column. The elution of the first run happened in Peak 1 and Peak 2. Fractions of Peak 1 had a yellow color typical for the FAD cofactor of the *Gc*GDH and die visible peak shoulder indicates again the presence of different glycosylation patterns of the *Gc*GDH. Peak 2 had a brown color which was also seen in the fractions of the IEX chromatography. This leads to the conclusion that SEC successfully separated the *Gc*GDH from the impurities. The impurities are likely remains of yeast extract from the expression media. The second run was aborted after the elution of the yellow-colored fractions of Peak 3, which contained the *Gc*GDH. Peak 1 and Peak 3 were then concentrated separately with Amicon centrifugal tubes (MWCO: 10 kDa).

Table 13: Results of the DCIP assay and Bradford assay after purification of GcGDH_WT.

***Gc*GDH_WT**						
Sample	**Volume [mL]**	**DCIP [U/mL]**	**Total units [U]**	**Protein concentration [mg/mL]**	**Total protein [mg]**	**Specific activity [U/mg]**
Peak 1	14.0	23.02	322	0.33	4.62	70
Peak 3	1.7	379.28	645	0.90	1.53	422

After SEC, protein concentration was measured with the Bradford method and GDH activity was determined by a DCIP assay (

Table 13). It also could be seen that the specific activity of Peak 1 is lower than in Peak 3 which lead to the conclusion that there were still impurities present in Peak 1. The specific activity of Peak 3 (422 U/mg) was similar to the pure fractions of *Gc*GDH_K480G (365 U/mg), which can be found in Table 15. In another study taken on this subject, a specific activity of 836 U/mg was achieved [13]. The lower specific activity of this fermentation might be traced back to the suboptimal culture conditions in the shaking flasks compared to the fermenter. The united and concentrated fractions of Peak 3 were used for further experiments.

3.1.3 *Gc*GDH_K480G

In a small-scale experiment, four positive colonies of *P. pastoris* X33 containing the *Gc*GDH_K480G gene were picked and in 100 mL baffled shaking flasks 20 mL of BMGY medium were inoculated with a single positive colony (cultivation and expression procedure as in 3.1.2). After cultivation and protein expression, a DCIP assay of the culture supernatants was performed. All four culture supernatants had an equally good GDH activity and Colony 1 was chosen for the inoculation of the precultures for the 5 L fermentation.

For the pre-preculture of the 5-L fermentation, 20 mL BMGY medium were again inoculated with the chosen colony. Cultivation was done in a baffled shaking flask at 120 rpm shaking at 30 °C. After 24 h, this culture was used entirely to inoculate 200

mL YPD medium containing zeocin. The preculture was cultivated in a baffled shaking flask at 120 rpm shaking at 30 °C for 65 h to reach a proper cell density.

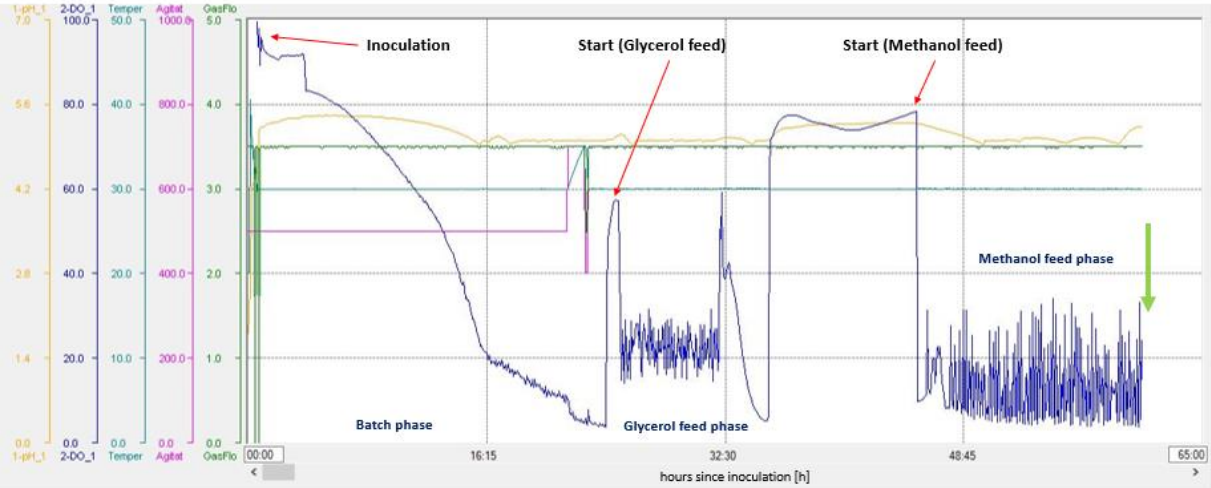

Figure 8: Process diagram of the GcGDH_K480G fermentation.

Figure 8 shows the process diagram of the fermentation with the DO curve in dark blue. After inoculation of the 5-L fermenter, DO started to decrease as the cells metabolize glycerol and use oxygen. The batch phase lasted for 25 h. After a spike in the DO was observed at the end of the batch phase indicating the complete consumption of the initial glycerol, the glycerol feed was started. The agitation was increased from 500 rpm to 700 rpm. As soon as DO surpassed the set limit, feed medium was pumped into the fermenter which led to a drop of DO again (spiky DO curve). The glycerol feed phase lasted for 21 h. This phase was followed by the methanol feed phase which induced the production of the *Gc*GDH_K480G and lasted for 28 h. After around 18 h of feed, the program stopped recording due to technical issues (green arrow), but fermentation continued. Both different feeds were programmed that feed medium is pumped into the fermenter with 0.5 mL/min once the DO rose above 20 %.

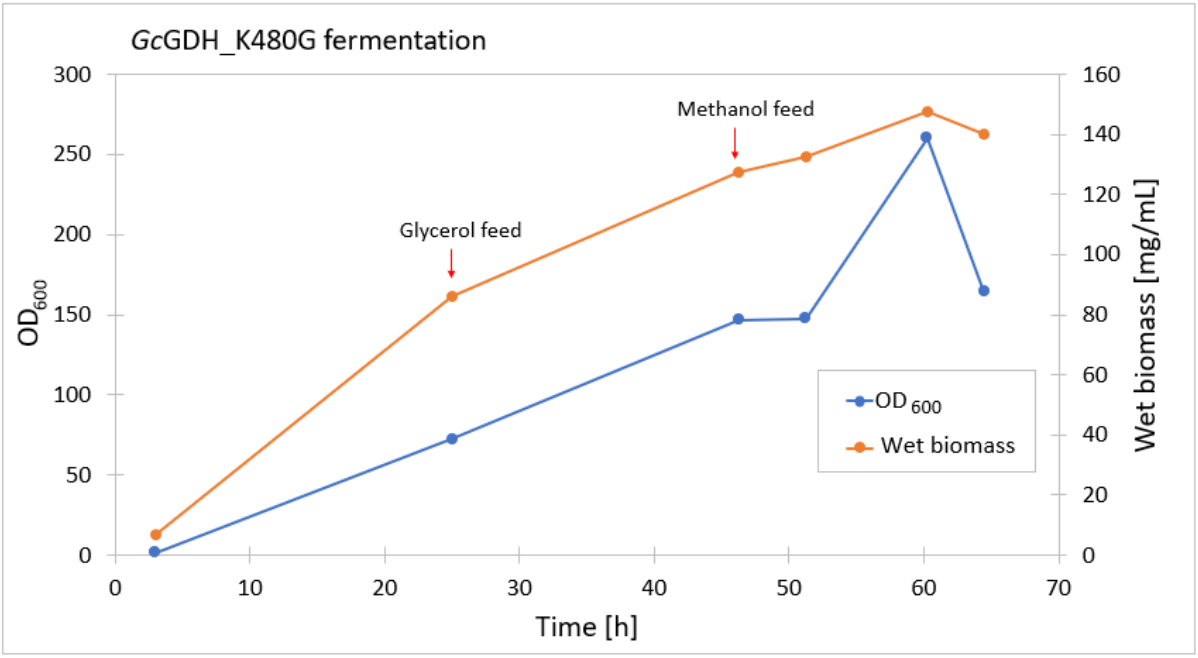

Figure 9: OD_{600} and wet biomass concentration during GcGDH_K480G fermentation.

Table 14: OD_{600}, wet biomass and DCIP assay data for GcGDH_K480G fermentation.

***Gc*GDH_K480G**			
Time since inoculation [h]	OD_{600}	Wet biomass [mg/mL]	DCIP [U/mL]
3.00	1.4	6.7	-
25.00	72.5	86.0	-
46.25	146.9	127.2	2.63
51.25	147.4	132.6	3.02
70.25	260.3	147.7	16.97
74.50	164.3	140.0	14.82

Figure 9 shows the trend of OD_{600} and wet biomass during *Gc*GDH_K480G fermentation. During the methanol feed phase, OD_{600} and wet biomass only slightly increased. There is GDH activity in the supernatant before induction with methanol

(Table 14). Because the activity did not increase between the last two samples anymore, the culture was harvested. The GDH activity was measured in triplicates.

After centrifugation and subsequent sterile filtration, the fermentation yielded 4.05 L supernatant. The supernatant was concentrated by ultrafiltration (MWCO: 10 kDa) to a total volume of 1.15 L and then rebuffered to the start buffer for the IEX chromatography. As the start buffer, 50 mM Tris pH 5.5 was used. The elution buffer additionally contained 500 mM NaCl. After loading of the concentrated culture supernatant onto the anion exchange column was completed, elution was done by gradually increasing the ionic strength in the column. Protein elution started with around 20 % of the elution buffer in the mixture which equals a concentration of around 100 mM NaCl.

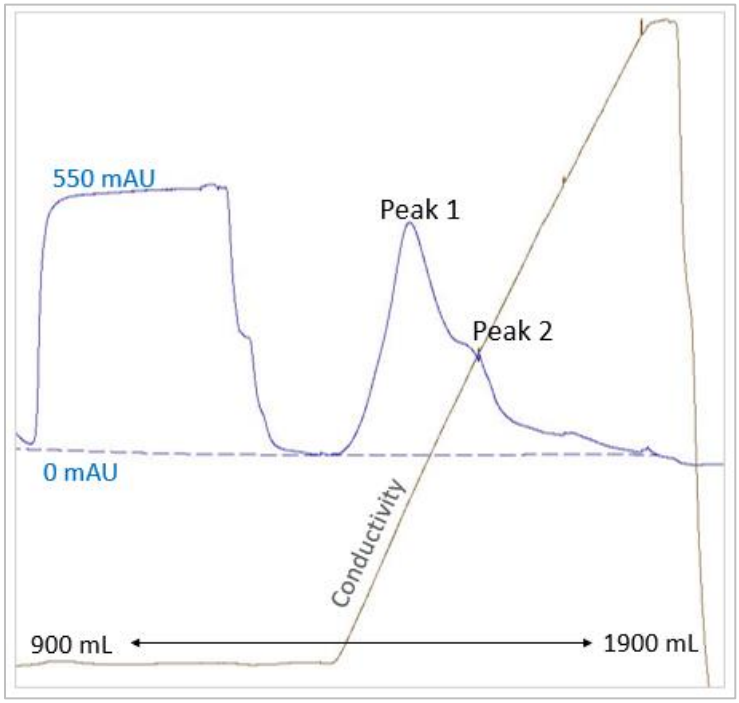

Figure 10: IEX chromatogram of GcGDH_K480G.

The elution happened in 2 peaks (Peak 1, Peak 2) which can be seen in Figure 10. The peaks were fractionated and pooled.

Table 15: Results of the DCIP assay and Bradford assay after purification of GcGDH_K480G.

***Gc*GDH_K480G**						
Sample	**Volume [mL]**	**DCIP [U/mL]**	**Total units [U]**	**Protein concentration [mg/mL]**	**Total protein [mg]**	**Specific activity [U/mg]**
Loading sample	1150	63.86	73438	0.572	658	112
Flow Through	400	0.41	162	0.160	64	3
Peak 1	9	1228.13	11053	3.362	30	365
Peak 2	110	2.98	328	0.125	14	24

Afterwards, a DCIP assay and determination of protein concentration by Bradford was done (Table 15). Because of the significantly higher activity, Peak 1 was further concentrated with Amicon centrifugal tubes (MWCO: 10 kDa) to a volume of 9 mL and was stored at -30 °C. The threefold increase of the specific activity compared to the loading sample indicates a good purification from other proteins in the sample. In the loading sample 63.9 U/mL could be measured which is comparable to 48.0 U/mL obtained in previous studies taken on this subject [13]. In the same study, final specific activity (836 U/mg) however is significantly higher than after this fermentation (365 U/mg). For purification, only anion exchange was performed in contrast to an additional hydrophobic interaction chromatography [13] which might explain the lower specific activity. Of 73430 U in the loading sample, only 11053 U could be recovered (15 %).

3.1.4 *Gc*GDH_A53P_N54D_K480G

In a small-scale experiment to check for the correct expression, the one positive colony of *P. pastoris* X33 containing the *Gc*GDH_A53P_N54D_K480G gene was used to inoculate 200 mL of BMGY medium containing zeocin in a baffled shaking flask. After cultivation, a DCIP assay of the culture supernatant was performed. For the pre-preculture of the 5 L fermentation, 2 x 20 mL BMGY medium containing zeocin were inoculated with the positive colony. Cultivation was done in baffled shaking flasks at 120 rpm shaking at 30°C. After 7 h, this cultures were used entirely to inoculate 2 x

200 mL BMGY medium containing zeocin. The precultures were cultivated in a baffled shaking flasks at 150 rpm shaking at 30 °C for 19 h.

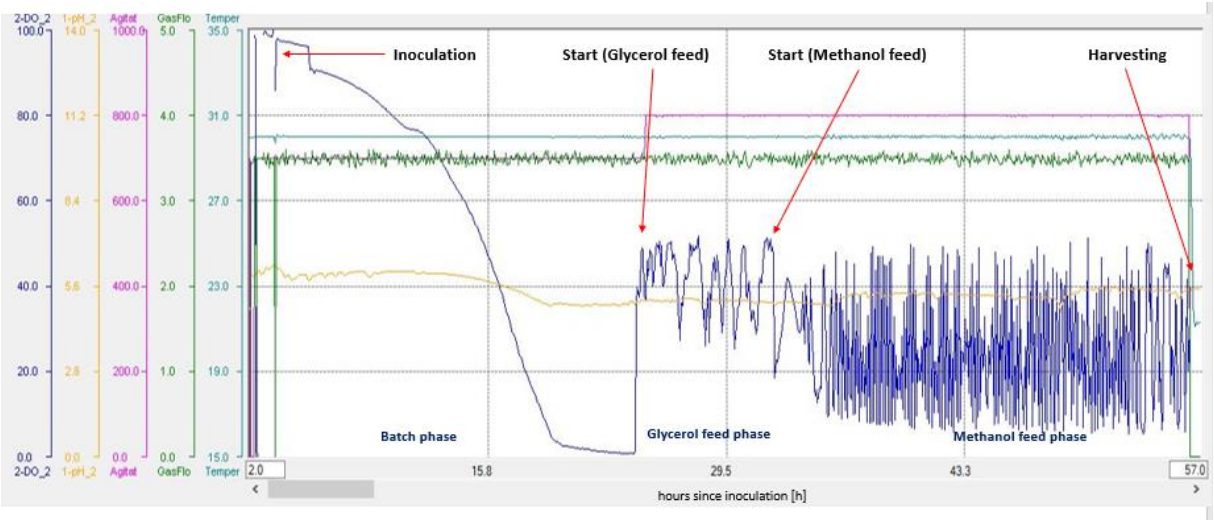

Figure 11: Process diagram of the GcGDH_A53P_N54D_K480G fermentation.

Figure 11 shows the process diagram of the fermentation with the DO curve in dark blue. After inoculation of the 5 L fermenter, the batch phase lasted for 21 h. After a spike in the DO was observed at the end of the batch phase, the glycerol feed was started. The agitation was increased from 700 rpm to 800 rpm for better aeration. The glycerol feed phase lasted for 8 h. This phase was followed by the methanol feed phase which induced the production of the *Gc*GDH_A53P_N54D_K480G and lasted for 24 h. Both different feeds were programmed that feed medium is pumped into the fermenter with 0.5 mL/min once the DO rose above 40 %.

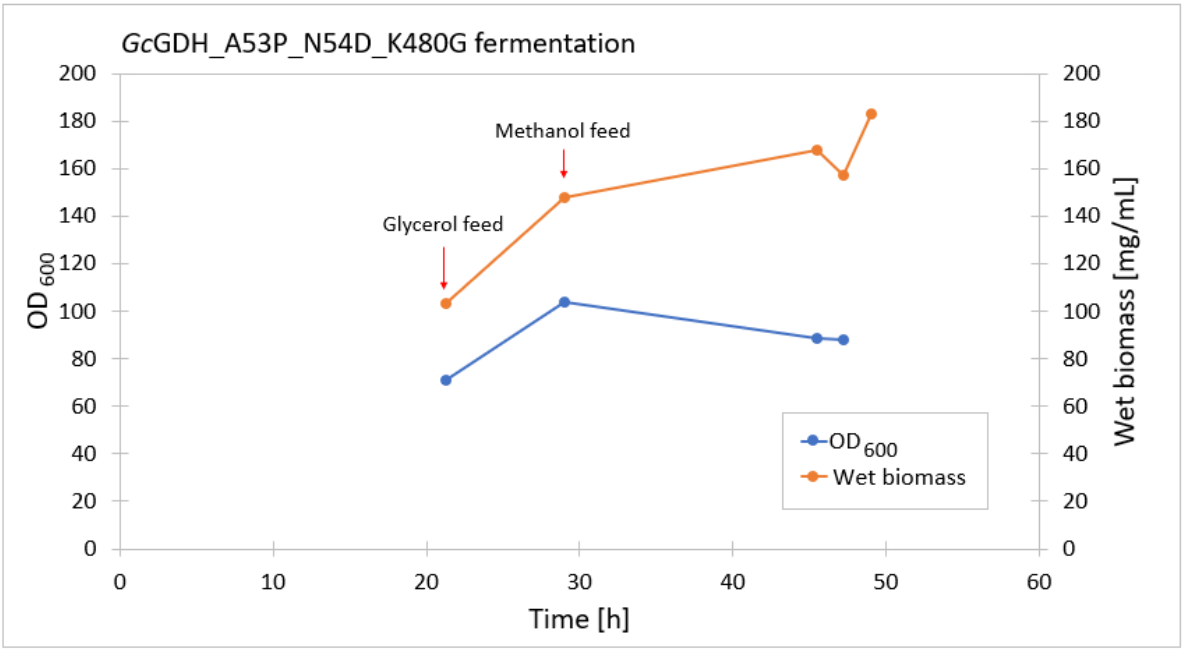

Figure 12: OD_{600} and wet biomass concentration during GcGDH_A53P_N54D_K480G fermentation.

Table 16: OD_{600}, wet biomass and DCIP assay data for GcGDH_A53P_N54D_K480G fermentation.

GcGDH_A53P_N54D_K480G			
Time since inoculation [h]	OD_{600}	Wet biomass [mg/mL]	DCIP [U/mL]
21.25	71.2	103.3	-
29.00	103.8	148.1	-
45.50	88.4	167.7	0.18
47.25	87.8	157.0	2.04
53.00	-	183.0	0.96

The trend of the OD_{600} and the wet biomass during *Gc*GDH_A53P_N54D_K480G fermentation is shown in Figure 12. It can again be seen, that after induction with methanol wet biomass only slightly increased while OD_{600} remained constant. The majority of biomass is produced during the batch phase. After 53 h of fermentation, the

production of foam unexpectedly increased drastically, and a large portion of the culture was spilled into the attached base trap. The culture was then immediately harvested, although the supernatant did not show satisfactory activity at this point (Table 16).

After centrifugation and subsequent sterile filtration, the fermentation yielded 2.5 L supernatant and a total wet biomass of 405 g. The volume was less than the start volume because the culture produced a lot of foam resulting in loss of culture to the base trap. A final wet biomass concentration of 162 mg/mL was determined. The supernatant was then concentrated and rebuffered to the start buffer by cross flow filtration (MWCO: 10 kDa) to a total volume of 700 mL for the IEX chromatography. As the start buffer, 50 mM Tris pH 6.5 was used. The elution buffer additionally contained 500 mM NaCl. After loading of the concentrated culture supernatant onto the anion exchange column was completed, elution was done by an increasing ionic strength in the column. Protein elution started with around 15 % of the elution buffer in the mixture which equals a concentration of around 75 mM NaCl.

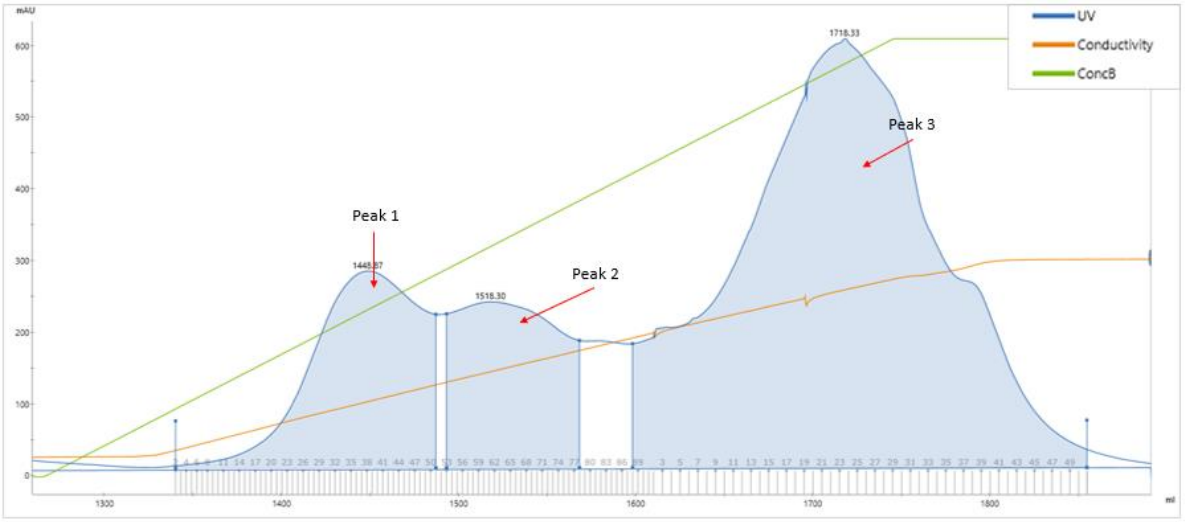

Figure 13: IEX chromatogram of GcGDH_A53P_N54D_K480G.

Elution happened over three peaks, shown in Figure 13. All peaks were fractioned, and the corresponding fractions were united. An SDS-PAGE was afterwards performed to check the protein composition of each peak.

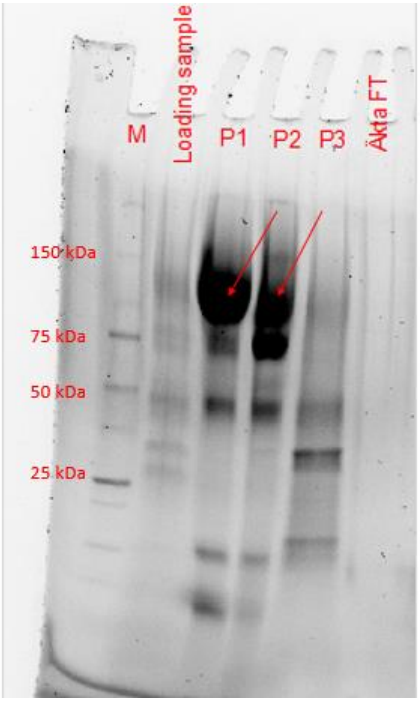

Figure 14: SDS PAGE after IEX chromatography of GcGDH_A53P_N54D_K480G.

Onto the SDS-PAGE (Figure 14) the three peaks of the IEX chromatography, the loading sample and the chromatography flow-through (Äkta FT) were applied. In Peak 1 and Peak 2 the expressed GDH domain is present, the typical smeared bands are clearly observable (red arrows). The smear comes from extensive but non-homogeneous glycosylation, which are typically for *P. pastoris* as an expression host. Because of these glycosylations, the GDH domain appears larger, the deglycosylated protein band would be found at around 65 kDa. Nevertheless, Peak 1 and 2 still showed impurities. To further purify the GDH domain, the peaks were united and SEC with a Superdex 200 column was performed. Because Peak 3 did not contain the GDH domain, it was discarded.

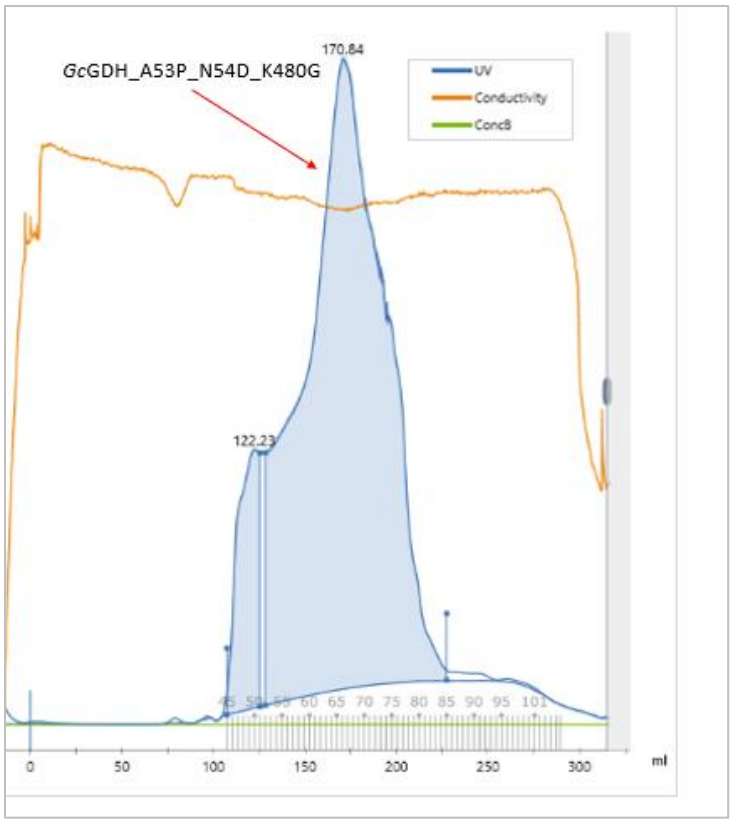

Figure 15: SEC chromatogram of GcGDH_A53P_N54D_K480G with Superdex200.

After SEC however, no active and functional protein could be recovered. The formerly yellow peaks completely lost their color. An SDS-PAGE was performed with the Fractions 48, 70 and 80 of the elution peak (Figure 15).

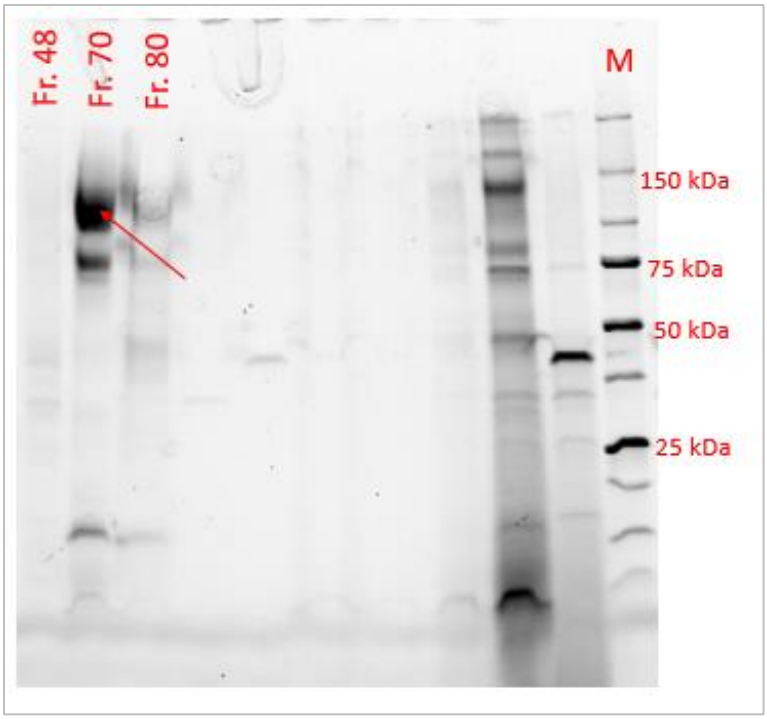

Figure 16: SDS PAGE after SEC of GcGDH_A53P_N54D_K480G.

The SDS-PAGE in Figure 16 showed that the GDH domain is still present after eluting of the Superdex 200 column (Fr. 70). However, the typical yellow color as well as the activity were lost. The loss of the color would indicate a loss of the FAD cofactor in the active site of the GDH domain which would also result in a loss of activity. The introduced mutation might therefore affect the binding of the FAD cofactor in the enzyme.

Table 17: Results of the DCIP assay and Bradford assay after purification of GcGDH_A53P_N54D_K480G.

***Gc*GDH_A53P_N54D_K480G**						
Sample	**Volume [mL]**	**DCIP [U/mL]**	**Total units [U]**	**Protein concentration [mg/mL]**	**Total protein [mg]**	**Specific activity [U/mg]**
Loading sample	700	5.95	4161	0.171	120	35
Flow Through	800	0.30	243	0.037	30	8

Because of the loss of all samples after SEC with the Superdex 200 column, only data for the loading sample and the flow through of the IEX chromatography could be measured (Table 17). GDH activity was measured with a DCIP assay and protein concentration was determined by Bradford. However, the loading sample of this variant showed a significantly lower specific activity than the other *Gc*GDH variants. It can be concluded that the removal of glycosylation sites by mutation of the protein chain promotes instability of the enzyme which goes hand in hand with a loss of activity. However, we cannot fully conclude yet that this is mostly attributed to the missing glycosylation or if the mutated residue itself results in improper folding of the protein. Because of its low specific activity and instability, this *Gc*GDH variant was not used for further experiments.

3.1.5 Comparison of the variants and discussion

When comparing the three expressed variants, it can be noticed that the removal of glycosylation sites had a negative impact on stability and activity (*Gc*GDH_A53P_N54D_K480G). *Gc*GDH_WT and *Gc*GDH_K480G showed similar specific activities after purification. Enzyme production in the fermenter is beneficial compared to the expression in shaking flasks for *Gc*GDH, as a sufficient and continuous supply of methanol and oxygen during the feed period can be guaranteed. Additionally, the fermenter provides a more sterile environment than the expression in shaking flasks.

3.2 Production of the cytochrome *cz* domain

3.2.1 Cloning

Production of the cytochrome *cz* domain was done in *E. coli* BL21. The cytochrome *cz* gene was ordered and arrived already cloned into a pET22b(+) plasmid. To facilitate purification, the cytochrome *cz* gene carries a polyhistidine-tag. Generally, purification of heme proteins with the cofactor ligated to a histidine with HisTag (imidazole in the elution buffer) is often not ideal. However, for this protein we know that imidazole does not interfere with the protein structure. Protein expression is controlled by the *lac* promoter which is inducible by IPTG. To allow expression of functional cytochrome *cz*, the pEC86 plasmid carrying eight additional proteins of the cytochrome *c* maturation cassette (ccmA-H) for periplasmic expression, is needed as well. The pEC86 plasmid also carries a gene for chloramphenicol resistance.

Both plasmids were transformed into *E. coli* BL21 simultaneously by heat shock. Heat shock competent cells were already available in the laboratory. After transformation, the cells were spread out on LB medium agar plates containing ampicillin and chloramphenicol to allow selection for colonies which carried both plasmids. Since the plasmids were ordered and did not have to be cloned, additional sequencing with Sanger was not necessary.

3.2.2 Fermentation in 2 L shaking flasks

For the pre-preculture, 50 mL LB medium containing ampicillin and chloramphenicol in a non-baffled shaking flask were inoculated with a single positive culture of E. coli BL21 carrying the pEC68 plasmid and the pET-22b(+) plasmid. The cultivation was done on a shaker with 160 rpm at 37 °C for 7 h. The preculture of 2 x 250 mL LB medium containing again both antibiotics was inoculated with equal amounts of the pre-preculture and cultivated under the same conditions for 18 h.

The main culture consisted of 8 x 2 L non-baffled shaking flasks with each carrying 600 mL LB medium containing ampicillin and chloramphenicol, leading to a total expression volume of 4.8 L. Each flask received 65 mL of preculture for inoculation. Until OD_{600} of

0.4 was reached, the flasks were cultivated on a shaker with 160 rpm at 37 °C. Then, a temperature of 30 °C was equilibrated for 30 min. Afterwards, cytochrome *cz* expression was induced with the addition of IPTG. Protein expression was done for 24 h. Figure 17 shows the shaking flasks during fermentation.

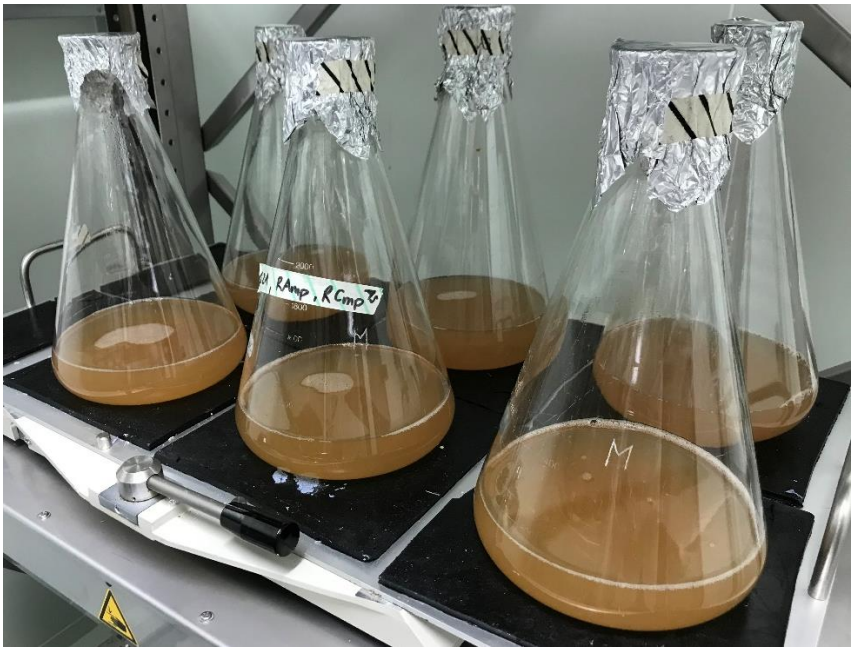

Figure 17: Fermentation of cytochrome cz in 2-L shaking flasks.

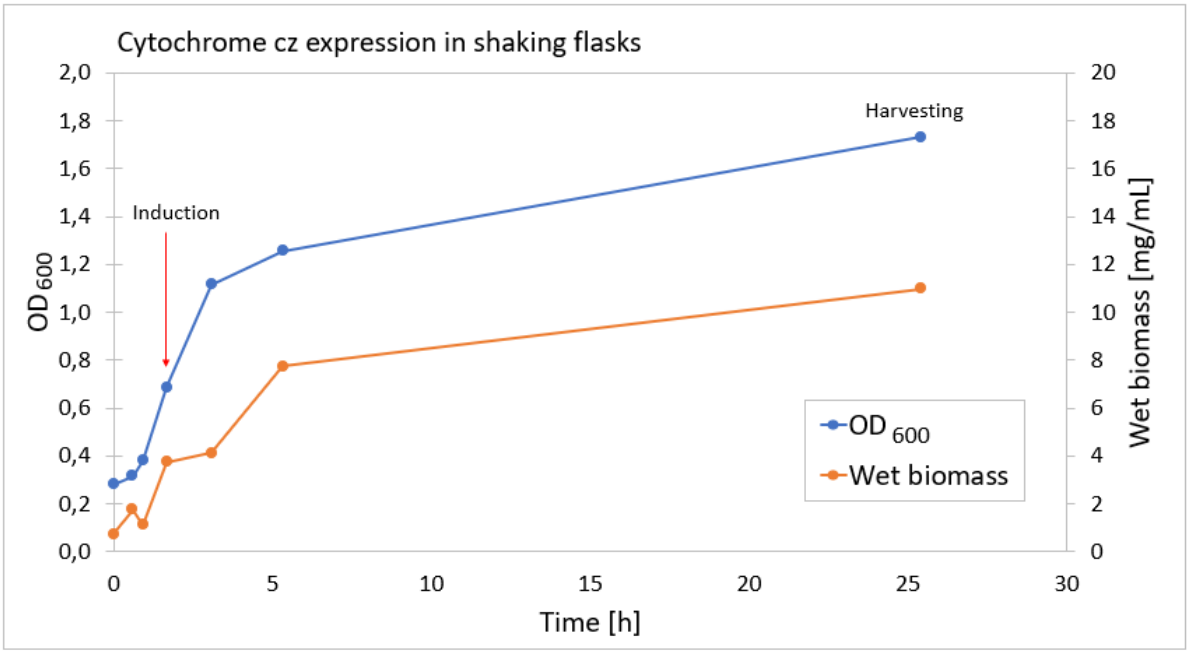

Figure 18: OD_{600} and wet biomass concentration during cytochrome cz expression in 2-L shaking flasks.

In Figure 18 the trend of the OD_{600} and wet biomass concentration in one of the shaking flasks is shown. In the first 3 h of the fermentation, an exponential increase of the OD_{600} is visible. After induction however, the curve starts to flatten because the cultivation temperature was decreased to 30 °C. Additionally, cell growth is slowed down drastically during protein expression. During expression, a total wet biomass of 37.4 g was reached (total cell pellet after harvesting).

After expression, the culture was harvested, and periplasmic extraction was performed. The deep pink supernatant of periplasmic fraction and the still reddish remaining pellet were frozen separately at -80 °C. The thawed cells were then disrupted by treatment with the French press for four cycles. Additionally, the cell lysate was then homogenized and filtered. Usually, the remaining cell pellet containing the cytoplasm would be discarded. However, because it also contained a big amount of cytochrome as judged based on the red color, we decided to also add this fraction. The filtered cell lysate was then united with the supernatant from the periplasmic extraction to have 350 mL loading sample for the following HisTrap chromatography. For HisTrap

chromatography, a 50 mM Tris pH 7.5 start buffer was used. For elution, a 50 mM Tris pH 7.5 buffer with 500 mM imidazole was used.

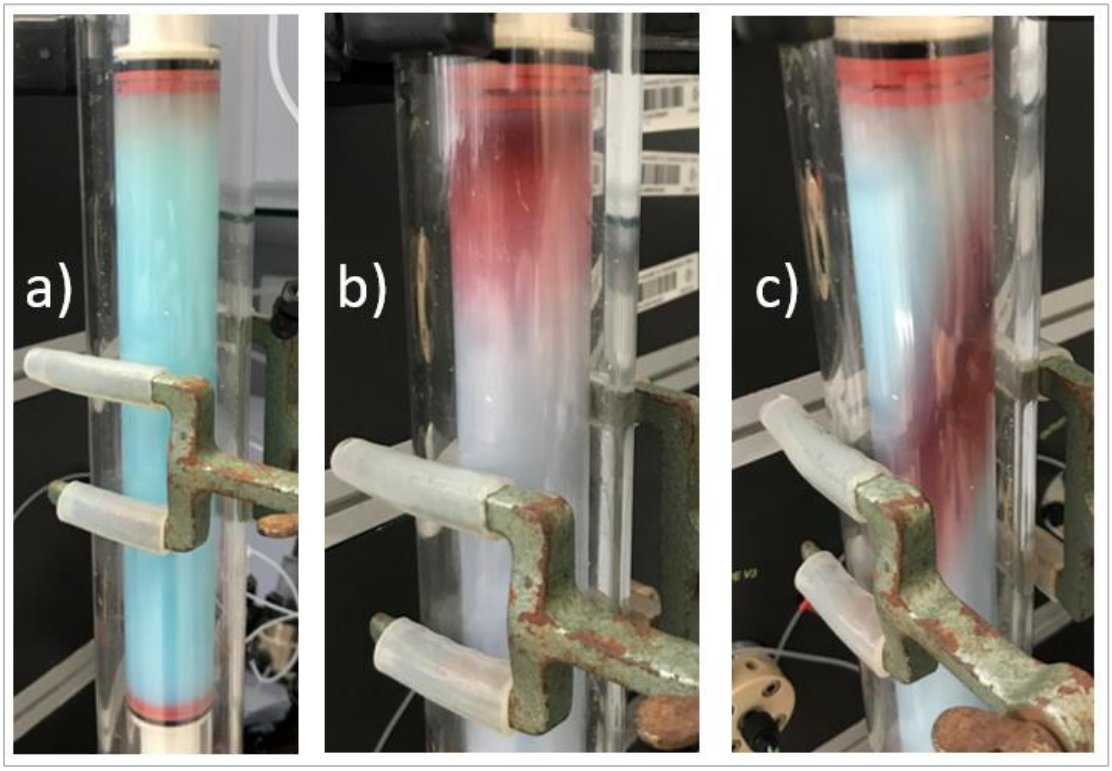

Figure 19: IMAC column during HisTrap chromatography.

Figure 19 shows the IMAC column before loading (a), during loading (b) and during elution (c).

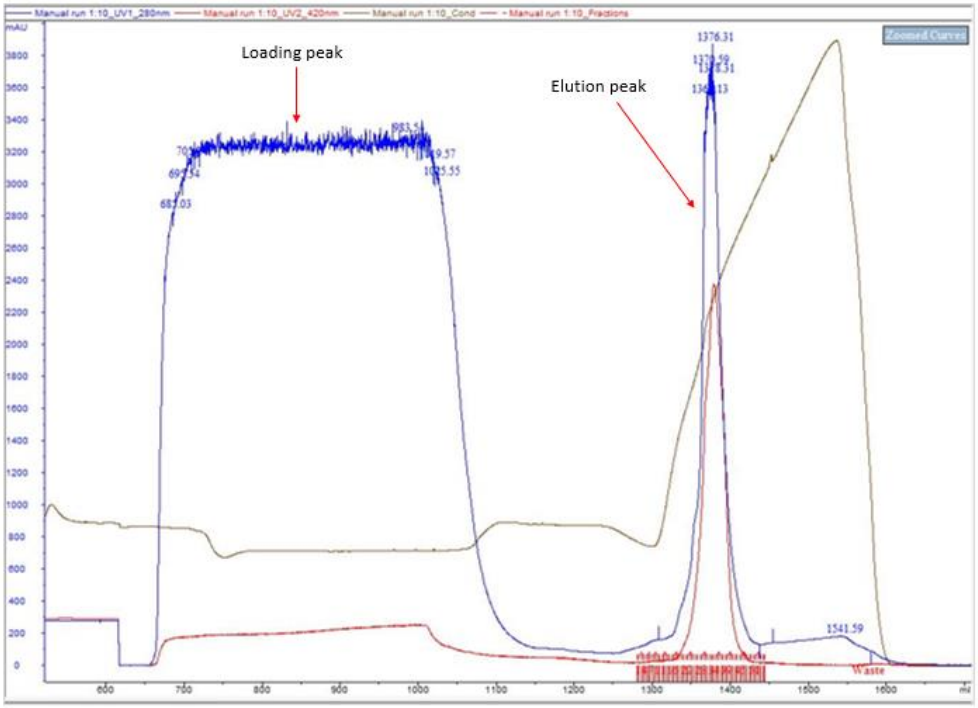

Figure 20: HisTrap chromatogram of cytochrome cz after 2 L shaking flask expression.

In Figure 20, the loading and elution of cytochrome *cz* is shown. The fractions of the elution peak were concentrated and rebuffered with Amicon centrifugal tubes (MWCO: 3 kDa) to decrease the sample volume as much as possible for the subsequently performed SEC. SEC had to be done in three runs because of the large overall sample volume. Per run, 2 mL of sample were applied through the loading loop. For a good peak separation, the volume of loaded sample for SEC should ideally be 1-2 % of the resin bed size but maximum 4 %.

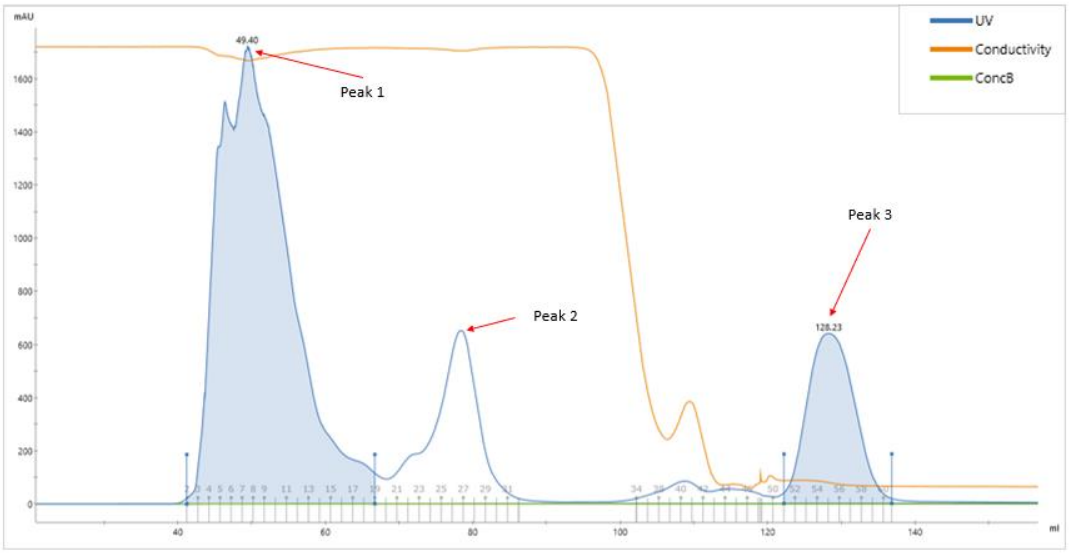

Figure 21: Run 1 of SEC of cytochrome cz after 2 L shaking flask expression.

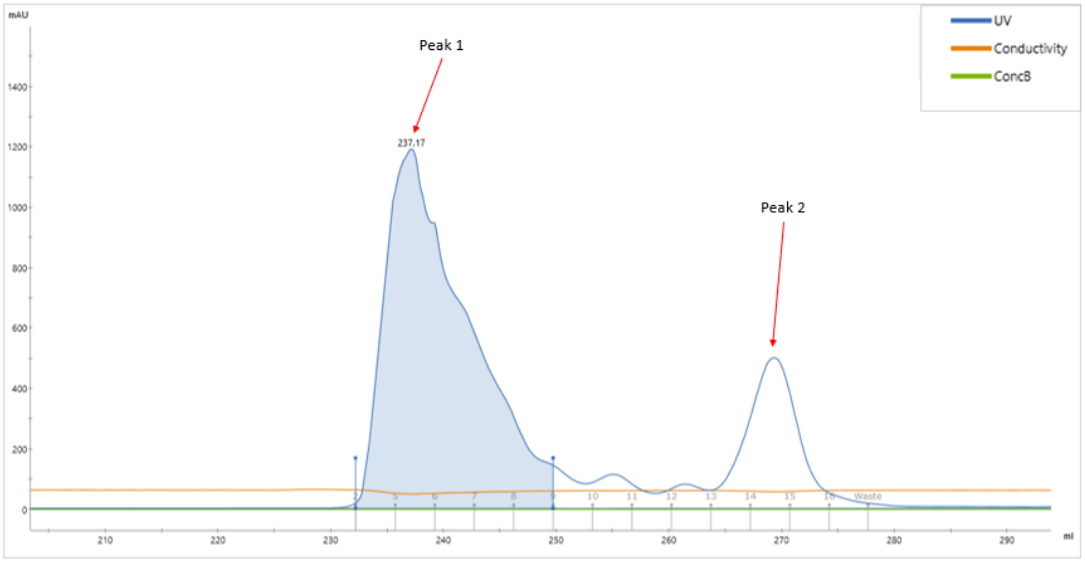

Figure 22: Run 2 of SEC of cytochrome cz after 2 L shaking flask expression.

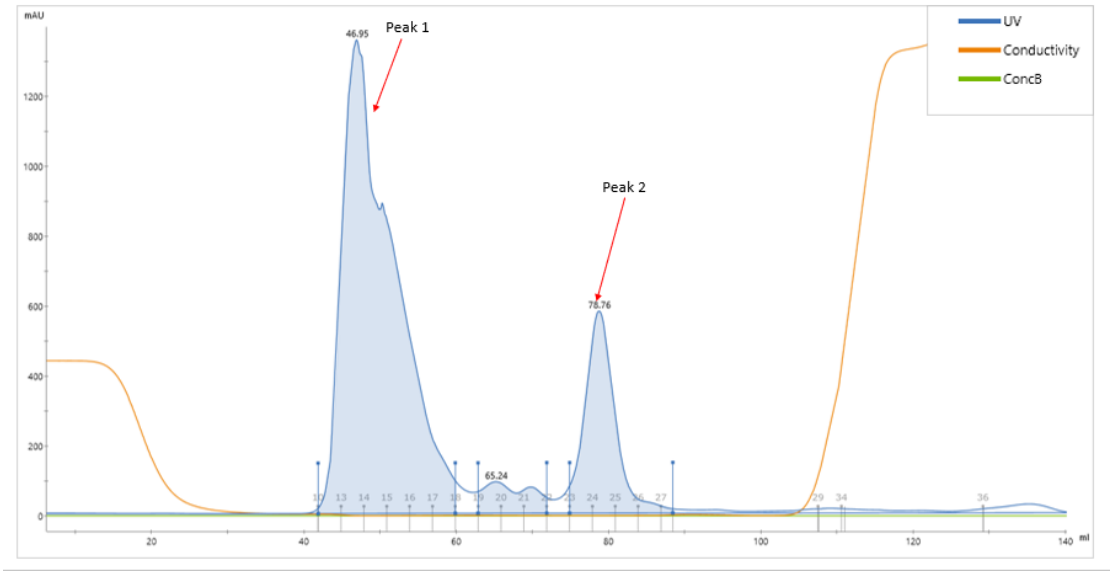

Figure 23: Run 3 of SEC of cytochrome cz after 2 L shaking flask expression.

After SEC, all Peak 1 fractions and Peak 2 fractions of all runs were united and concentrated separately with Amicon centrifugal tubes (MWCO: 3 kDa). Peak 3 of SEC Run 1 did not contain any cytochrome *cz* because it did not have a reddish color and was therefore not used for further treatment.

Table 18: Purification scheme of the cytochrome cz expression in 2-L shaking flasks.

Cytochrome *cz* expression in 2-L shaking flasks				
Sample	**Protein concentration [mg/mL]**	**Total Volume [mL]**	**Total protein [mg]**	**Yield [%]**
Crude extract	3.79	350	1325	-
SEC Peak 1	0.53	71	38	2.9
SEC Peak 2	0.56	47	26	2.0

Table 18 shows the protein concentration after the different purification steps. Protein concentration of the crude extract was measured with Bradford, concentration after SEC was determined based on absorption in the diode array spectrophotometer at 551 nm (ε_{551} = 30 mM^{-1} cm^{-1}).

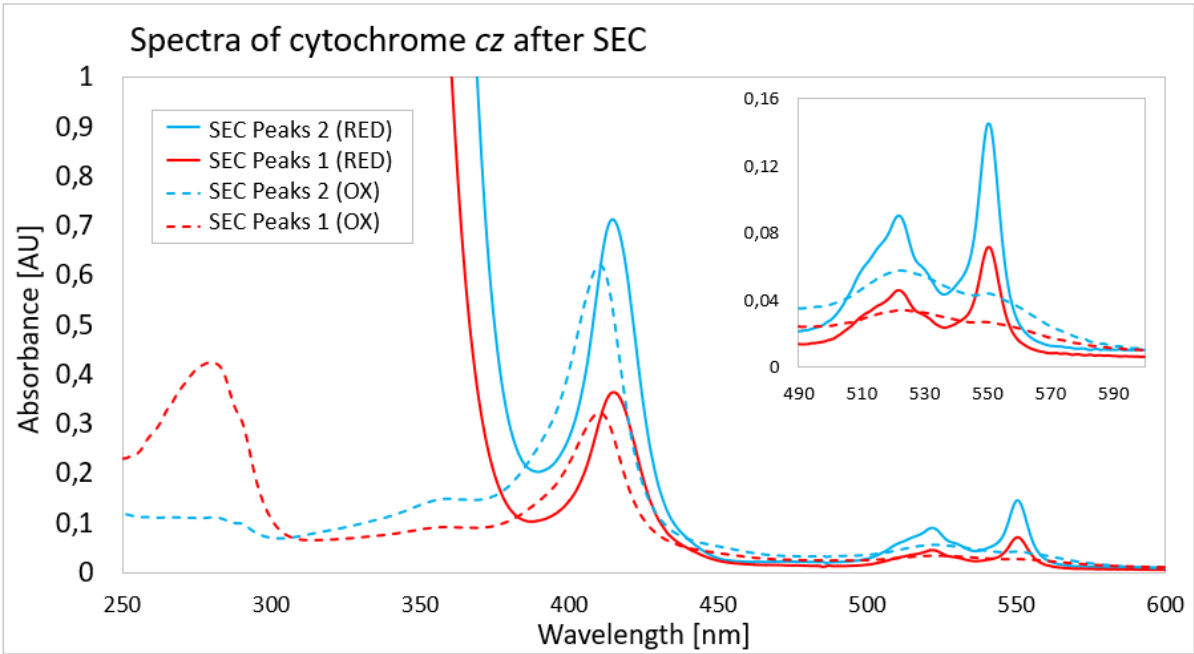

Figure 24: Spectra of the cytochrome cz peaks.

Figure 24 showed the spectra of the two united and concentrated peaks after SEC. The reduced spectra are measured after the addition of solid disodiumdithionite. When comparing the oxidized spectra (punctuated lines), Peak 1 from the SEC (red lines) showed a higher absorbance at 280 nm than Peak 2 from the SEC (blue lines). Because cytochrome *cz* has a very low content of aromatic amino acids, Peak 1 from the SEC contained other protein impurities. Between the oxidized and reduced spectra, a shift of the Soret band at around 420 nm could be observed. Additionally, in the reduced spectra the peaks at 522 nm and 551 nm were visible. They can be traced back to the reduction of the heme complex. The inset in the upper right corner shows a zoom of the spectra between 490 and 600 nm.

3.2.3 Fermentation of cytochrome *cz* in the 5-L fermenter

A different approach to cytochrome *cz* production was the expression in a 5-L Eppendorf BioFlo 120 fermenter with a feed to keep the cells at a constant growth rate. For the preculture, a non-baffled shaking flask with 200 mL LB medium containing ampicillin and chloramphenicol was inoculated with a single positive colony of *E. coli* BL21 cells and was put on a shaker with 130 rpm at 37 °C for around 24 h. For the fermentation in the fermenter, ampicillin and chloramphenicol were added.

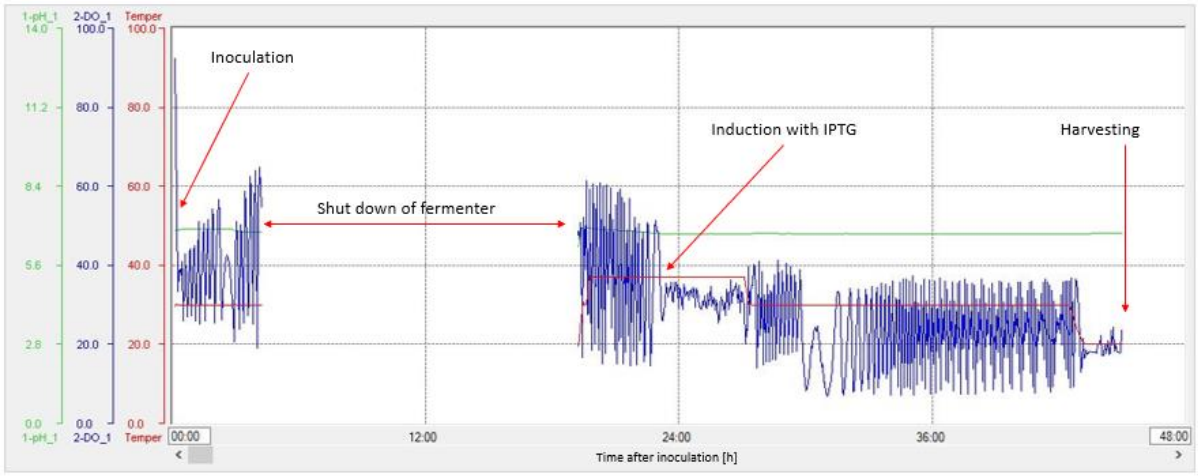

Figure 25: Process diagram of the cytochrome cz fermentation in the 5 L fermenter.

Figure 25 show the DO concentration and the temperature during the fermentation. After the initial glucose in the start media was used up in the batch phase, the glucose feed was started. The feed was automatically controlled by the Eppendorf BioFlo 120 system. Overnight, the fermenter shut down unexpectedly and in the morning all cells have sunk to the bottom of the vessel. The fermenter was restarted and the cells were brought back into suspension. Additionally, the temperature was increased to 37 °C to ensure proper growth conditions. After induction, the cells needed some time to adapt at the new conditions. When the DO signal was stable again, temperature was decreased to 30 °C for the protein expression phase. Shortly before harvesting, the temperature was again decreased to 20 °C.

After harvesting by centrifugation, a total wet biomass of 95 g could be obtained. The supernatant was discarded and periplasmic extraction of the cytochrome *cz* was attempted. Because the extraction was not successful, likely because remaining contents of the media on the cell pellet were interfering with the osmotic shock, the cell pellet was frozen at -80 °C until further processing. After thawing, the cells were lysed and additionally, ultrasonication was performed to break open all the still intact cells. The suspension was centrifuged, and the supernatant was filtered. The crude extract was then purified by HisTrap chromatography.

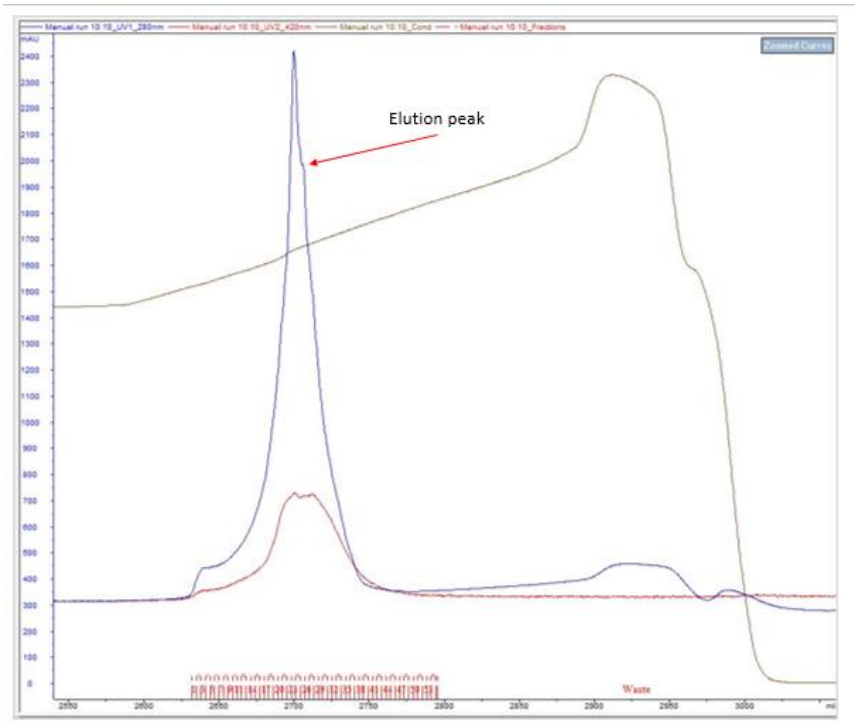

Figure 26: HisTrap chromatogram of cytochrome cz fermentation in the 5 L fermenter.

As a start buffer for HisTrap, 50 mM Tris with 150 mM NaCl, pH 7.5 was used. For the elution buffer, this buffer was supplemented with 500 mM imidazole. Figure 26 shows the elution peak of the HisTrap chromatogram. After HisTrap purification, SEC was performed to separate apo- and holoform of cytochrome *cz.*

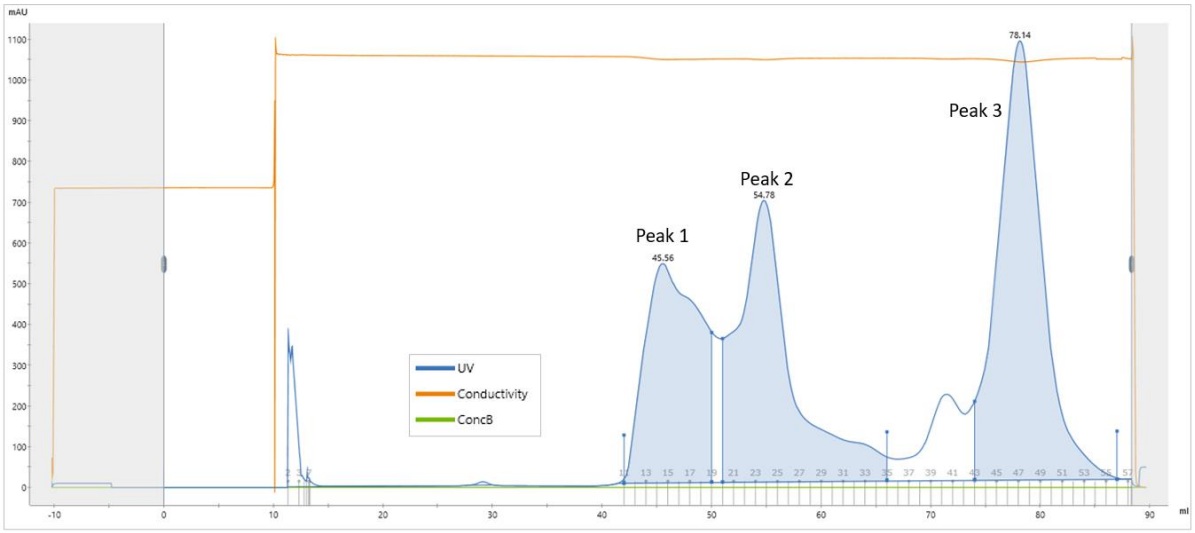

Figure 27: SEC of cytochrome cz after expression in the 5-L fermenter.

In Figure 27, it is shown that protein elution happened in three peaks. The fractions of Peak 3 however were not colored, fractions of Peaks 1 and 2 had a pink color. All fractions of the first two peaks were united, concentrated and rebuffered.

Table 19: Purification scheme of cytochrome cz expression in a 5-L fermenter.

Cytochrome *cz* expression in a 5-L fermenter				
Sample	**Protein concentration [mg/mL]**	**Total Volume [mL]**	**Total protein [mg]**	**Yield [%]**
Crude extract	0.12	1650	198	-
After HisTrap	6.43	3	19.29	9.7
After SEC	1.98	5	9.90	5.0

Table 19 shows the protein concentration after the different purification steps. Protein concentration of the crude extract was measured with Bradford, concentration after SEC and after HisTrap were measured with the diode array at 551 nm (Abs.coeff. 30 mM^{-1} cm^{-1}) because of the high purity of cytochrome *cz*. A lot of protein was lost between the HisTrap purification and the SEC. It can be assumed that the majority of this lost protein was apo form of the cytochrome *cz.* This leads to the conclusion that the production in the fermenter produced more unusable apo form then during the

shaking flask expression. A reason for this might be the overnight shut-off of the fermenter which resulted in a loss of the pEC86 plasmid leaving *E. coli* unable to fully mature cytochrome *cz*.

3.2.4 Comparison and discussion of cytochrome *cz* production

When comparing the two different production strategies mentioned in 3.2.2 and 3.2.3, the fermenter production of cytochrome *cz* is very time consuming, needs a lot of preparation and a complicated media and feed preparation. The protocol used for production in the fermenter was supposed to be a high-density fermentation with a theoretical final wet biomass of up to 200 g/L. However, this does only work if the cells are constantly kept in a growth phase but in our case, the fermenter had technical issues and stopped overnight. Thereby only a low wet biomass comparable to the shaking flask expression was achieved. This protocol seems to be more beneficial for *E. coli* with integrated genes and not for expression from a plasmid. The shaking flask expression on the other hand is a lot more convenient for the laboratory-scale production because of the easier handling and simpler media. When cytochrome *cz* needs to be produced in greater amount than needed in the laboratory, the expression in the fermenter poses a good alternative to the shaking flask expression because parameters can be controlled more easily and the handling of larger culture volumes is easier as well. However, optimal expression conditions still need to be evaluated and investigated further for this method.

When comparing the cytochrome *cz* yields of the two methods, the shaking flask expression has greater total cytochrome yield (holo- and apo form) than the expression in the fermenter. We observed that it is important to have antibiotics present in the fermentation media, otherwise a great amount of cytochrome will not have heme inserted due to the loss of the pEC86 plasmid. The overall culture volumes of both methods were similar. This lower yield of holo protein (38 mg in shaking flasks expression compared to 9.90 mg after fermenter expression, Table 18 and Table 19) could partly be explained by the assumed loss of the pEC86 plasmid during cultivation.

3.3 Sortagging and chimeric protein purification

Of the three *Gc*GDH variants produced, so far only *Gc*GDH_K480G was fused with the cytochrome *cz* domain to create a chimeric enzyme. The reaction was carried out while centrifuging in an Amicon centrifugal tube (MWCO: 3 kDa) to remove cut off tags from the cytochrome. The small C-terminal ends of cytochrome *cz* can go through the membrane. This prevents backreaction with the free glycine end of the tag and pushes the reaction product more towards the chimeric protein. After the reaction, SEC was performed to separate sortase and the remaining free cytochrome *cz* domains from the mixture.

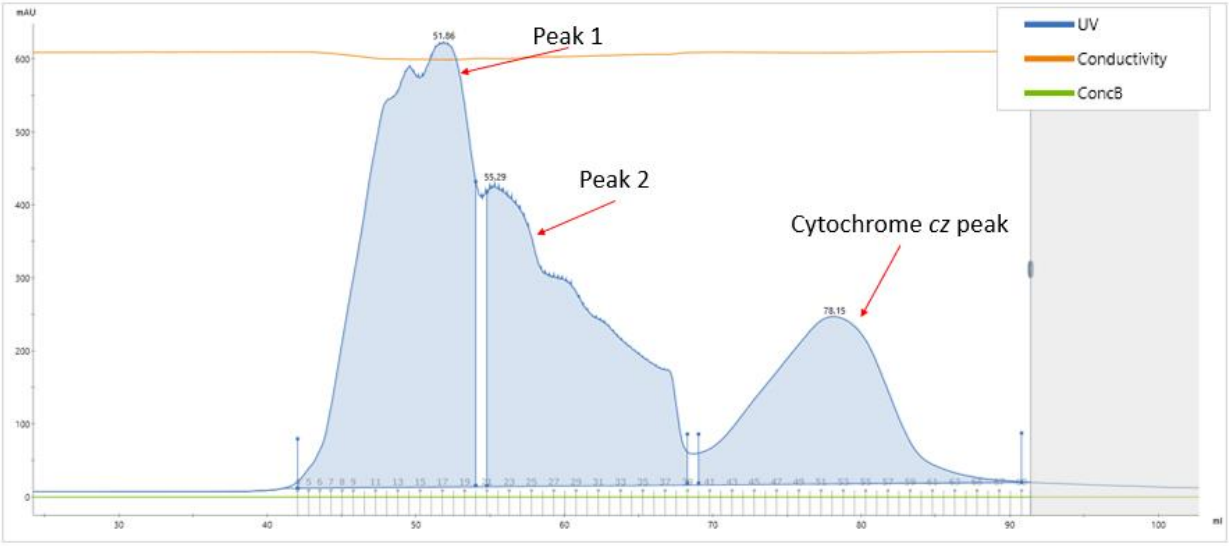

Figure 28: SEC of the sortagging reaction mixture.

Figure 28 shows the elution of the SEC. Peak 1 and Peak 2 had an orange color, which indicates a mixture the chimeric enzyme and the remaining *Gc*GDH domain. Cytochrome *cz* and sortase A eluted separately, all fractions of this peak were red.

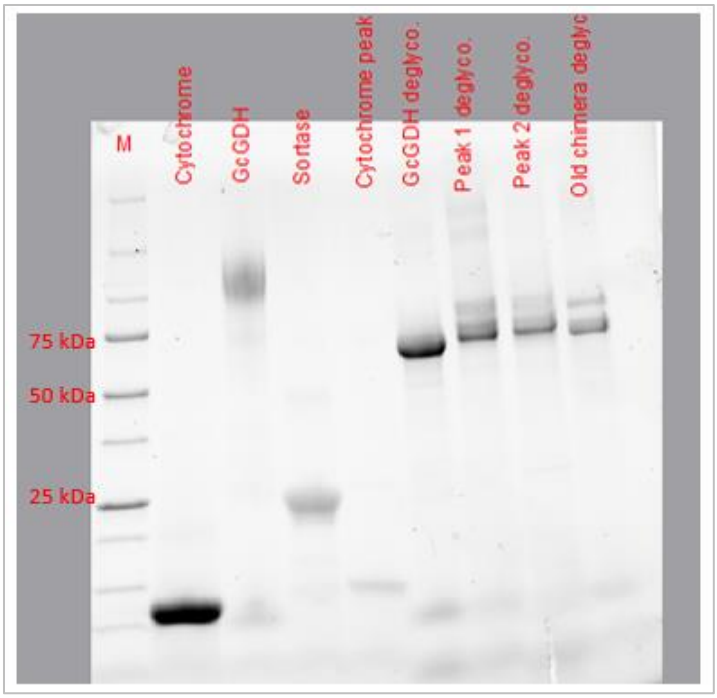

Figure 29: SDS PAGE after SEC of the GcGDH_K480G fusion enzyme.

With selected fractions of all peaks, a SDS PAGE was performed (Figure 29). For better comparability of the content of all fractions, cytochrome *cz*, wild-type *Gc*GDH, deglycosylated wildtype *Gc*GDH and sortase were applied to the gel as well as a control sample. Because Peak 1 and Peak 2 were separated insufficiently with DEAE Sepharose which might be attributed to glycoforms of different sizes, the fractions were united and concentrated. Afterwards cation exchange chromatography (Source 15S resin) was performed. Sortase and cytochrome *cz* were believed to elute in the same peak of SEC because of their similar size. Sortase related bands could also not be seen on the SDS PAGE because only a small amount was added to the mixture.

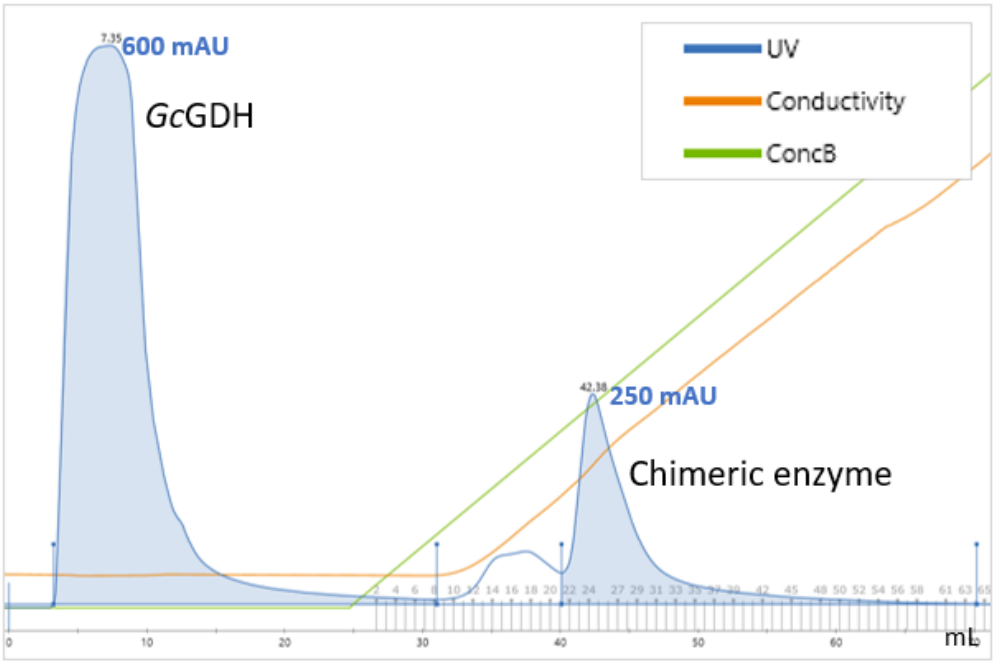

Figure 30: IEX chromatography (Source 15S) of the sortagging reaction mixture.

In Figure 30, the separation of the remaining *Gc*GDH domain and the chimeric enzyme is visible. A 50 mM sodium acetate buffer pH 4 was used as running buffer. The *Gc*GDH (pI of 4.59) went through the column without binding, whereas the chimeric enzyme bound to the column because of the attached cytochrome *cz* domain which has a pI above 9. Elution was carried out with the same buffer supplemented with 750 mM NaCl. The fractions were united and concentrated with Amicon centrifugal tubes (MWCO: 10 kDa). Because of the extremely low yield of chimeric enzyme, neither determination of protein concentration nor a DCIP assay were done after purification. The enzyme was frozen until used for pH profile, photometer assay and chronoamperometry.

3.4 Activity pH profiles of the *Gc*GDH domains

With the different obtained *Gc*GDH variants, as well as with the chimeric enzyme, pH profiles within a range between pH 3 and pH 9 were recorded to investigate, whether introduced mutations or fusion with the cytochrome *cz* domain affects pH optimum or overall activity of the *Gc*GDH domain. For better comparability, the wild-type *Gc*GDH

and the deglycosylated *Gc*GDH were included in the measurements. Activity was measured with a DCIP assay.

To investigate the influence of the buffer on the activity and determine the pH optimum of the protein, three different systems were used. Firstly, a 100 mM potassium phosphate buffer was used between pH 6 and pH 8. Secondly, a McIlvaine buffer system was used between pH 3 and pH 8. Furthermore, a phosphate-borate-citrate buffer system was used from pH 3 to pH 9 [21]. All samples were adjusted to a protein concentration of 0.02 mg/mL, measurements were performed with a spectrometer. Measurements of the DCIP assay were carried out in triplicates.

3.4.1 Activity pH profile of wildtype *Gc*GDH (deglycosylated and glycosylated)

Deglycosylation was done with an enzymatic digestion reaction using EndoHF where 85 µL of the wildtype *Gc*GDH sample, 5 µL of EndoHF and 10 µL 10x EndoHF buffer were incubated for 1.5 h at 30 °C. Wildtype *Gc*GDH was taken from the laboratory´s storage.

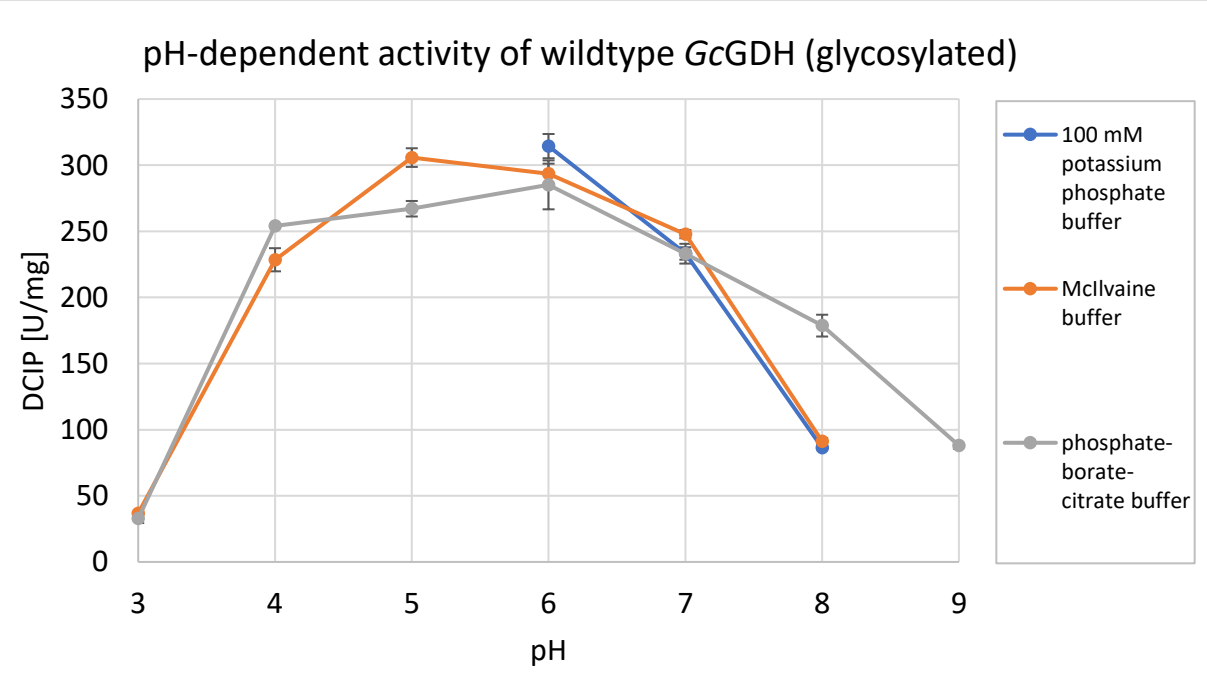

Figure 31: pH-dependent activity of wildtype GcGDH in three buffer systems.

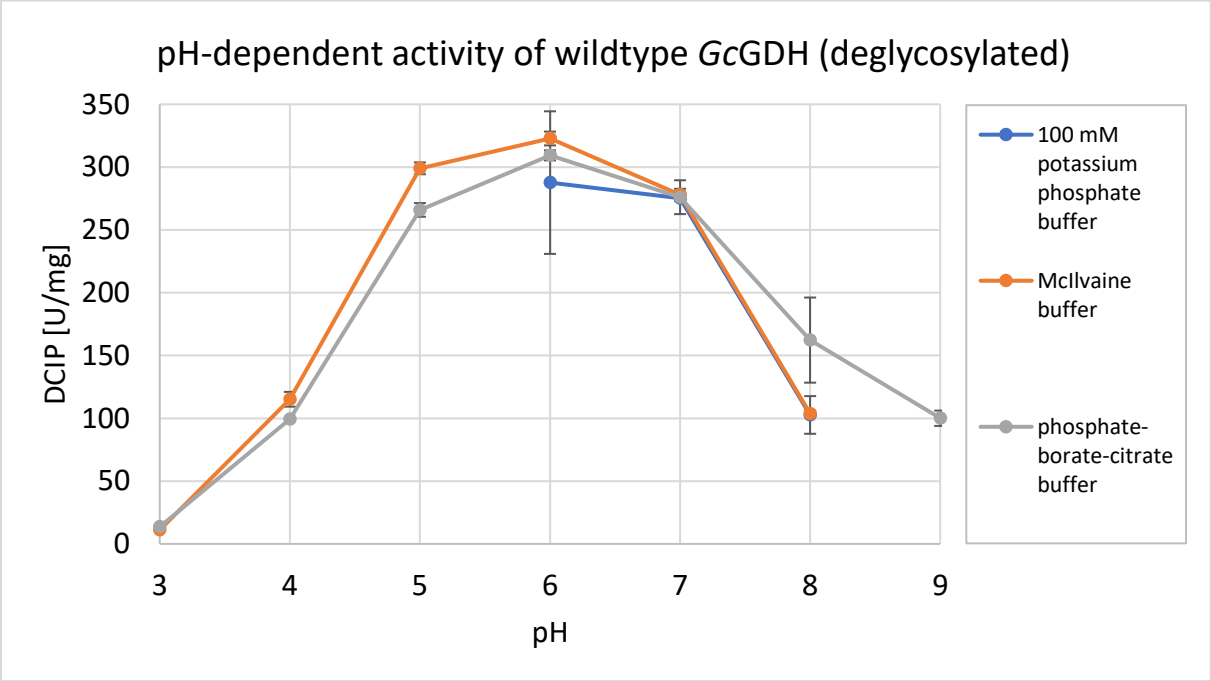

Figure 32: pH-dependent activity of deglycosylated wildtype GcGDH in three buffer systems.

According to Figure 31, wild-type *Gc*GDH has a pH activity optimum of pH 6.0 with a maximum activity of 314 U/mg (in 100 mM potassium phosphate buffer). When deglycosylating the enzyme (Figure 32), the observed pH optimum as well as the maximum specific activity stayed almost constant with 323 U/mg (in McIlvaine buffer). Nevertheless, the deglycosylated *Gc*GDH showed a depression in specific activity in the acidic pH range around pH 4 from around 240 U/mg (glycosylated) to around 100 U/mg (degylcosylated). Despite that, the deglycosylation did not affect the activity significantly. The buffer system only has a minor influence on the overall specific activity, but it can be observed that both enzymes showed higher specific activity above pH 7 in the phosphate-borate-citrate buffer, while specific activity dropped significantly above pH 7 in the other two systems.

3.4.2 Activity pH profile *Gc*GDH_K480G and *Gc*GDH_WT (with attached linker domain)

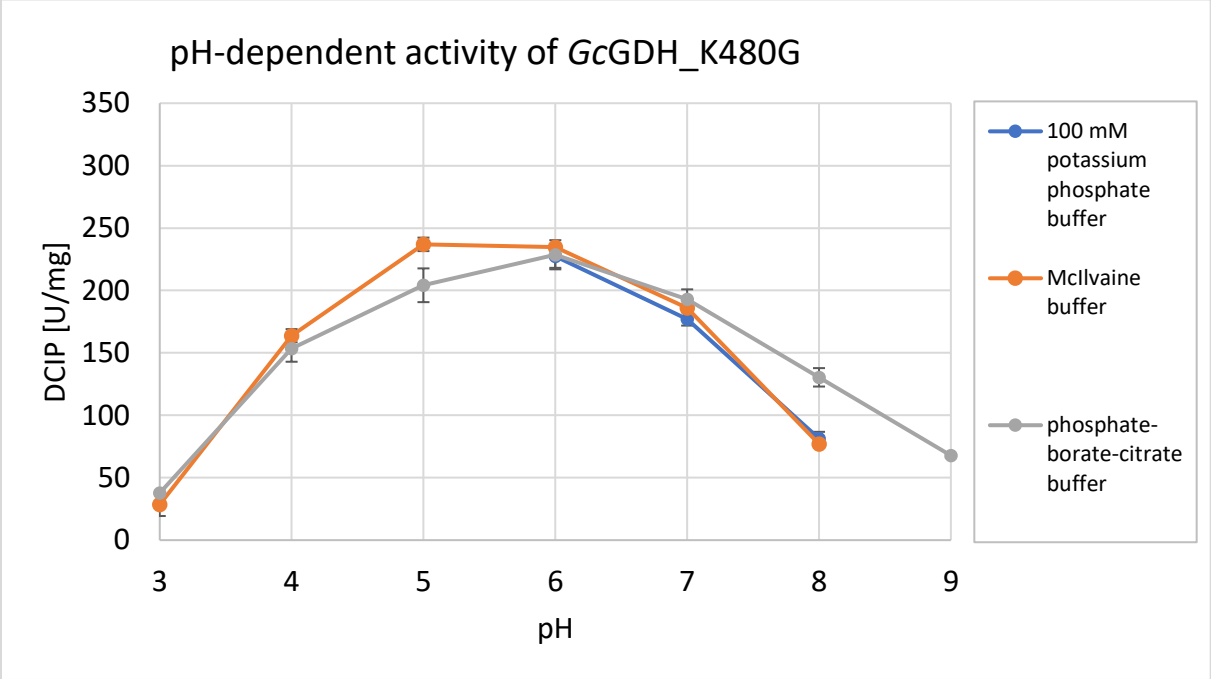

Figure 33: pH-dependent activity of GcGDH_K480G in three buffer systems.

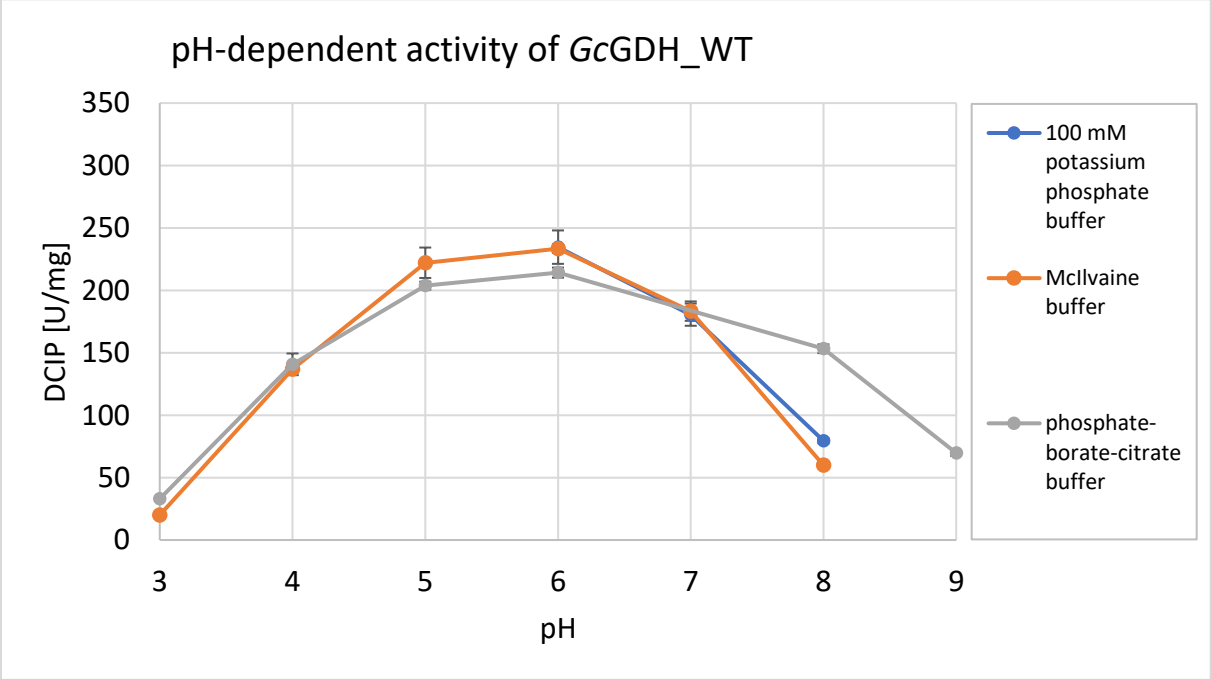

Figure 34: pH-dependent activity of GcGDH_WT in three buffer systems.

When comparing *Gc*GDH_K480G (Figure 33) and *Gc*GDH_WT (Figure 34) to the wildtype *Gc*GDH (Figure 31), a slight shift in pH activity optimum occurred for *Gc*GDH_K480G to pH 5 (in McIlvaine buffer), whereas for *Gc*GDH_WT, the pH optimum stayed at pH 6. The optimum shift of *Gc*GDH_K480G however was minor and negligible when comparing the average activity values of all buffer systems. However, it can be observed that the overall specific activity of both modified enzymes at pH 6 decreased from 314 U/mg (wildtype *Gc*GDH; in 100 mM potassium phosphate) to 235 U/mg (*Gc*GDH_K480G; in McIlvaine buffer) and 233 U/mg (*Gc*GDH_WT; in McIlvaine buffer), respectively. Because wildtype *Gc*GDH and *Gc*GDH_WT only differed in the attached linker domain, it could be concluded that the artificial linker domain decreased specific activity of the enzyme. However, the difference is more likely attributed to differences in the protein concentration and we assume that the linker sequence has no negative effect on the enzyme activity. As mentioned in 3.4.1, a higher specific activity in the phosphate-borate-citrate buffer system above pH 7 could be observed.

3.4.3 Chimeric *Gc*GDH_K480G fusion enzyme

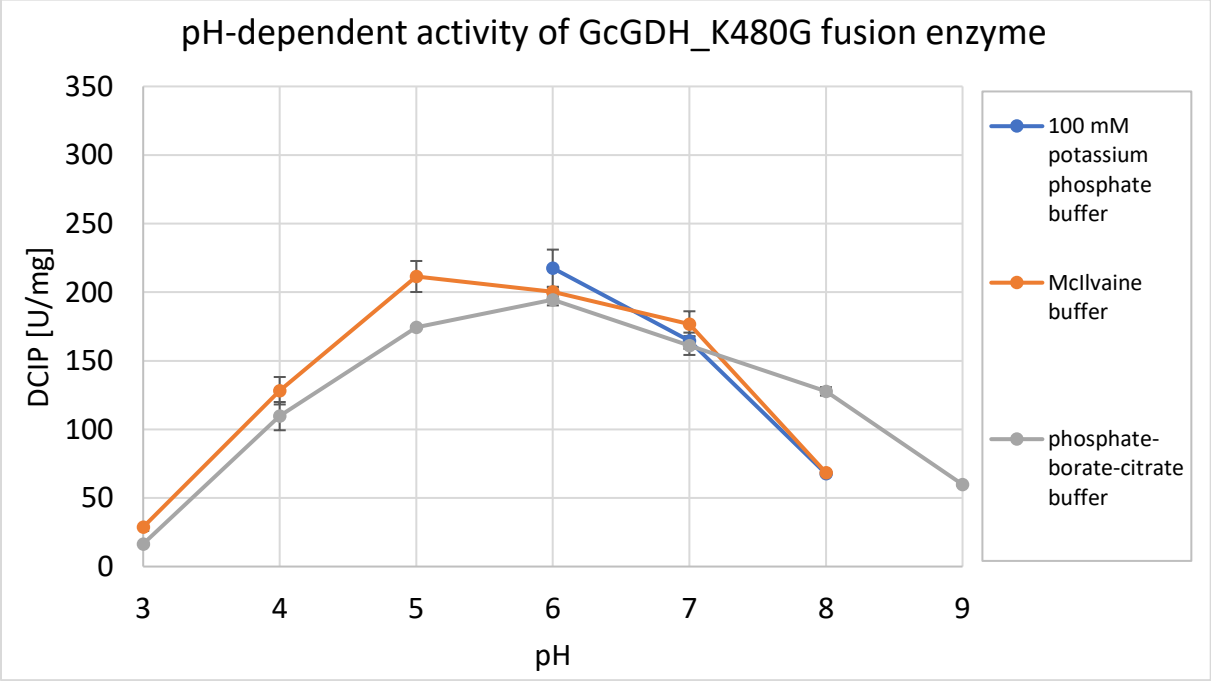

Figure 35: pH-dependent activity of chimeric GcGDH_K480G fusion enzyme in three buffer systems.

When comparing the pH profile of the chimeric enzyme (Figure 35) to the *Gc*GDH_K480G without the attached cytochrome *cz* (Figure 33), specific activity was only slightly lower for the chimeric enzyme (217 U/mg; in 100 mM potassium phosphate buffer) at the pH optimum of pH 6. This might be since also for this pH profile a protein concentration of 0.02 mg/mL was used, but because of the attachment, the chimeric enzyme has a higher molar mass. This resulted in a lower molar concentration of chimeric protein which would explain the lower specific activity. The cytochrome *cz* attachment did not have an impact on the activity pH optimum when compared to the wildtype *Gc*GDH (Figure 31). Additionally, the same increased specific activity in the phosphate-borate-citrate buffer above pH 7 could again be observed as mentioned in 3.4.1 and 3.4.2.

3.5 Reduction of cytochrome *cz* by *Gc*GDH in solution

A qualitative photometer assay was performed with the unlinked species of wildtype *Gc*GDH, the deglycosylated wildtype *Gc*GDH, *Gc*GDH_K480G and *Gc*GDH_WT. All variants were combined equimolarly (both 5 µM) with cytochrome *cz*, having both the GcGDH domain and the cytochrome *cz* domain free in solution. Additionally, the chimeric *Gc*GDH_K480G fusion enzyme (5 µM) was tested with this method as well. All reactions were carried out in a phosphate-borate-citrate buffer at pH 6 [21] and 30 °C and in the presence of oxygen. As soon as the signal at 551 nm was stable, 50 mM glucose was added. The *Gc*GDH converts glucose, whether electrons harvested from this reaction can be transferred to the cytochrome (and to what extent) is tested in this assay. To see the full extent of the reduction, disodiumdithionite (here abbreviated DDT) was added when no rise in absorbance after glucose addition was visible anymore. The spectra were normalized at 600 nm, because there is no absorbance expected at this wavelength. In the spectra overlay diagrams, a zoomed-in picture of spectra between 490 nm and 600 nm is shown for a more detailed view on the heme peaks.

3.5.1 Wild-type *Gc*GDH and deglycosylated wild-type *Gc*GDH

Deglycosylation of *Gc*GDH was performed as mentioned in 3.4.1.

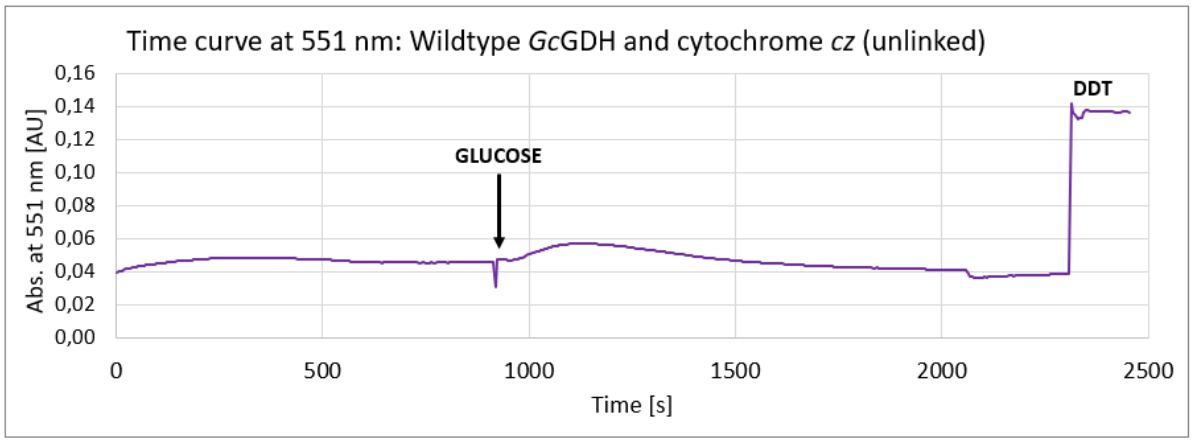

Figure 36: Absorbance at 551 nm over time of wildtype GcGDH and cytochrome cz (unlinked) upon glucose addition.

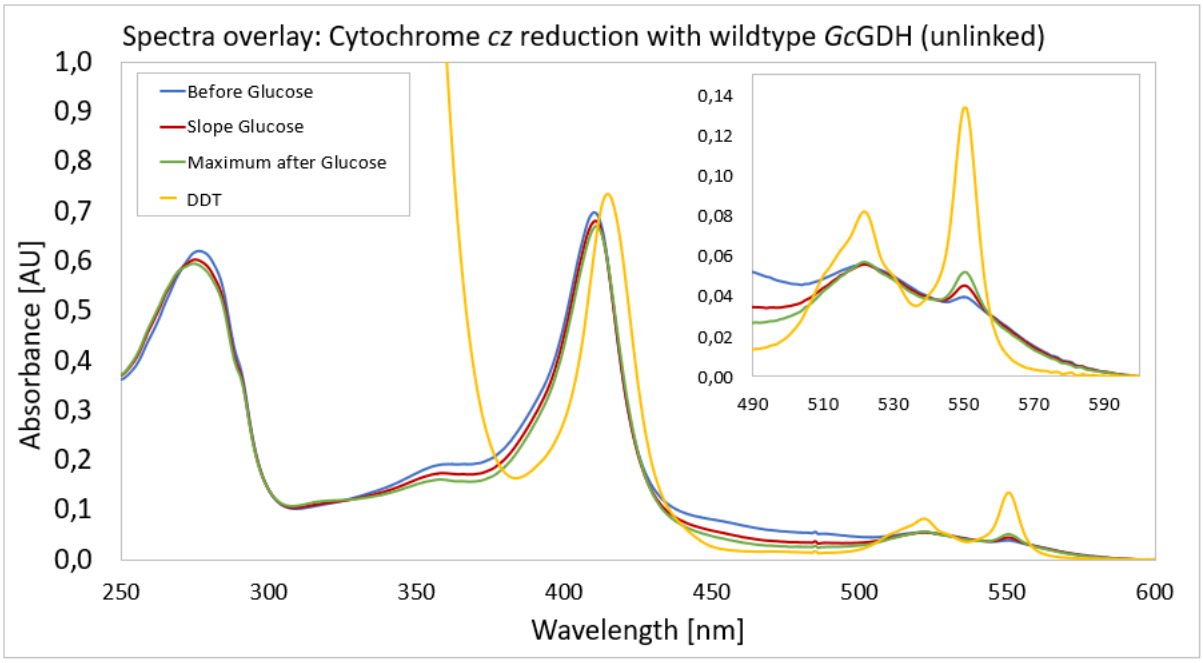

Figure 37: Spectra overlay of cytochrome cz reduction with wildtype GcGDH (unlinked) before glucose addition (blue), during the rise of absorbance after glucose addition (red), the maximum absorbance after glucose addition (green) and the maximum possible absorbance after addition of DDT (yellow).

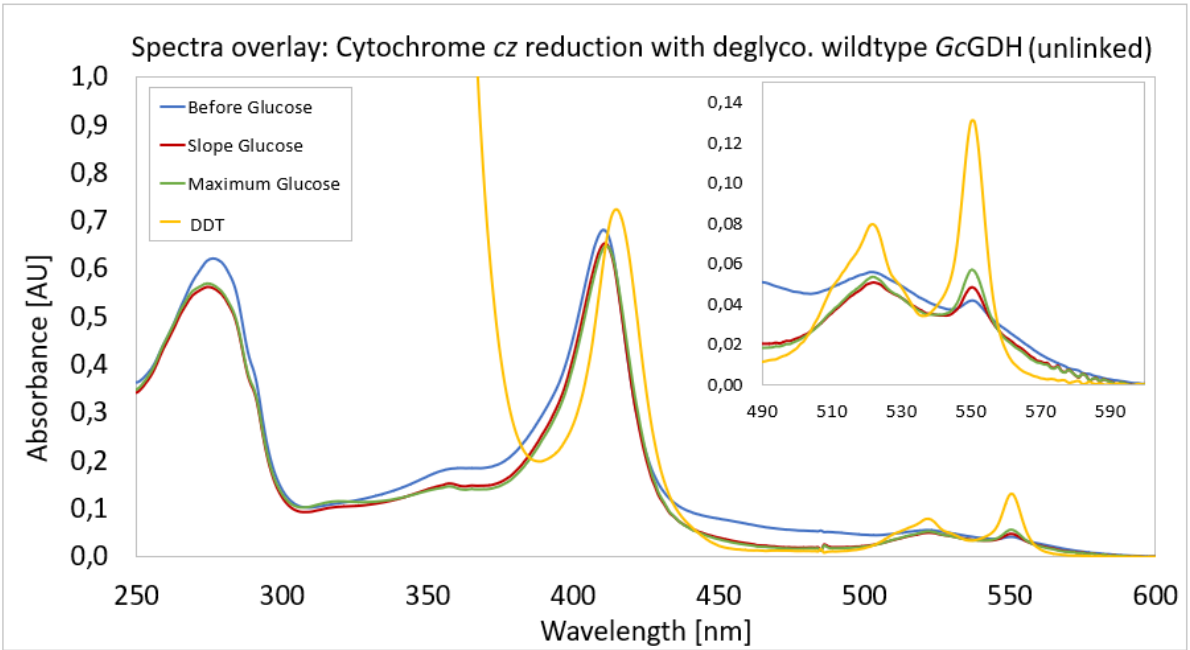

Figure 38: Spectra overlay of cytochrome cz reduction with deglycosylated wildtype GcGDH (unlinked) before glucose addition (blue), during the rise of absorbance after glucose addition (red), the maximum absorbance after glucose addition (green) and the maximum possible absorbance after addition of DDT (yellow).

In Figure 36 the absorbance at 551 nm during the reaction can be seen as an indication of reduced cytochrome. A similar curve was obtained for all variants. After the addition of glucose, absorbance at 551 nm starts to rise because *Gc*GDH transfers electrons to the cytochrome. After some time, the absorbance again starts to decrease, likely because of the reduced cytochrome *cz* is oxidized in the presence of oxygen.

Figure 37 and Figure 38 show the overlayed spectra at different time points during the reaction with glucose. A clear rise of absorbance at the two distinctive peaks of cytochrome *cz* (522 nm, 551 nm) could be observed. This led to the conclusion that cytochrome *cz* was capable of oxidizing the *Gc*GDH (wildtype and deglycosylated wildtype) after its reaction with glucose. This led to the reduction of the cytochrome *cz* domain. Because there is a great difference between the maximum absorbance after glucose addition and the maximum possible absorbance with disodiumdithionite (DDT), reduction of cytochrome *cz* only happened to a certain extent. The reaction was carried out in the presence of oxygen which leads to the oxidation of cytochrome *cz* by oxygen.

With this method it was not possible to determine the extent of cytochrome *cz* reduction quantitatively, but we could demonstrate that the concept of chimeric proteins for biosensors is possible. We clearly see that the GDH interacts with the cytochrome and reduces it. Considering that the domains are not linked and the GDH and cytochrome first must interact with each other in a way that the interfaces of the redox centers come in proximity, a slow reduction rate of cytochrome was expected. For later sensor design, proteins will be linked with an optimized linker to optimize the interaction of the domains.

The second issue to address is the oxygen sensitivity of the cytochrome. This effect can also be limited by linking the domains with an optimal linker where the reduction of the cytochrome occurs faster than the transfer of the electrons to oxygen. Also, the cytochrome did never reach 100 % reduction. However, in sensors a high enough potential will be applied to collect the electrons at the electrode, cytochromes will be immediately reoxidized at the electrode and will then be available again for reduction by GDH.

3.5.2 *Gc*GDH_K480G and *Gc*GDH_WT (with attached linker domain)

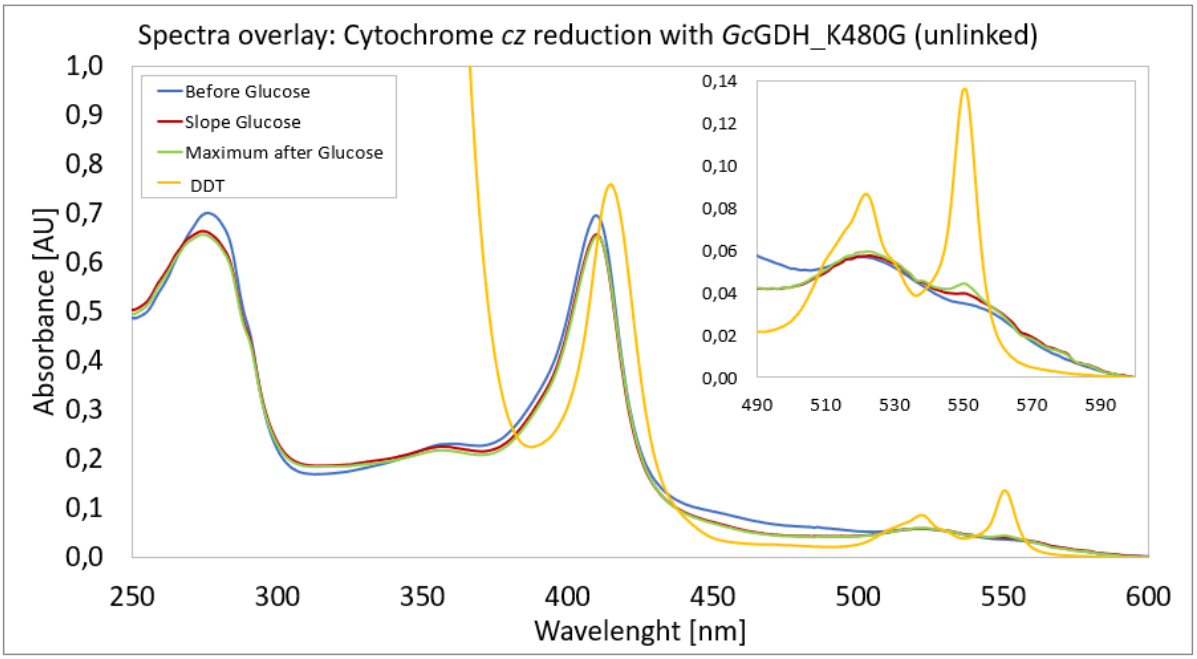

Figure 39: Spectra overlay of cytochrome cz reduction with GcGDH_K480G (unlinked) before glucose addition (blue), during the rise of absorbance after glucose addition (red), the maximum absorbance after glucose addition (green) and the maximum possible absorbance after addition of DDT (yellow).

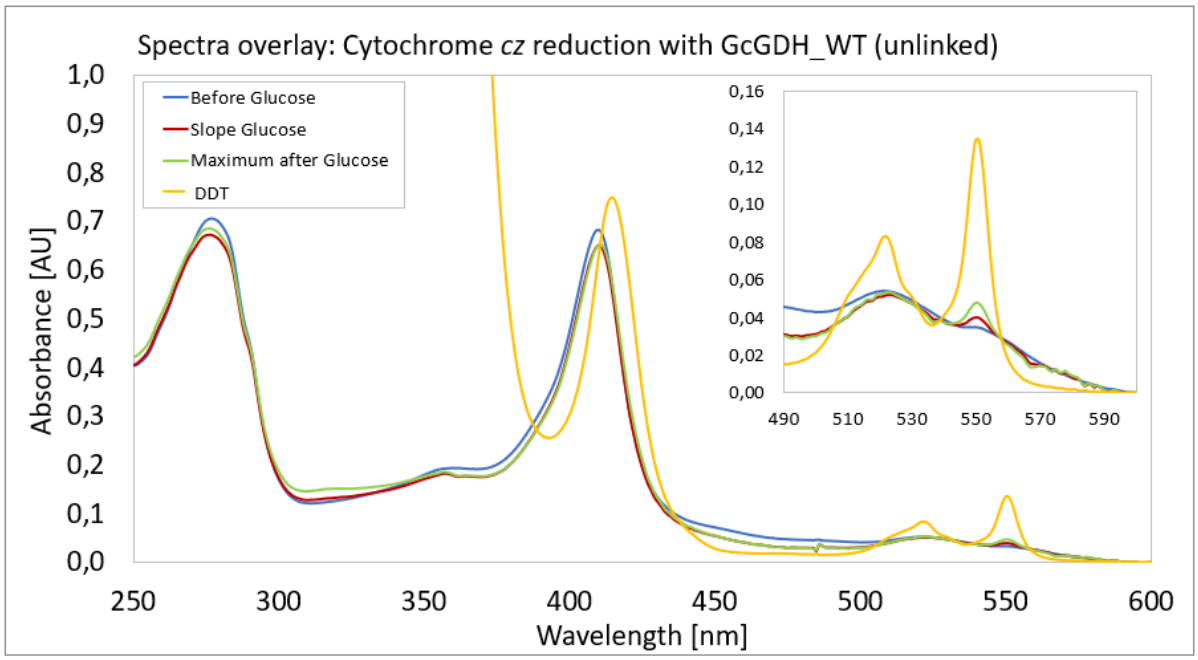

Figure 40: Spectra overlay of cytochrome cz reduction with GcGDH_WT (unlinked) before glucose addition (blue), during the rise of absorbance after glucose addition (red), the maximum absorbance after glucose addition (green) and the maximum possible absorbance after addition of DDT (yellow).

When comparing the spectra overlay of the wildtype and the deglycosylated wildtype *Gc*GDH (Figure 37, Figure 38) to the spectra overlay of the two *Gc*GDH variants (Figure 39, Figure 40), similar extents of reduction after DDT addition can be observed. In Figure 39 and Figure 40 again the peaks at 522 nm and 551 nm increase during the reaction with glucose with again indicates a successful reduction of free cytochrome *cz* by the withdrawal of electrons from the *Gc*GDH domains. This led to the conclusion that the introduced mutations in the *Gc*GDH domains did not hinder the electron transfer between the two free species. A facilitation of electron transfer could not be observed with this method.

3.5.3 Chimeric *Gc*GDH_K480G fusion enzyme

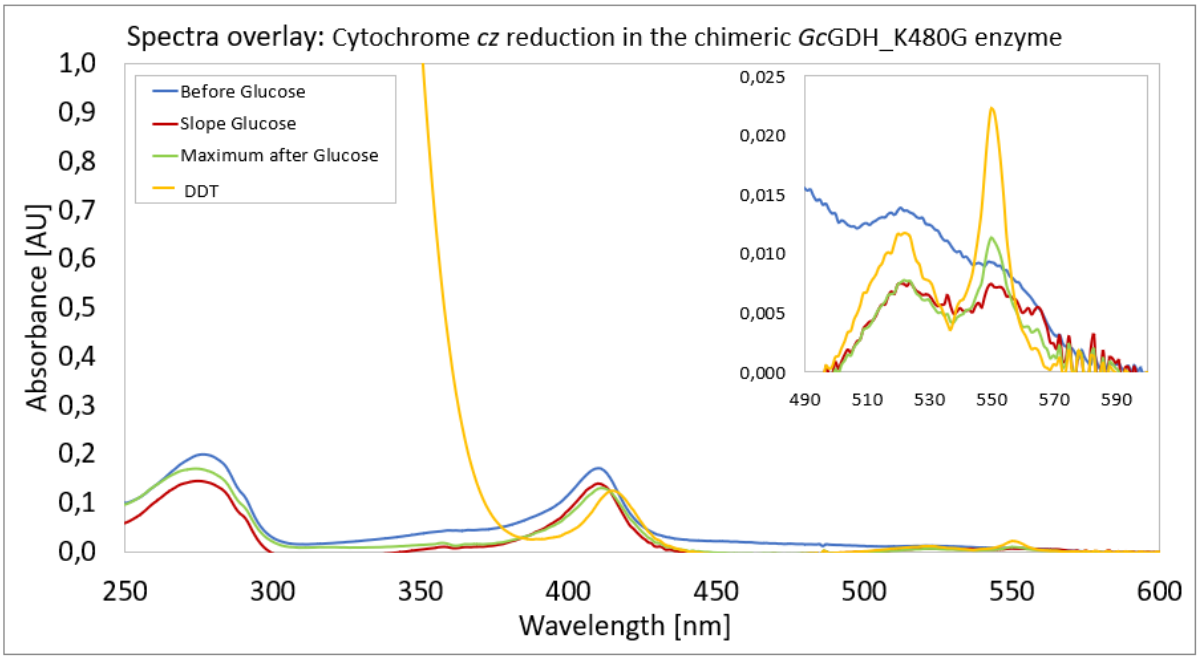

Figure 41: Spectra overlay of cytochrome cz reduction in the chimeric GcGDH_K480G enzyme before glucose addition (blue), during the rise of absorbance after glucose addition (red), the maximum absorbance after glucose addition (green) and the maximum possible absorbance after addition of DDT (yellow).

When the spectra overlay of the chimeric enzyme (Figure 41) was compared to all spectra overlays of the other unlinked variants, the absorbance over the complete recorded wavelength spectrum was lower for the chimeric enzyme. This could be traced back to a dilution error when setting the protein concentration for the assay. It can be seen, that reduction of cytochrome *cz* occurs. It is unclear however, if the cytochrome *cz* withdraws the electrons from its linked *Gc*GDH domain or another *Gc*GDH domain in proximity. From this data it is difficult to say whether the intra-electron transfer between domains in the linked protein is faster and because the reaction is so slow it is impossible to determine statistically solid rates. Generally, we cannot conclude from this data whether or to what extent linkage of the domain has an effect and we can also not make a solid statement whether the mutation of the lysine in the interface on the GDH is beneficial or counterproductive. Additional experiments will be required such as a detailed electrochemical characterization of all linked and

unlinked proteins. As the cytochrome production results in rather low protein yields, the number of possible experiments is limited.

Table 20: Comparison of the time to reach the maximum reduction upon glucose addition (red) and extent of reduction compared to full reduction with DDT (green).

Variant	Time to maximum [s]	Level of reduction [%]
Wildtype *Gc*GDH	205	39
Deglyco. wildtype *Gc*GDH	190	43
*Gc*GDH_K480G	175	33
*Gc*GDH_WT	220	35
Chimeric *Gc*GDH_K480G	868	50

In Table 20, the time to reach maximum reduction after glucose addition and the extent of reduction reached compared to full reduction of cytochrome *cz* with DDT is shown. For the chimeric *Gc*GDH_K480G enzyme, more time is needed to reach maximum reduction (868 s) compared to the other variants (around 200 s). Nevertheless, all variants reached approximately the same level of reduction.

3.6 Cyclic voltammetry of cytochrome *cz*

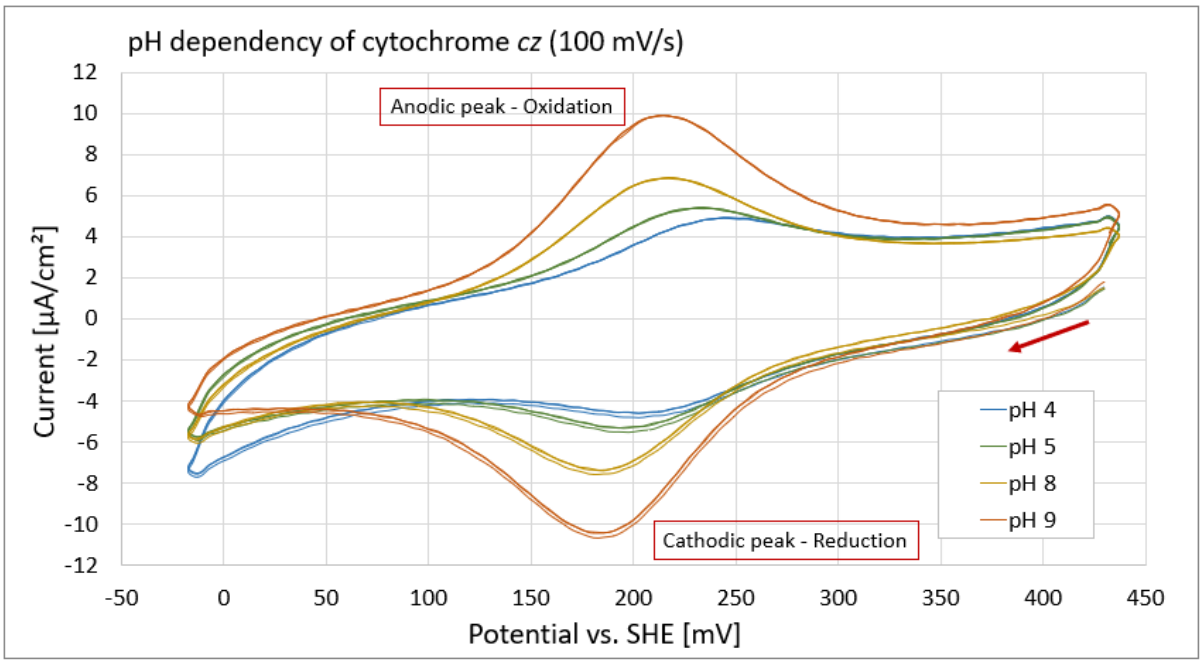

Figure 42: pH dependency of cytochrome cz (100 mV/s) between pH 9.0 and 4.0.

In Figure 42, the pH dependency of the resulting current during cyclic voltammetry of cytochrome *cz* can be observed. The cyclic scans are started from high potential going to low potential and back. When measuring from high to low potentials, the cytochrome is reduced which results in the cathodic peak. At the switching point, the potential is reversed and the cytochrome is again oxidized. For each scan rate, triplicates are measured. With decreasing pH, the resulting current also decreases for cytochrome *cz*.

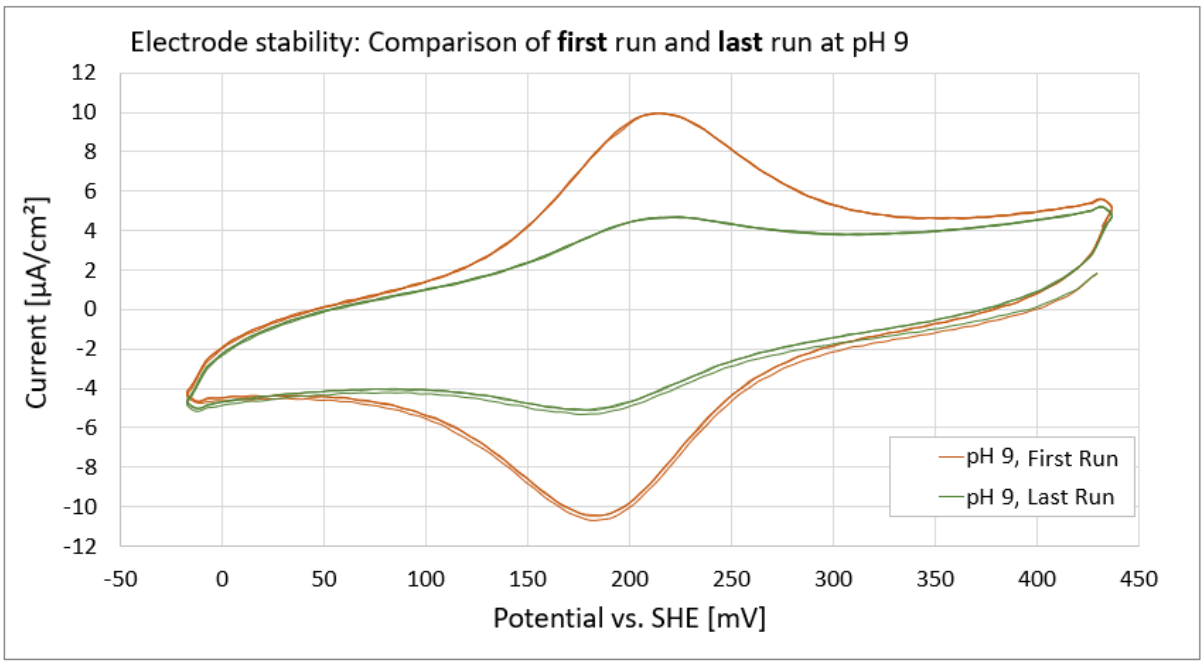

Figure 43: Comparison of first and last run at pH 9.0.

Cyclic voltammetry measurements were done from pH 9.0 to pH 4.0. After all measurements, pH 9.0 was scanned again and the results were compared to the first run at pH 9.0 (Figure 43). It could clearly be seen that the resulting current of the last run at pH 9.0 was significantly lower than during the first run. This led to the conclusion that cytochrome *cz* lost its stability on the electrode most likely because of diffusion through the dialysis membrane, resulting in lower currents.

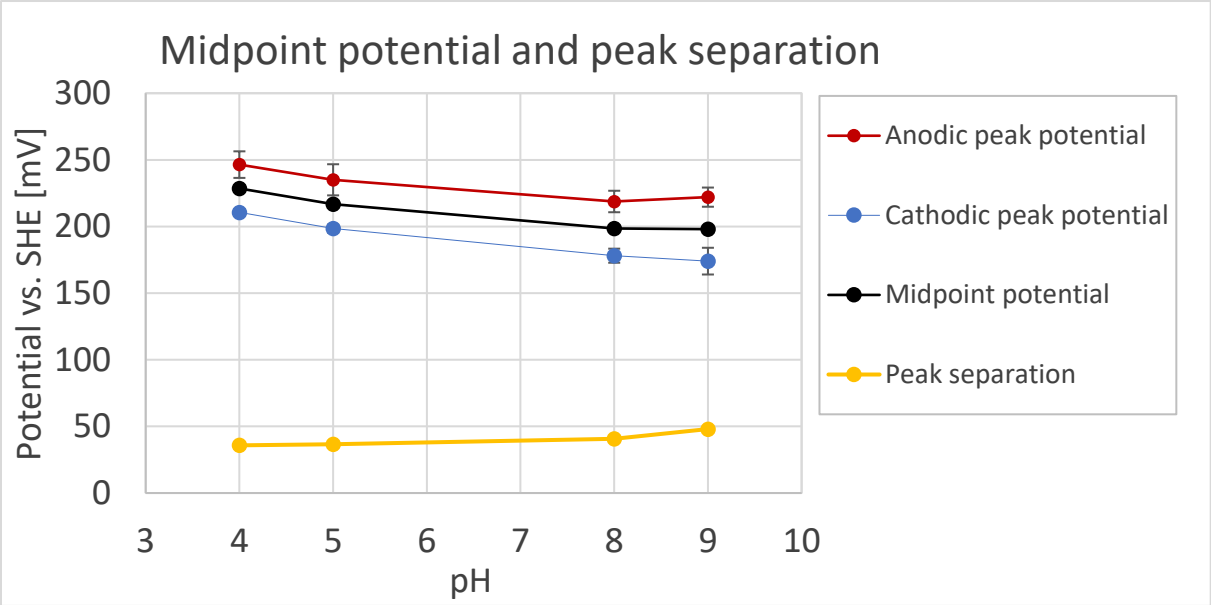

Figure 44: Midpoint potential and peak separation.

In Figure 44, the calculated midpoint potential and peak separation in dependence of the pH are shown. When pH is decreased from pH 9 to 4, the midpoint potential increased from 198 mV vs. SHE to 228 mV vs. SHE, showing that cytochrome *cz* has a pH dependent redox potential. Peak separation stayed constant in the applied pH range. A peak separation below roughly 50-60 mV indicates an electrochemically reversible process.

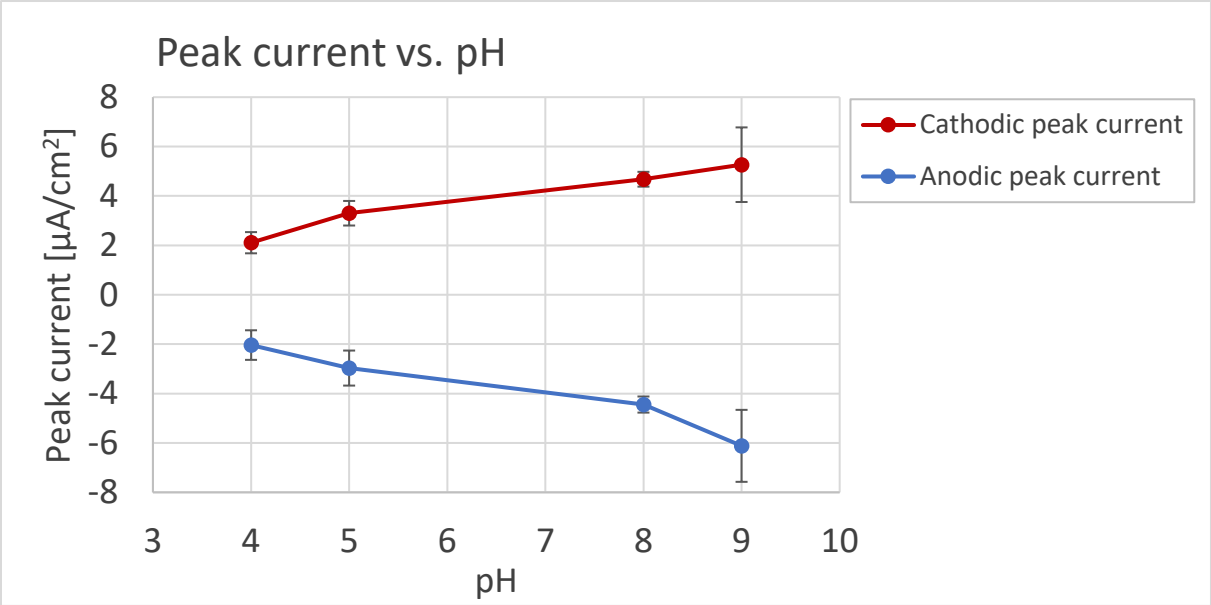

Figure 45: pH dependency of peak currents.

As already indicated by Figure 42, Figure 45 again shows the dependency of the resulting peak currents on pH. The resulting peak currents decreased with decreasing pH. This could be a function of the reduced potential but also be caused by a cytochrome loss through the dialysis membrane.

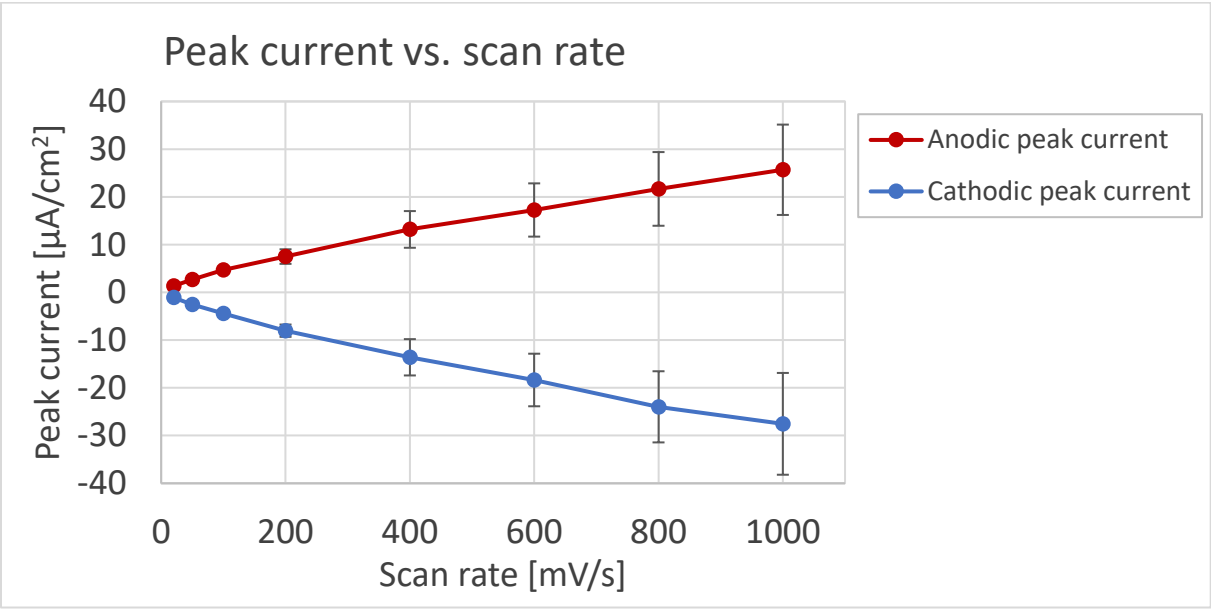

Figure 46: Scan rate dependency of peak currents.

In Figure 46, the resulting peak currents were plotted against the used scan rates. The resulting currents increased at higher scan rates due to a reduced mass transfer limitation of the protein in close vicinity of the electrode.

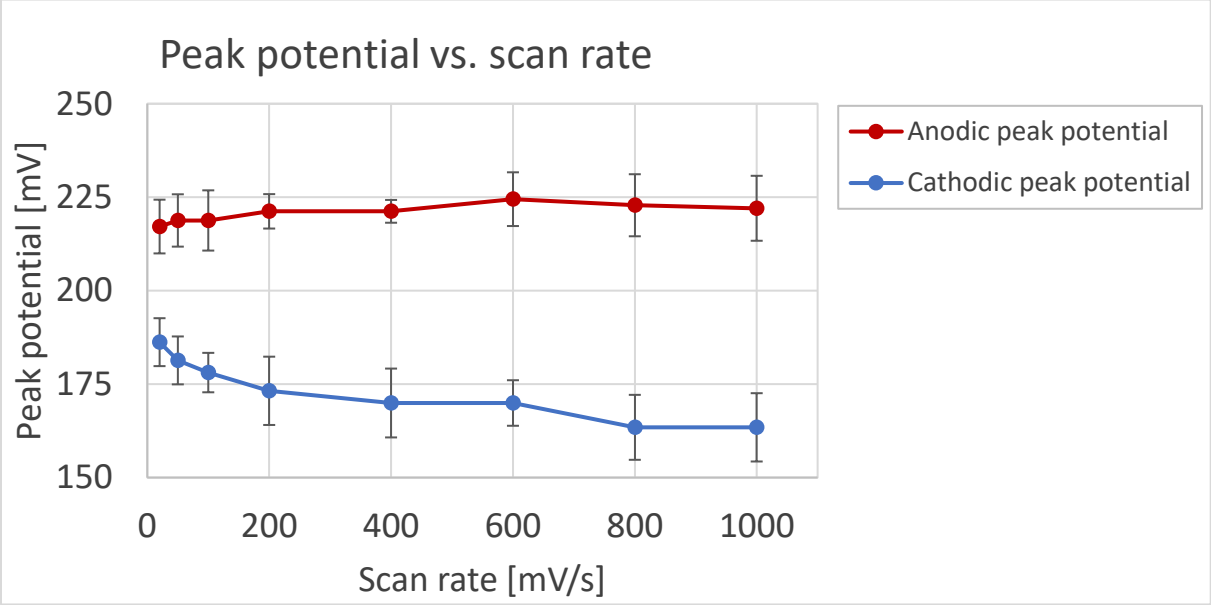

Figure 47: Scan rate dependency of peak potentials.

As shown in Figure 47, the anodic peak potential stayed constant over the applied scan rates, whereas the cathodic peak potential slightly increased with higher scan rates. Whether peak current and scan rate correlate linearly or not, reveals more detail about the electrochemical behavior of a protein (e.g. how many electrons and protons are transferred). However, for proteins such statements should be made only with great caution. The linear behavior further indicates an electrochemically reversible process.

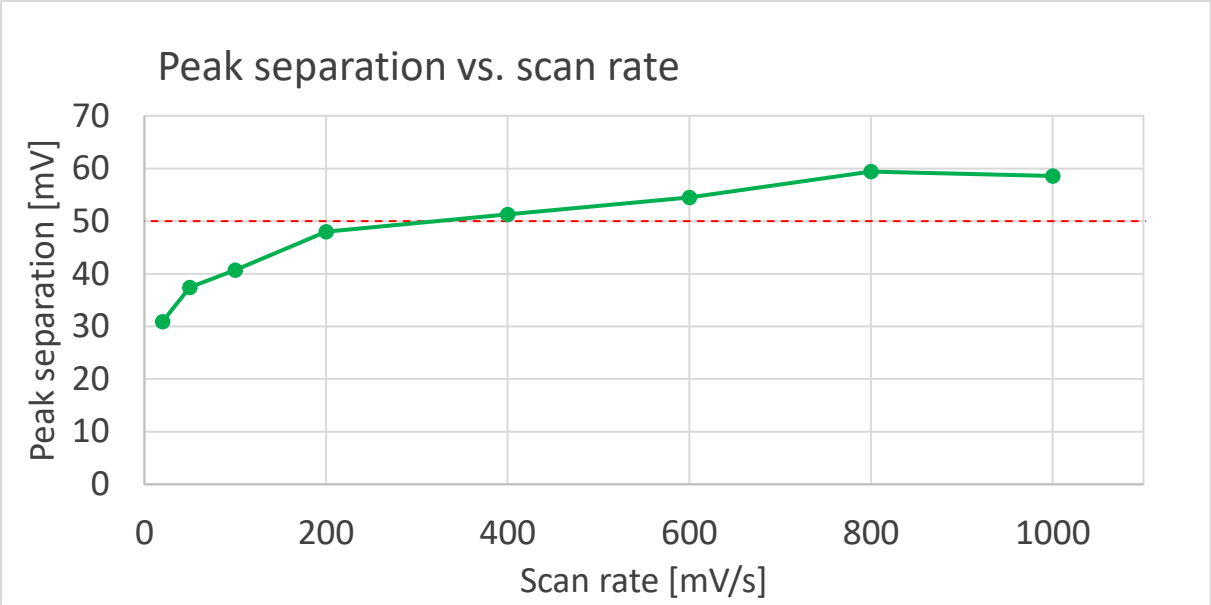

Figure 48: Scan rate dependency of peak separation.

When comparing peak separation and scan rate (Figure 48), the peak separation increased with increasing scan rates. When the peak separation stays below 50 mV, the process is considered electrochemically reversible. We see that at scan rates above 100 mV/s the process becomes more and more irreversible which can be mostly attributed to mass transfer, diffusion effects and adsorption effects of the protein on the electrode.

3.7 Amperometry

In amperometry, a fixed potential – often around 100 mV above the midpoint potential – is applied and currents originating from catalytic reactions are detected. Once the signal is stable at 0 mV, glucose is added. GDH converts the glucose, electrons become free. Because of the buried redox center, GDH cannot interact directly with the electrode. First, the electrons are transferred to the cytochrome domain and then from the cytochrome domain directly to the electrode. The amount of catalytic current is limited by various factors. Firstly, the interface of the GDH should be optimal for the cytochrome to accept the electron, however, the proteins should also not interact for too long. Secondly, the correct orientation of the cytochrome towards the electrode is

crucial to enable electron transfer from the cytochrome to the electrode. As a combination of the above, the linker must ensure fast interaction and must also be flexible enough to ensure correct orientation of the cytochrome when interacting with the electrode.

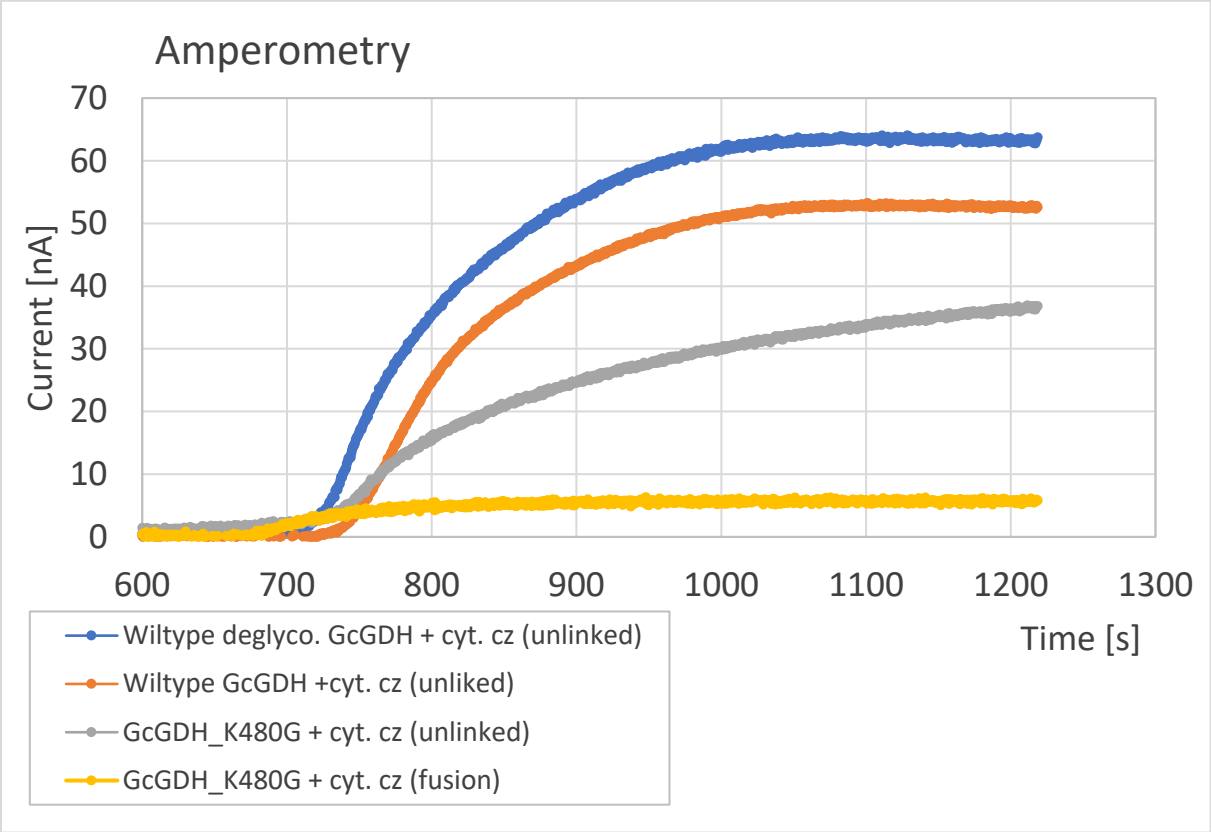

Figure 49: Amperometry of the unlinked and fused species (200 µM) with 50 mM glucose.

Table 21: Achieved currents of the chronoamperometric measurements

Achieved currents	
Wildtype *Gc*GDH (deglyco.) + cyt. *cz* (unlinked)	**62.5 nA**
Wildtype *Gc*GDH + cyt. *cz* (unlinked)	**52.4 nA**
*Gc*GDH_K480G + cyt. *cz* (unlinked)	**35.0 nA**
*Gc*GDH_K480G fusion enzyme	**5.1 nA**

In Figure 49, the rise in current after the addition of 50 mM glucose to the cell can be observed with all four investigated variants. After the addition, the rise did not occur immediately. It took around 200 sec after a rise of current could be observed, glucose needed to diffuse through the membrane of the prepared electrode. In Table 21, the resulting currents are calculated. Wild-type *Gc*GDH (52.4 nA) and the deglycosylated wild-type *Gc*GDH (62.5 nA) yielded significantly more current than the unlinked and fused *Gc*GDH_K480G variant (35.0 nA). This led to the conclusion that the introduced mutation in the interface of the *Gc*GDH was not beneficial when it came to electron transfer from the *Gc*GDH to the cytochrome *cz*. Although a current could be measured with the fusion enzyme (5.1 nA), it was extremely low compared to all the unlinked species. The linker did not have an advantage for the electron transfer to the cytochrome *cz* domain. It is worth mentioning that all these results were obtained in an environment where oxygen was present.

4 Conclusions and outlook

Fermentation of *Gc*GDH in *P. pastoris* in the 5-L Eppendorf BioFlo 120 bioreactor is a well-established standard procedure and has the benefit of automated feeding and process monitoring. This approach to expression is therefore more beneficial than expression in shaking flasks where pH control is difficult and a feed has to be executed manually without control over the methanol concentration in the flask or the viability of the cells. Also, purification with ion exchange is more tedious for culture supernatants of shaking flask expressions as they contain yeast extract which needs more purification steps to be eliminated. Nevertheless, shaking flasks expression is a decent and resource-friendly possibility when only small amounts of protein are needed or for expression screening.

When it comes to cytochrome *cz* expression in *E. coli*, we experienced that large-scale shaking flask expression is more suitable for laboratory-scale production in terms of cytochrome yield and overall handling. The high-density fermenter expression however poses a very good alternative when the process is optimized and better established.

The sortagging of the different protein species works, but the reaction has very low yields of chimeric enzyme. Even though purification is very labor-intensive, we could successfully develop a strategy to purify the chimeric enzyme.

When comparing the pH dependent activity profiles, all tested *Gc*GDH enzymes (wild-type, modified and chimeric enzyme) showed a pH optimum around pH 6. The two rtecombinantly expressed enzymes (*Gc*GDH_K480G and *Gc*GDH_WT) showed significantly lower maximum specific activity (235 U/mg and 233 U/mg) compared to the wild-type (314 U/mg) at pH 6.0. The chimeric *Gc*GDH_K480G enzyme´s specific activity at pH 6 (217 U/mg) is slightly lower than the activity of *Gc*GDH_K480G domain which can be explained by sample preparation (3.4.3).

The data spectroscopically obtained from the reduction of cytochrome *cz* by *Gc*GDH proved, that the cytochrome *cz* can be reduced by the *Gc*GDH indicated by the emerging peaks at 522 nm and 551 nm. This happened when the two species were combined unlinked as well as with the chimeric fusion enzyme. However, no quantitative conclusion can be drawn from the outcome of this experiment. It also needs to be further investigated if the emerging heme peaks approximate the level of

full cytochrome reduction with DDT when carried out in an oxygen-free environment. As we experienced, absorbance at 522 nm and 551 nm starts to decrease because of the cytochrome oxidation by oxygen.

In the amperometric experiments, currents were yielded from every tested *Gc*GDH and cytochrome *cz* combination (unlinked and fused), whereas all unlinked species yielded higher currents than the chimeric fusion enzyme. Wildtype *Gc*GDH (52.4 nA) combined with a non-fused cytochrome achieved more current than the *Gc*GDH_K480G combined with the non-fused cytochrome in solution (35.0 nA) which indicates that the introduced mutation is not beneficial for the interaction of GDH with the cytochrome leading to inferior electron transfer behavior. When comparing the unlinked *Gc*GDH_K480G (35.0 nA) with the chimeric *Gc*GDH_K480G fusion enzyme (5.1 nA), the achieved current of the chimeric enzyme is significantly lower than the current of the unlinked species. This leads to the conclusion that the current linker design is not of advantage for DET. We also cannot say whether the electrons from the GDH are transferred to its attached cytochrome or if the electrons are taken up by a neighboring cytochrome. More data must be generated to make statistically solid conclusions.

In order to achieve higher currents in the future, optimized linker designs, GDH enzymes originating from different organisms, or a different electrode design could be tried. Although, production, purification and testing of the different species and the chimeric enzyme itself are very tedious and time consuming, we successfully showed that the concept of chimeric *Gc*GDH enzymes with a cytochrome *cz* domain worked on biosensors and low currents could be achieved.

5 References

[1] A. Heller and B. Feldman, “Electrochemical glucose sensors and their applications in diabetes management,” *Chem. Rev.*, vol. 108, no. 7, pp. 2482–2505, 2008, doi: 10.1021/cr068069y.

[2] J. Okuda-Shimazaki, N. Kakehi, T. Yamazaki, M. Tomiyama, and K. Sode, “Biofuel cell system employing thermostable glucose dehydrogenase,” *Biotechnol. Lett.*, vol. 30, no. 10, pp. 1753–1758, 2008, doi: 10.1007/s10529-008-9749-7.

[3] C. Sygmund, M. Klausberger, A. K. Felice, and R. Ludwig, “Reduction of quinones and phenoxy radicals by extracellular glucose dehydrogenase from Glomerella cingulata suggests a role in plant pathogenicity,” *Microbiology*, vol. 157, no. 11, pp. 3203–3212, 2011, doi: 10.1099/mic.0.051904-0.

[4] K. Sode *et al.*, “Novel fungal FAD glucose dehydrogenase derived from Aspergillus niger for glucose enzyme sensor strips,” *Biosens. Bioelectron.*, vol. 87, pp. 305–311, 2017, doi: 10.1016/j.bios.2016.08.053.

[5] S. Tsujimura *et al.*, “Novel FAD-dependent glucose dehydrogenase for a dioxygen-insensitive glucose biosensor,” *Biosci. Biotechnol. Biochem.*, vol. 70, no. 3, pp. 654–659, 2006, doi: 10.1271/bbb.70.654.

[6] A. Baumstark, C. Schmid, S. Pleus, C. Haug, and G. Freckmann, “Influence of partial pressure of oxygen in blood samples on measurement performance in glucose-oxidase-based systems for self-monitoring of blood glucose,” *J. Diabetes Sci. Technol.*, vol. 7, no. 6, pp. 1513–1521, 2013, doi: 10.1177/193229681300700611.

[7] Z. Tang, R. F. Louie, J. H. Lee, D. M. Lee, E. E. Miller, and G. J. Kost, “Oxygen effects on glucose meter measurements with glucose dehydrogenase- and oxidase-based test strips for point-of-care testing.,” *Crit. Care Med.*, vol. 29, no. 5, pp. 1062–1070, May 2001, doi: 10.1097/00003246-200105000-00038.

[8] A. E. G. Cass *et al.*, “Ferrocene-Mediated Enzyme Electrode for Amperometric

Determination of Glucose," *Anal. Chem.*, vol. 56, no. 4, pp. 667–671, 1984, doi: 10.1021/ac00268a018.

[9] H. Yoshida, G. Sakai, K. Mori, K. Kojima, S. Kamitori, and K. Sode, "Structural analysis of fungus-derived FAD glucose dehydrogenase," *Sci. Rep.*, vol. 5, pp. 1–13, 2015, doi: 10.1038/srep13498.

[10] K. Ito *et al.*, "Designer fungus FAD glucose dehydrogenase capable of direct electron transfer," *Biosens. Bioelectron.*, vol. 123, no. July 2018, pp. 114–123, 2019, doi: 10.1016/j.bios.2018.07.027.

[11] T. Yanase, J. Okuda-Shimazaki, K. Mori, K. Kojima, W. Tsugawa, and K. Sode, "Creation of a novel DET type FAD glucose dehydrogenase harboring Escherichia coli derived cytochrome b562 as an electron transfer domain," *Biochem. Biophys. Res. Commun.*, vol. 530, no. 1, pp. 82–86, 2020, doi: 10.1016/j.bbrc.2020.06.132.

[12] F. Schachinger, H. Chang, S. Scheiblbrandner, and R. Ludwig, "Amperometric biosensors based on direct electron transfer enzymes," *Molecules*, vol. 26, no. 15, 2021, doi: 10.3390/molecules26154525.

[13] C. Sygmund *et al.*, "Heterologous overexpression of Glomerella cingulata FAD-dependent glucose dehydrogenase in Escherichia coli and Pichia pastoris," *Microb. Cell Fact.*, vol. 10, 2011, doi: 10.1186/1475-2859-10-106.

[14] Y. Y. Londer, "Expression of recombinant cytochromes c in E. coli," *Methods Mol. Biol.*, vol. 705, no. May, pp. 123–150, 2011, doi: 10.1007/978-1-61737-967-3_8.

[15] E. Arslan, H. Schulz, R. Zufferey, P. Künzler, and L. Thöny-Meyer, "Overproduction of the Bradyrhizobium japonicum c-type cytochrome subunits of the cbb3 oxidase in Escherichia coli," *Biochem. Biophys. Res. Commun.*, vol. 251, no. 3, pp. 744–747, 1998, doi: 10.1006/bbrc.1998.9549.

[16] P. Hamel, V. Corvest, P. Giegé, and G. Bonnard, "Biochemical requirements for the maturation of mitochondrial c-type cytochromes," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1793, no. 1, pp. 125–138, 2009, doi: 10.1016/j.bbamcr.2008.06.017.

[17] N. Pishesha, J. R. Ingram, and H. L. Ploegh, “Sortase A: A Model for Transpeptidation and Its Biological Applications.,” *Annu. Rev. Cell Dev. Biol.*, vol. 34, pp. 163–188, Oct. 2018, doi: 10.1146/annurev-cellbio-100617-062527.

[18] A. Geerlof, “High cell-density fermentation of Escherichia coli,” 2008. http://www.helmholtz-muenchen.de.

[19] B. Li, S. Willard, K. Voll, I. Pabbathi, and M. Sha, “Push-Button Simplicity: Automatic Fermentation with the BioFlo 120 Auto-Culture Mode,” *Bioprocess Int.*, no. Industry Yearbook 2017-2018, p. 14, 2017.

[20] I. Chen, B. M. Dorr, and D. R. Liu, “A general strategy for the evolution of bond-forming enzymes using yeast display,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 28, pp. 11399–11404, 2011, doi: 10.1073/pnas.1101046108.

[21] W. R. Carmody, “An easily prepared wide range buffer series,” *J. Chem. Educ.*, vol. 38, no. 11, pp. 559–560, 1961, doi: 10.1021/ed038p559.