Production and purification of chimeric enzymes and establishment of deflavination method

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PRODUCTION AND PURIFICATION OF CHIMERIC ENZYMES AND ESTABLISHMENT OF DEFLAVINATION METHOD

Experimental work for this Graduate thesis was done at the Department of Food Science and Technology, BOKU – University of Natural Resources and Life Sciences, Vienna. The thesis was made under the guidance of associate professor Roland Ludwig, Ph.D., and with the help of assistant professor Su Ma, Ph.D., and Dipl. Ing. Marie-Christin Viehauser.

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PRODUCTION AND PURIFICATION OF CHIMERIC ENZYMES AND ESTABLISHMENT OF DEFLAVINATION METHOD

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Abstract: Glucose biosensors are important analytical devices, which most often use glucose dehydrogenase or glucose oxidase, both from the GMC oxidoreductases family, as a biological recognition element. The use of enzymes in biosensors is hampered in many ways, e.g. by low activity and stability of enzymes, substrate specificity and most importantly by an insufficient connection of the bioelement with an electrode. In order to achieve direct electron transfer between the enzyme and electrode, two glucose dehydrogenases were connected to the heme *b*-containing cytochrome domain from cellobiose dehydrogenase IIA, from *Neurospora crassa,* which is naturally capable of DET. Chimeric enzymes were produced in *Pichia pastoris* and then purified. Moreover, flavoenzymes with loss of FAD cofactor by dissociation immediately lose their activity. In order to determine the FAD dissociation constant of GMC oxidoreductases and further improve their FAD loading, a method for deflavination and reconstitution of flavoenzymes was established. Two different GMC oxidoreductases, used in glucose biosensors, were produced in the *Pichia pastoris* GlycoSwitch strain and the native and reconstituted enzymes were characterized in comparison.

Keywords: biosensors, chimeric enzymes, DET, deflavination, GMC oxidoreductases

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PROIZVODNJA I PROČIŠĆAVANJE KIMERNIH ENZIMA I USPOSTAVA METODE DEFLAVINACIJE

Karlo Koraj, 1169/BPI

Sažetak: Biosenzori za mjerenje glukoze su važni analitički uređaji koji najčešće koriste glukoza dehidrogenazu ili glukoza oksidazu, iz obitelji GMC oksidoreduktaza, kao biološki element. Upotreba enzima u biosenzorima ograničena je niskom aktivnosti i stabilnosti enzima, specifičnosti enzima prema supstratu te najvažnije nezadovoljavajućom povezanosti bioelementa s elektrodom. Kako bi se postigao direktni prijenos elektrona s enzima na elektrodu dvije glukoza dehidrogenaze su povezane sa citokrom domenom, koja sadrži hem *b*. Korištena citokrom domena je iz enzima celobioza dehidrogenaza IIA, iz plijesni *Neurospora crassa*, koja je prirodno sposobna za direktni transfer elektrona. Kimerni enzimi su proizvedeni u kvascu *Pichia pastoris* te zatim pročišćeni. Nadalje, flavoenzimi disocijacijom FAD kofaktora automatski gube svoju aktivnost. Uspostavljena je metoda za deflavinaciju i rekonstituciju flavoenzima s ciljem određivanja konstante disocijacije FAD-a za odabrane GMC oksidoreduktaze te poboljšanje enzimskog vezanja FAD-a. Dvije različite GMC oksidoreduktaze, koje se koriste u biosenzorima, su proizvedene u soju kvasca *Pichia pastoris* GlycoSwitch te su nativni i rekonstituirani enzimi karakterizirani.

Ključne riječi: biosenzori, kimerni enzimi, DET, deflavinacija, GMC oksidoreduktaze

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1. INTRODUCTION

Biosensors, a concept that originated from the guidance of nature is a subject of great interest in recent years. A biosensor is an analytical device comprising of a biological recognition element connected to a signal transducer, which together relates the concentration of an analyte, or group of analytes, to a measurable response. They are of particular interest because of practical advantages, such as simple instrumentation, formidable selectivity, low prices and easy automation (Mehrotra, 2016).

The use of enzymes in biosensors is hampered in many ways, e.g. by low activity and stability of enzymes, substrate specificity and most importantly by an insufficient connection of the bioelement with the transducing electrode (Heller and Feldman, 2008). Several methods to connect enzymes to electrodes have been developed. First-generation biosensors measure the concentration of substrates or products of the enzymatic reaction. Second-generation biosensors couple the oxidative or reductive half-reaction of an enzyme via redox mediators to the electrode (mediated electron transfer – MET). Third-generation biosensors establish a direct electron transfer (DET) from enzymes to electrodes (Zhang and Li, 2004). Cellobiose dehydrogenase (CDH) is one of the best documented enzymes capable of DET and is used for applications in biosensors and biofuel cells. CDHs are composed of two domains: a heme *b*-binding cytochrome domain and a FAD-binding dehydrogenase catalytic domain which are connected by a flexible linker. Recent protein engineering efforts mimic naturally evolved electron transfer proteins and enzymes by fusing a cytochrome domain to glucose dehydrogenases and other oxidoreductases. A good interaction of the cytochrome domain with the active site cofactor (FAD) is important to obtain high IET (interdomain electron transfer) and DET rates and consequently a high current density for glucose biosensors (Ma and Ludwig, 2019).

As a part of this thesis selected glucose dehydrogenases (genes from *Aspergillus flavus* and *Glomerella cingulata*) were linked to the cytochrome domain of CDH IIA from *Neurospora crassa* which is used as an electron transferring domain. The aim was to produce two different chimeric enzymes in shaking flasks using *Pichia pastoris* KM71H strain and then establish a purification scheme.

As already mentioned, low activity and stability of enzymes decrease their applicability in biosensors. That's especially the case with flavoenzymes, from GMC (Glucose-Methanol-Choline) oxidoreductases family, which have non-covalently bound FAD. The loss of FAD by

dissociation leads to an immediate loss of activity and destabilizes the apoenzyme. In order to determine the FAD dissociation constant of GMC oxidoreductases and further improve their FAD loading, the method for deflavination and reconstitution of flavoenzymes was established. Two different enzymes from GMC oxidoreductases family (glucose oxidase from *Aspergillus niger* and glucose dehydrogenase from *Glomerella cingulata*) used in glucose biosensors were produced in *Pichia pastoris* GlycoSwitch strain. Both native and reconstituted enzymes were characterized in comparison.

2. THEORY

2.1. BIOSENSORS

According to the IUPAC definition, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (McNaught and Wilkinson, 1997). The field of biosensors is very versatile due to a vast number of possible analytes, enzymes, electrodes, and electrode modifications. They are of particular interest because of practical advantages such as operation simplicity, the low expense of fabrication and suitability for real-time detection (Zhang and Li, 2004).

The biosensor concept was introduced by Leland C. Clark in 1962 (Clark and Lyons, 1962). He used his previously developed oxygen-sensitive electrode detecting oxygen and modified it with a membrane containing enzyme glucose oxidase. The readout was the amount of oxygen consumed by glucose oxidase during the enzymatic reaction with the substrate glucose. Such biosensors detecting changed levels of enzyme substrates or products are called first-generation biosensors.

The first-generation biosensors have many drawbacks – the applied potential is too high, which increases the chance of possible interference. In addition, if oxygen is used as a redox indicator there are many problems such as maintaining an air-tight sample chamber. Moreover, the concentration of dissolved oxygen is fluctuant and the tenuity of dissolved O_2 significantly decreases electrical currents, which influences the detection limit.

The idea of artificial mediators was, therefore, proposed to overcome the inherited drawbacks of natural mediators. If one of the enzyme's substrates or products is replaced by an artificial redox molecule being able to shuttle electrons between enzyme and electrode this type of electrochemical biosensor is called second-generation biosensor. Second-generation biosensors use so-called mediated electron transfer. Some small redox-active molecules can diffuse in and react with the active site of the enzyme and diffuse out and react with the electrode surface, consequently shuttling the electrons between the enzyme and the electrode. These mediators can efficiently decrease the applied potential of the biosensors and, therefore, decrease the interference from electrochemically oxidizable compounds present in real samples (Zhang and Li, 2004). However, the redox mediators used in conjunction with redox proteins are in no way selective, but rather general redox catalysts, facilitating not only the electron

transfer between electrode and enzyme, but also various interfering reactions (Heller, 1992). Since most “electrochemical” mediators lack selectivity, researchers have continued to look for better ways to accomplish electronic coupling between oxidoreductases and electrodes.

The ability of an enzyme to directly communicate with an electrode surface is called a direct electron transfer. The very first reports on direct electron transfer with a redox-active proteins were published in 1977 when it was shown that cytochrome c on gold and tin-doped indium oxide electrodes, respectively, exhibited virtually reversible electrochemistry (Yeh and Kuwana, 1977; Eddowes and Hill, 1977). Cytochrome c is a small redox protein that is active in biological electron-transfer chains, but it has no enzyme properties. These first publications were soon followed by reports that direct electron transfer was also possible for larger oxidoreductases with enzymatic activity.

For biosensors based on direct electron transfer – third-generation biosensors, the absence of mediators is the main advantage, providing them with superior selectivity, both because they should operate in a potential window closer to the redox potential of the enzyme and are, thus, less prone to interfering reactions, but also because of the lack of yet another reagent in the reaction, which simplifies the reaction system. However, it is unfortunate that most enzymes cannot exhibit direct electron transfer at normal electrode surfaces. Great efforts have been taken in direct protein electrochemistry, but only a few enzymes have been proved to exhibit direct electrochemistry. To achieve direct electron transfer has been a “bottleneck” in developing third-generation biosensors. Up to now, extensive studies have been carried out toward finding novel surface functionalization, new electrode materials, and new enzymes that are capable of DET.

Another attractive feature of the system, based on DET, is the possibility of modulating the desired properties of an analytical device by using protein modification with genetic or chemical engineering techniques. Enzymes that are known to exhibit direct electrochemistry at the electrode can be exploited to give that property to enzymes that are not naturally capable of direct electron transfer (Ito et al., 2019).

2.2. GMC OXIDOREDUCTASES

Oxidoreductases are a major enzyme class with broad application in biosensors because their substrates are physiologically and industrially relevant and they can be coupled to electrodes via mediated or direct electron transfer. The GMC (Glucose-Methanol-Choline) superfamily is a large and functionally diverse family of oxidoreductases that share a common structural fold. Fungal members of this superfamily that are characterized and relevant for lignocellulose degradation include aryl-alcohol oxidoreductase, alcohol oxidase, cellobiose dehydrogenase, glucose oxidase, glucose dehydrogenase, pyranose dehydrogenase, and pyranose oxidase (Sützl et al., 2019). All GMC oxidoreductases share a common structure and covalently or non-covalently bound flavin adenine dinucleotide (FAD) cofactor.

Commonly known electron donor substrates for GMC oxidoreductases range from various sugars and alcohols to cholesterol and choline. Despite this broad range of chemically diverse substrates, the overall reaction mechanism is similar for these FAD-dependent oxidoreductases. The mechanism can be separated into a reductive (reduction of FAD with the oxidation of the electron donor substrate) and an oxidative half-reaction (re-oxidation of $FADH_2$) and relies on a highly conserved catalytic His/His or His/Asn pair in the active site. As the final electron acceptor, GMC oxidoreductases can employ oxygen or alternative electron acceptors such as different quinones, phenol radicals, or metal ions. Varying preferences for these electron acceptors separate GMC enzymes into oxidases (which can utilize O_2 as an electron acceptor) and dehydrogenases (which show negligible or very low reactivity with O_2) (Sützl et al., 2019).

For the most prominent biosensor – the glucose biosensor – the published scientific and patent literature is almost impossible to follow. Researchers have employed several enzymes, but especially glucose 1-oxidase (GOX) and glucose 1-dehydrogenase (GDH), from the GMC oxidoreductases superfamily, for this purpose.

Both, GOX and GDH are FAD-dependent enzymes which specifically oxidize β-D-glucose at the anomeric carbon to δ-gluconolactone (D-glucono-1,5-lactone). GOX and GDH are catalytically and phylogenetically closely related but differ in their preference for the electron acceptors employed in their oxidative half-reactions. While GOX preferentially reduces molecular oxygen to H_2O_2, GDH shows very low activity with O_2 and utilizes a range of alternative electron acceptors (Sützl et al., 2018).

GOX (EC 1.1.3.4) is a homodimeric glycoprotein with a non-covalently but tightly bound FAD cofactor. The first description of GOX from *A. niger* dates back to 1928. GOX shows a very high preference for β-D-glucose, and hardly any other sugars are oxidized with significant catalytic efficiency. This specificity towards β-D-glucose stems from a highly specialized active-site architecture resulting in the formation of hydrogen bonds to all five hydroxyl groups of β-D-glucose (Yoshida et al., 2015). *An*GOX is the currently best-characterized glucose oxidase and is also most widely used in industrial applications. *An*GOX shows a catalytic efficiency of up to 1.5×10^6 M^{-1} s^{-1} for D-glucose (Roth and Klinman, 2003).

Attention towards GDHs (EC 1.1.5.9) developed only lately for its possible application in glucose biosensors independent of O_2. The first crystal structure of *Aspergillus flavus* GDH was solved recently (Yoshida et al., 2015). GDHs are found either as monomeric or homodimeric proteins. They are phylogenetically and structurally very closely related to GOX, showing both the same domain architecture and conserved catalytic residues. Together with structural features, GDH shares most of the active site composition and, therefore, the high-substrate specificity towards D-glucose (Sützl et al., 2018). GDH from *Glomerella cingulata* is a monomeric protein with non-covalently bound FAD and its preferred substrates are glucose and xylose (Sygmund et al., 2011).

2.3. CHIMERIC ENZYMES CAPABLE OF DET

Major efforts are taken to improve the sensitivity, selectivity, and stability of enzyme-modified electrodes. The critical factor is the electrical coupling of the oxidoreductase's activity to the transducing electrode as mentioned above. Free or immobilized redox mediators can introduce several problems of their own, e.g. diffusion limitation, stability, poisoning by matrix compounds, unfitting redox potentials, etc. (Turner, 2013). Enzymes that are capable of direct electron transfer are independent of redox mediators. However, the electron transfer rate is restricted by the electron transfer distance. At a distance bigger than 1.5 nm the electron transfer rate between the enzymes cofactor and the electrode quickly falls below the technically useful limit of 1 s^{-1} (Patolsky et al., 2004). For the optimization of direct electron transfer rates, substrate specificity and suppression of interfering species protein engineering is a powerful tool (Lambrianou et al., 2008). Protein engineering can also be used to assemble domains from different enzymes into the chimeric enzymes.

As a product of recombinant DNA technology, fusion proteins have been developed as a class of novel biomolecules with multi-functional properties (Chen et al., 2013). Fusion or chimeric enzymes are proteins created by fusing two or more genes that originally coded for separate proteins. Translation of this fusion gene results in a single polypeptide with functional properties derived from each of the original proteins.

The combination of a cytochrome domain and GMC oxidoreductases into chimeric flavocytochromes can be used to reroute the flow of electrons from the catalytic center to an electrode. The enzymes' dependence on cosubstrates like oxygen, quinones or redox mediators is being replaced by direct electron transfer to an electrode. Cytochrome modified GMC oxidoreductases with efficient DET are highly interesting for biosensors and bioelectrocatalytic processes. The electron transfer efficiency in the existing flavocytochrome cellobiose dehydrogenase, but also in the generated chimeric enzymes, is governed by the cytochrome domain mobility, which is modulated by the interdomain protein linker length.

The function of the cytochrome domain of cellobiose dehydrogenase as an electron transferring domain for oxidoreductases was studied by Ito et al. (2019). In this study, the designer chimeric enzyme was constructed using CDH structural information. Glucose dehydrogenase from *Aspergillus flavus* (*Af*GDH) was selected as the catalytic domain and was linked to the heme *b*-containing cytochrome domain with a native linker from *Phanerochaete chrysosporium* CDH. The recombinant production of the designer chimeric enzyme resulted in flavin to heme *b* IET during the oxidation of glucose. In addition, this enzyme showed DET ability to the electrode and maintained the original substrate specificity of *Af*GDH.

The aim of this thesis was to attach cytochrome domain from *Neurospora crassa* CDH IIA to the several selected GMC oxidoreductases to generate chimeric enzymes with a built-in redox mediator. This principle occurs naturally in CDH (EC 1.1.99.18), which is a fungal, extracellular flavocytochrome and one of the few oxidoreductases capable of DET (Ortiz et al., 2012). The catalytic dehydrogenase domain of CDH belongs to the GMC oxidoreductases and contains one non-covalently bound FAD. It is connected to an electron transferring heme *b*-containing cytochrome domain by a flexible interdomain linker (Ludwig et al., 2010). Two different cellobiose dehydrogenases are found in *Neurospora crassa* - CDH IIA with a C-terminal cellulose-binding module (CBM) and CDH IIB without a CBM (Harreither et al., 2011). Both CDHs differ in various aspects e.g. pH optima, substrate specificity, and turnover rates, but the most important difference lies in the different intramolecular electron transfer

rates of the cytochrome domains (Zhang et al., 2013). CDH IIA, which is shown in figure 1, can reach IET rates of up to 40 s^{-1}, whereas CDH IIB only reaches 0.5 s^{-1}.

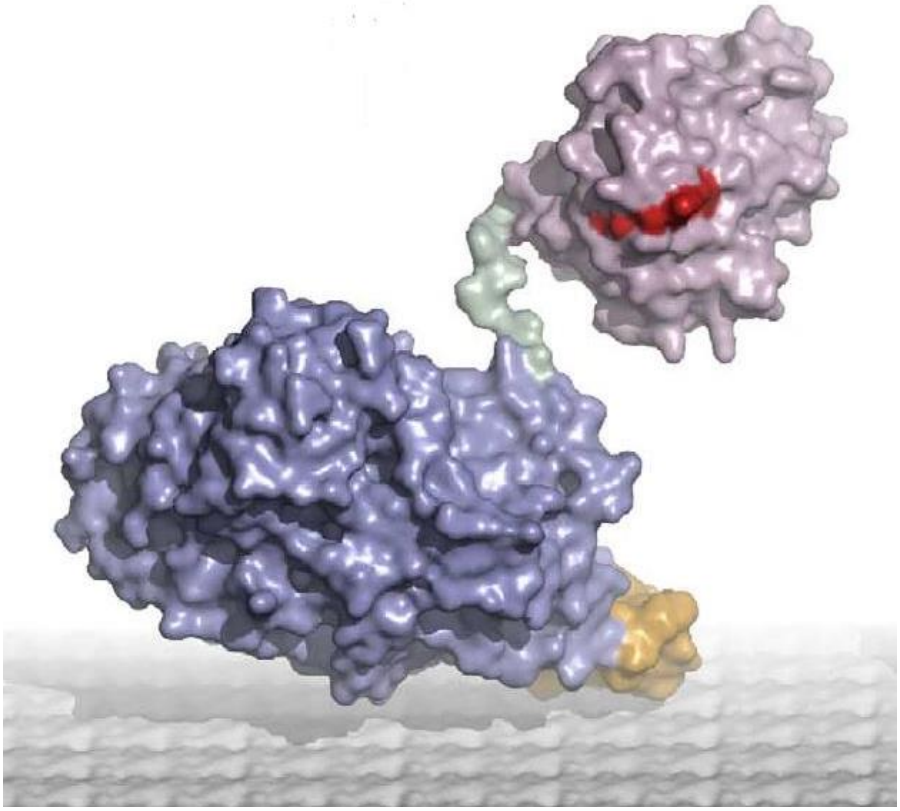

Figure 1. The crystal structure of *Neurospora crassa* CDH IIA modelled on an idealized crystalline cellulose surface – CBM (orange), dehydrogenase domain (blue), interdomain linker (green) and cytochrome domain (magenta) (adapted from Tan et al., 2015)

In contrast to many other oxidoreductases, GDH from *Glomerella cingulata* barely uses oxygen as a cosubstrate, which makes it a perfect choice to replace the cosubstrate by the cytochrome domain to transfer electrons directly to the electrode (Sygmund et al., 2011). Studies have shown the feasibility of this strategy, but little is published because of ongoing commercialization efforts. *G. cingulata* GDH has a high catalytic rate and substrate specificity which makes it very suitable for a glucose biosensor. In addition, as part of this paper GDH from *Aspergillus flavus* will be used to connect to the cytochrome domain of CDH IIA as well. In summary, two different GMC oxidoreductases - glucose dehydrogenases from *G. cingulate* and *A. flavus* as part of this thesis will be connected to heme *b* containing-cytochrome domain and native linker of CDH IIA from *Neurospora crassa* in order to achieve IET between the dehydrogenase domain and the cytochrome domain. And, furthermore, to achieve DET between the chimeric enzymes and an electrode.

2.4. STABILITY OF FLAVOENZYMES

The use of enzymes in biosensors is hampered in many ways. Most importantly by an insufficient connection of the bioelement with the transducing electrode, which is mentioned above, but also by low activity and stability of enzymes, low substrate specificity, etc. (Heller and Feldman, 2008). Many proteins in nature require the binding of cofactors to perform their biological functions. These molecules often fold *in vivo* in cellular environments where their cofactors are present and thus may bind to the appropriate polypeptide before folding (Caldinelli et al., 2008). Activity and stability of flavoenzymes (selected GMC oxidoreductases) is studied as part of this thesis.

Flavins are key components of many oxidoreductases by serving as prosthetic groups and lending their redox capability to the enzyme. From the FAD-dependent family of catalytically interesting GMC oxidoreductases, glucose oxidase (GOX) and glucose dehydrogenase (GDH) have been widely used in glucose biosensors. The loss of FAD by dissociation leads to an immediate loss of activity and destabilizes the apoenzyme. This effect is commonly observed in biocatalysis and biosensing and greatly influences the applicability of flavoenzymes. Only 10% of flavoproteins have a covalently bound FAD and GOX and GDH do not belong in this group (Hefti et al., 2003). Covalent binding of FAD could prevent its dissociation and improve enzyme stability.

Use of native GOX and GDH in bioanalytical applications may be expanded by modifications in its structure such as replacement of native cofactor by its derivative or, in a specific case, by removal of the native cofactor (Posthuma-Trumpie et al., 2007). These modifications involve a preparation of enzymes apo-form, which is not a trivial task. FAD cofactor is buried in the polypeptide chain and, therefore, for its removal, one must use relatively harsh conditions such as extreme pH and/or presence of denaturants. On the other hand, this approach may lead to irreversible perturbation of the apo-form structure with losing the ability to bind the cofactor (Garajová et al., 2017).

Diaziridine functionalization of FAD at the ribityl or adenine group provides universal coupling to the protein scaffold based on specificity and size, without interfering with the redox chemistry of the isoalloxazine group. In figure 2 is shown the structure of FAD and planned diaziridine modifications. Photochemical formation of radical species could lead to the covalent bond formation of the cofactor with amino acids in proximity in the correct orientation (LudwigLab, 2018).

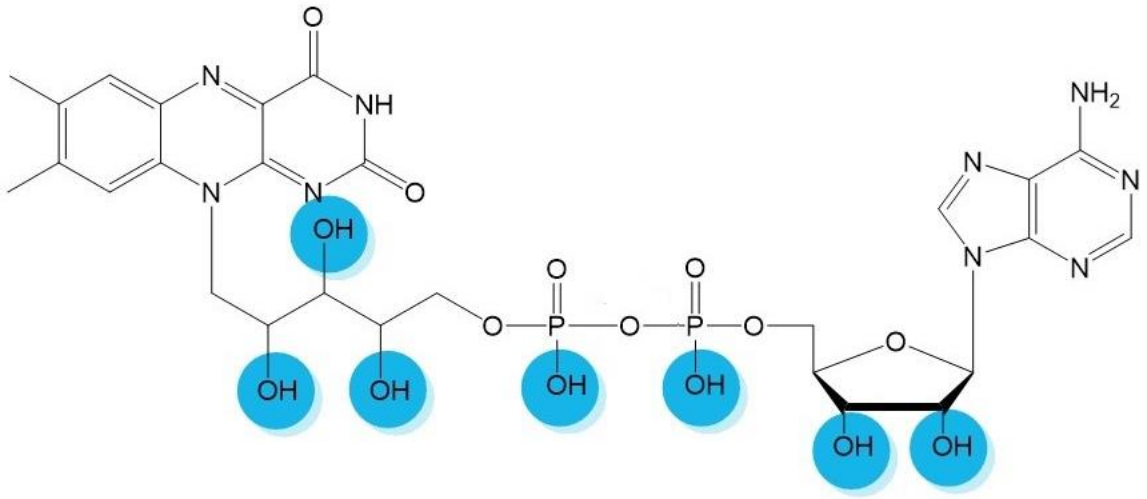

Figure 2. Structure of FAD and planned diaziridine modifications (blue) (adapted from LudwigLab, 2018)

As mentioned before, FAD is buried deeply inside flavoenzymes so deflavination is not an easy task. For the preparation of deflavinated form of flavoenzymes, many different methods exist, but Swoboda method, described below, is the most efficient regarding reversible deflavination of GOX (Garajová et al., 2017).

2.5. EXPRESSION SYSTEM

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which separates toxic byproducts away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia*. Two genes in *Pichia pastoris* are coding for alcohol oxidase – *AOX1* and *AOX2*. The majority of alcohol oxidase activity in the cell is attributable to the product of the *AOX1* gene. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high levels, typically > 30% of the total soluble protein in cells grown with

methanol (Invitrogen, 2010). The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein (Koutz et al., 1989). While *AOX2* is about 97% homologous to *AOX1*, growth on methanol is much slower than with *AOX1* (Cregg et al., 1989).

Expression of the *AOX1* gene is controlled at the level of transcription. The regulation of the *AOX1* gene is a two-step process: a repression/derepression mechanism plus an induction mechanism. Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth on glycerol is recommended for optimal induction with methanol. Growth on glycerol alone (derepression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for even detectable levels of *AOX1* expression (Koutz et al., 1989). Loss of the *AOX1* gene, and thus a loss of most of the cell's alcohol oxidase activity, results in a strain that is phenotypically Mut^S (Methanol utilization slow). This results in a reduction in the cells' ability to metabolize methanol. The cells, therefore, exhibit poor growth on methanol medium. Mut^+ (Methanol utilization plus) refers to the wild type ability of strains to metabolize methanol as the sole carbon source.

However, the *P. pastoris* expression system has some drawbacks, such as low cofactor loading and overglycosylation (Ludwig et al., 2013). *Pichia pastoris* have a majority of N-linked glycosylation of the high-mannose type and very little O-glycosylation has been observed. In recent years, much effort has gone to engineering the N-glycosylation pathway of *Pichia pastoris* to mimic the human N-glycosylation pathway. This can be of crucial importance to generate the appropriate glycoforms of therapeutically relevant glycoproteins or to gain a better understanding of structure-function relationships (Laukens et al., 2015).

Glycosylation can influence protein folding, stability, and protein-protein interactions. So, GlycoSwitch was engineered to generate proteins with more “human-like” glycosylation to produce better pharmaceuticals, as well as, improve other classes of recombinant proteins. The GlycoSwitch technology consists of the disruption of the endogenous *Pichia pastoris* glycosyltransferase gene (*OCH1*) and the stepwise introduction of heterologous glycosidase and glycosyltransferase activities. Furthermore, the expression of the alpha-1,2-mannosidase in the SuperMan5 strain results in Man5GlcNAc glycans. Therefore, the SuperMan5 strains have more uniform, limited glycosylation and are in a position to acquire modifying enzymes for subsequent glycan engineering (Jacobs et al., 2009).

3. MATERIALS AND METHODS

3.1. CHEMICALS AND MEDIA

3.1.1. Chemicals

All chemicals used during the course of this research were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Fluka (Vienna, Austria) or Carl Roth (Karlsruhe, Germany) and were of the analytical grade or of the highest purity available.

For the preparation of aqueous solutions distilled water (dH_2O) or ultrapure water (HQ-H_2O) filtered by a Siemens Ultra Clear Basic UV SG system were used.

3.1.2. Media for *Escherichia coli*

- LB (Luria broth) (+ Zeocin) agar
  - 10 g L^{-1} NaCl
  - + 5 g L^{-1} Yeast extract
  - + 10 g L^{-1} Peptone
  - + 15 g L^{-1} Agar-agar

The medium was autoclaved after dissolving and cooled to approximately 50 ºC after which pouring to sterile Petri dishes was carried out.

For media with Zeocin, LS-LB (low salt-Luria broth) was prepared with 5 g L^{-1} NaCl and after sterilization and cooling down 25 mg L^{-1} Zeocin was added.

For liquid media, the recipe is the same without the addition of agar-agar.

- SOC (Super optimal broth with catabolite repression) medium (New England Biolabs, Ipswich, USA):
  - 20 g L^{-1} Vegetable Peptone
  - + 5 g L^{-1} Yeast Extract
  - + 10 mM NaCl
  - + 2.5 mM KCl
  - + 10 mM $MgCl_2$
  - + 10 mM $MgSO_4$
  - + 20 mM Glucose

3.1.3. Media for *Pichia pastoris*

- YPD (+ Zeocin) agar

  10 g L^{-1} Yeast Extract
  + 20 g L^{-1} Peptone
  + 4 g L^{-1} Glucose
  + 15 g L^{-1} Agar-agar

The medium was autoclaved after dissolving and cooled to approximately 50 ºC after which pouring to sterile Petri dishes was carried out.

For media with antibiotic, YPD was prepared and after sterilization and cooling down 100 mg L^{-1} Zeocin was added.

For liquid media, the recipe is the same without the addition of agar-agar.

- BMY, BMMY and BMGY

  10 g L^{-1} Yeast Extract
  + 20 g L^{-1} Peptone
  + 100 mM Potassium phosphate buffer, pH 6
  + 13.4 g L^{-1} YNB
  + 4×10^{-4} g L^{-1} Biotin
  (+ 1% Glycerol or 0.5% Methanol)

After the addition of yeast extract and peptone to the appropriate volume of water sterilization by autoclaving was carried out.

Stock solutions were prepared:

- 10× YNB (134 g L^{-1} Yeast Nitrogen Base with Ammonium Sulfate without amino acids)
- 500× Biotin (0.2 g L^{-1} Biotin)
- 10× Potassium phosphate buffer (1 M, pH 6)

It is crucial to sterilize stock solutions of YNB, Biotin, and Methanol only by filtration.

BMY doesn't contain glycerol or methanol. BMMY contains 0.5% (v/v) methanol and BMGY contains 1% (v/v) glycerol.

- BYPD

  10 g L^{-1} Yeast Extract
  + 20 g L^{-1} Peptone from casein
  + 100 mM Potassium phosphate buffer, pH 6
  + 4 g L^{-1} D-Glucose

3.2. ENZYMES

Some enzymes were produced and purified by other lab members, but their characterization and comparison are part of this thesis. Two such enzymes are *An*GOX and *Gc*GDH produced in *Pichia pastoris* X-33 strain. In addition, glucose oxidase produced in *Aspergillus niger*, which was obtained from Sigma-Aldrich (St. Louis, Missouri, USA), was used for comparison.

Restriction enzyme PmeI, obtained from New England Biolabs (Ipswich, Massachusetts, USA), was used for the linearization of plasmids prior to the transformation of yeast.

Endoglycosidase H_f, which was used for deglycosylation of proteins, was obtained as well from New England Biolabs (Ipswich, Massachusetts, USA).

Peroxidase from horseradish, which was used for ABTS assay, was obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

3.3. TRANSFORMATION AND SCREEENING

3.3.1. Strains

3.3.1.1. *Bacteria*

For the propagation of constructed plasmids NEB 5-alpha competent *Escherichia coli* (High Efficiency) cells were used. The cells were obtained from New England Biolabs (Ipswich, Massachusetts, USA).

Genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)*M*15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

3.3.1.2. *Yeast*

- *Pichia pastoris* KM71H

For the production of chimeric enzymes *Nc*IIA_*Af*GDH and *Nc*IIA_*Gc*GDH, *Pichia pastoris* KM71H strain was used. The original strain was acquired from Invitrogen (Carlsbad, California, USA) and electrocompetent cells were produced in the lab according to the procedure described in paragraph 3.2.7. The KM71H strain is Mut^s (Methanol utilization slow) phenotype caused by the loss of alcohol oxidase activity encoded by the *AOX1* gene. A strain with a Mut^S phenotype has a mutant *aox1* locus but is wild type for *AOX2*. This results in the slow-growth phenotype on methanol medium.

Genotype: *arg4 aox1::ARG4*

- *Pichia pastoris* X-33 and *Pichia pastoris* GlycoSwitch SuperMan5

For the production of *An*GOX and *Gc*GDH, two different strains of *Pichia pastoris* were used. Both strains are Mut^+ (Methanol utilization plus) phenotype. Mut^+ refers to the wild type ability of strains to metabolize methanol as the sole carbon source.

Pichia pastoris X-33 is wild type and as such has a majority of N-linked glycosylation of the high-mannose type. X-33 strain was obtained from Invitrogen (Carlsbad, California, USA) and electrocompetent cells were produced in the lab according to the procedure in paragraph 3.2.7.

Pichia pastoris GlycoSwitch SuperMan5 was acquired from VTU Technology (Graz, Austria). The *Pichia* GlycoSwitch system is based on yeast strains that have been engineered to perform specific glycosylation steps at high fidelity. With *Pichia* GlycoSwitch, the yeast's own hyperglycosyl N-glycans are switched to the more human biantennary complex-type N-glycans. By mutating the *Pichia pastoris OCH1* gene and introducing heterologous enzyme activities, *Pichia* has been engineered to produce more human-like glycoproteins (Jacobs et al., 2009).

3.3.2. Vector

For the expression in *Pichia pastoris* and the proliferation of plasmids in *E. coli* the vector pPICZ A (Invitrogen, Carlsbad, California, USA), which is 3329 bp in length, was used (Figure 3). The regulation of the *AOX1* gene is a two-step process: a repression/derepression

mechanism plus an induction mechanism. Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. The BleoR gene (also known as *Sh ble* gene – *Streptoalloteichus hindustanus* bleomycin gene) expresses a protein which allows the binding of the antibiotic Zeocin (Invitrogen, Carlsbad, California, USA) and is used as a positive selection marker in *E. coli* and *Pichia pastoris*. The vector also contains an origin of replication (ori) which allows replication and maintenance of the plasmid in *E. coli* and a polyhistidine tag that binds divalent cations like Ni^{2+} to facilitate purification.

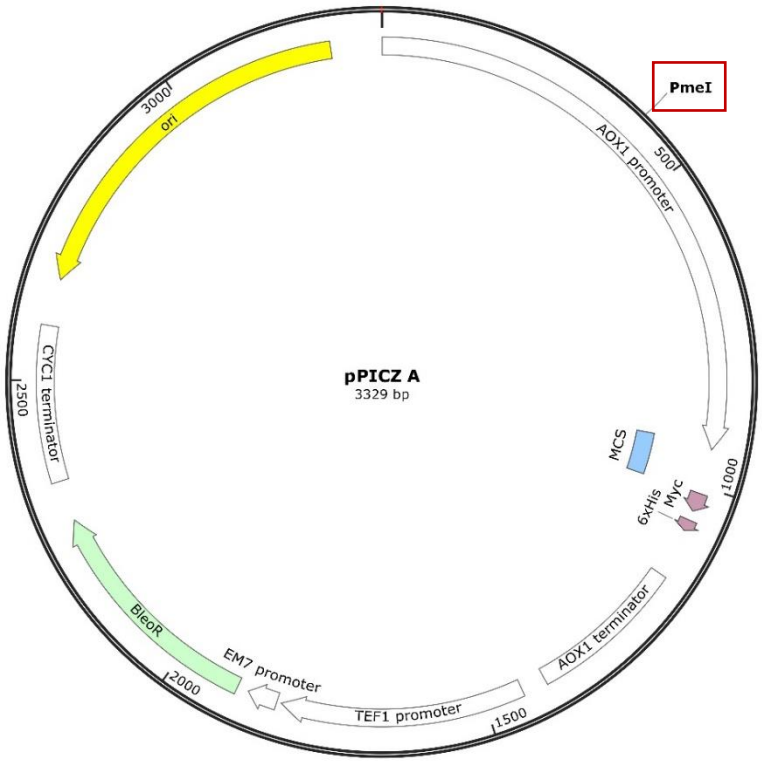

Figure 3. pPICZ A vector used for the expression of chimeric enzymes and flavoenzymes

The gene coding for CDH IIA from *Neurospora crassa* was isolated previously and inserted into the multiple cloning site of the pPICZ A vector. Additionally, the dehydrogenase domain of CDH IIA was deleted from the constructed plasmid. Therefore, prior to the insertion of selected GMC oxidoreductases, vector contained the sequence for the cytochrome domain and the native linker. In other words, the catalytically active domain (the dehydrogenase domain of *Neurospora crassa* CDH IIA) was replaced by other dehydrogenases of the GMC oxidoreductases family. In the experiment, two different genes for glucose dehydrogenase were inserted. One was from *Glomerella cingulata* (*Nc*IIA_*Gc*GDH) and the other one was from *Aspergillus flavus* (*Nc*IIA_*Af*GDH).

The same pPICZ A vector was used for the production of flavoenzymes as well. Two different flavoenzymes, from GMC oxidoreductases family, were produced – glucose oxidase from *Aspergillus niger* (*An*GOX) and glucose dehydrogenase from *Glomerella cingulata* (*Gc*GDH). Genes *An*GOX and *Gc*GDH were isolated previously and inserted into the multiple cloning site of the pPICZ A vector by other lab members.

3.3.3. Chemical transformation of *E. coli* NEB 5-alpha cells

The whole transformation process was done following the "High Efficiency Transformation Protocol" provided by the supplier of the *E. coli* NEB 5-alpha cells in order to achieve a high number of transformed cells carrying the circular vector (NEB, 2017). 50 µL of chemically competent *E. coli* NEB 5-alpha cells were thawed on ice and mixed with 1-5 µL DNA. After an incubation for 30 minutes on ice, the suspension was heat-shocked at 42 °C for 30 seconds and immediately placed back on the ice for 2 minutes. 950 µL of room temperature SOC media was added and the suspension was incubated for one hour at 37 °C and 250 rpm. The transformed cells were plated on LS-LB plates containing 25 mg L^{-1} of Zeocin as a selective marker. 100 µL of the cell suspension was pipetted to one plate and spread out with glass spreader or glass beads. The rest of the cell suspension was spun down at 10,000 × g for 1 minute. The cell pellet was resuspended in 100 µL supernatant and spread out on another LS-LB selective plate. The plates were incubated overnight at 37 ºC.

3.3.4. Plasmid isolation

To isolate cloned plasmids, five single colonies of *E. coli* were picked with a sterile toothpick and transferred to individual vials containing 3 mL LS-LB medium with Zeocin. The vials were incubated overnight at 37 ºC and 150 rpm. The plasmid isolation was done using a "Monarch Plasmid DNA Miniprep Kit Protocol" provided by New England Biolabs (2016). 1.5 mL of the cell suspension was pipetted into an Eppendorf tube and centrifuged for 1 minute at 16,000 × g. The supernatant was discarded, and the pellet was resuspended in 200 µL plasmid resuspension buffer. The lysis of bacterial cells was achieved by adding plasmid lysis buffer, after which the tube was gently inverted five times until the color of the solution turned dark pink. After a brief incubation period of one minute 400 µL of cold plasmid neutralization buffer was added and the tube was inverted until the color turned yellow. The solution was incubated for two minutes after which the lysate was clarified by centrifuging the tube for 3 minutes at

16,000 × g. The resulting supernatant was transferred to a spin column and spun down for 1 minute. The flow-through was discarded and 200 µL of plasmid wash buffer 1 was added. The solution was incubated for 5 minutes before centrifuging for one minute and discarding the flow-through. 400 µL of plasmid wash buffer 2 was added and the solution was centrifuged for 1 minute. The column was inserted into a clean 1.5 mL Eppendorf tube, 30 µL of sterile HQ-H_2O was added and the tube was spun down for 1 minute at 16,000 × g after two minutes of incubation. The solution was stored at -20 ºC.

The absorption of produced plasmids with DNA sequence for expressing chimeric enzymes was measured by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Plasmid solutions that had characteristic spectrum with a peak at 260 nm and satisfactory DNA purity were sent to sequencing to confirm that plasmids had incorporated genes of interest in the reading frame.

3.3.5. Preparation of electrocompetent *Pichia pastoris*

Two different strains of *Pichia pastoris* electrocompetent cells were produced – KM71H and GlycoSwitch SuperMan5. A single colony of each was used for the inoculation of 10 mL YPD medium. Incubation took place for 18 hours at 30 °C and 120 rpm. This culture was further used for the inoculation of 250 mL YPD medium and grown to a final OD_{600} of approximately 1.0. The cells were pelleted at 2,830 × g at 4 °C and resuspended in 50 mL YPD containing 2 mL of 1 M HEPES buffer, pH 8.0. 1.25 mL of 1 M DTT was added and the suspension was incubated at 30 °C and 100 rpm for 15 minutes. The suspension was brought to a final volume of 200 mL with sterile cold dH_2O and pelleted again at 1,590 × g for 5 minutes at 4 °C. The resulting pellet was washed with dH_2O twice and resuspended in 10 mL sterile cold 1 M sorbitol. The cells were finally pelleted at 3,220 × g for 10 minutes at 4 °C, resuspended by the addition of 0.5 mL sorbitol and aliquots of 50 µL were prepared. The aliquots were frozen at -80 °C until further usage.

3.3.6. Transformation of *Pichia pastoris* strains by electroporation

Prior to the transformation of *Pichia pastoris* the plasmids had to be linearized and this was achieved by using the restriction enzyme PmeI (New England Biolabs, Ipswich, Massachusetts, USA). The reaction mixture was prepared according to the supplier of the enzyme and incubated at 37 °C for 1 hour. After the inactivation of the restriction enzyme for

20 minutes at 65 °C, the mixture was directly used for transformation without further purification of the DNA from the reaction mixture.

50 µL of the electrocompetent *Pichia pastoris* suspension was thawed on ice and mixed with approximately 100 ng (2-4 µL) of the linearized plasmid DNA. After an incubation for 5 minutes on ice, the suspension was transferred into a sterile 1 mm electroporation cuvette. Electroporation was conducted at 1.5 kV and 125 Ω for 3 msec. Immediately after electroporation, 500 µL of ice-cold 1.0 M sorbitol and 500 µL of YPD medium were added, and the suspension was incubated at 30 °C and 100 rpm horizontal shaking for 3-4 hours. Afterward, 100 µL of the cell suspension was plated on YPD plates containing 100 mg L^{-1} Zeocin and incubated at 30 °C for 2 days. 1:10 dilution of cell suspension was made which was used for plating as well.

Electrocompetent *Pichia pastoris* KM71H cells were transformed with plasmids that had inserted genes for chimeric enzymes (*Nc*IIA_*Af*GDH and *Nc*IIA_*Gc*GDH). On the other hand, electrocompetent *Pichia pastoris* GlycoSwitch SuperMan5 cells were transformed with plasmids that had inserted genes for two different flavoenzymes – *An*GOX and *Gc*GDH.

3.3.7. Cryo-culture

A single *E. coli* colony was cultivated in LB medium at 37 °C overnight. 750 µL cell culture was mixed with 750 µL sterile glycerol (30%) and frozen and stored at -80 °C.

A single *Pichia pastoris* colony was cultivated in YPD medium at 30 °C for 24 hours. 750 µL cell culture was mixed with 750 µL sterile glycerol (30%) and frozen and stored at -80 °C.

3.3.8. Deep well plate screening

This method was applied for the screening of enzyme variants produced in *Pichia pastoris* GlycoSwitch SuperMan5. The transformed cells, expressing selected enzymes under the control of the inducible *AOX* promoter, were cultivated in a deep well plate and the secreted proteins were harvested and further analyzed.

For each protein of interest eight colonies of transformed *Pichia pastoris* cells were chosen from YPD selective plates. With cells from the same colonies, new agar plates were made which were later used for expression in shaking flasks. 250 µL of the BYPD medium was

transferred to selected wells and inoculated with a single colony from the YPD selective plate. The 96-well plate, which is shown in figure 4, was sealed with Breathe-Easy sealing membrane (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated for 65 hours at 30 °C and 300 rpm. After 65 hours of incubation, the protein expression was induced by adding 250 µL of BMMY medium. 50 µL of methanol feed medium was added after further 8, 24 and 48 hours. The cultivation was stopped after 136 hours, and cells were pelleted at 3,500 rpm for 15 minutes in the centrifuge. The supernatant containing the enzyme was used for the screening procedure.

Figure 4. 96-deep well plate (adapted from Stellar Scientific)

For the screening of *An*GOX and *Gc*GDH produced in *P. pastoris* SuperMan5 different assays were applied. Protein concentration was determined by the Bradford assay for both enzymes, but the activity of *An*GOX was determined by the ABTS assay and the activity of *Gc*GDH was determined by the DCIP assay. Cells from colonies that gave the highest specific activity of expressed protein were used for the production in shaking flasks. Production and screening of chimeric enzymes were carried out by other lab members.

3.4. PRODUCTION

After the screening procedure, the most promising producers were chosen for the production of enzymes of interest. Altogether there were four productions in shaking flasks – two chimeric enzymes (*Nc*IIA_*Gc*GDH and *Nc*IIA_*Af*GDH in *Pichia pastoris* KM71H) and two flavoenzymes (*An*GOX and *Gc*GDH in *P. pastoris* GlycoSwitch SuperMan5). The procedure for the expression was based on the ″EasySelect *Pichia* Expression Kit″ by Invitrogen (2010).

3.4.1. Expression of two chimeric enzymes in *Pichia pastoris* KM71H – Mut^s

For the preparation of a pre-culture, 50 mL of YPD with 25 mg L^{-1} of Zeocin was inoculated with a single colony from the screening plate in a 300 mL baffled shaking flask and incubated overnight at 30 °C and 150 rpm. For each enzyme, the pre-culture was produced in quintuplets yielding 10 baffled flasks. After 24 hours, cells were harvested by centrifugation at 2,000 × g for 5 minutes. The supernatant was discarded and cells were resuspended in 200 mL of BMGY medium in a 1 litre baffled flasks. One of the 5 flasks that were used for the production of *Nc*IIA_*Af*GDH was a PreSens flask shown in Figure 5.

Figure 5. PreSens baffled flask that allows online monitoring of dissolved oxygen, biomass and pH (adapted from PreSens)

The PreSens flask has integrated sensors that are used to provide online information about biomass growth, dissolved oxygen, and pH. All of the cultures were incubated for 16 hours at 30 °C and 150 rpm and, once again, cells were harvested by centrifugation at 2,000 × g for 5 minutes. To induce expression, cells were resuspended in 150 mL of BMMY medium to an $OD_{600} \approx 3$. After every 24 hours methanol was added to a final concentration of 1% or 1.5%, and 1 mL of the expression culture was taken to analyze expression levels and to determine the optimal time to harvest. Samples were centrifugated and the supernatants were analyzed for protein expression by SDS-PAGE and activity assay. In addition, protein concentration was determined using the Bradford assay.

3.4.2. Expression of two GMC oxidoreductases in *P. pastoris* GlycoSwitch SuperMan5 – Mut^+

For the preparation of a pre-culture, 3 × 50 mL of YPD with 100 mg L^{-1} of Zeocin was inoculated with cells from screening agar plate in a 300 mL baffled shaking flask and incubated overnight at 30 °C and 150 rpm. 25 mL of the pre-culture was used to inoculate 250 mL of BMY medium in 1 litre baffled flask. Each enzyme was produced in quadruplets so in total there were 8 baffled flasks used for expression (4 × *An*GOX and 4 × *Gc*GDH). Methanol was used for the induction and it was added twice a day – to the concentration of 0.5% in the morning and 0.8% in the evening. The flasks were closed with cotton caps for better oxygen dissolution. Samples were taken every 24 hours to analyze expression levels and to determine the optimal time to harvest. Samples were centrifugated and the supernatants were analyzed for protein expression by SDS-PAGE and activity assays. In addition, protein concentration was determined using the Bradford assay.

3.5. PURIFICATION OF CHIMERIC ENZYMES

3.5.1. Cell removal and ammonium sulfate addition

The cells were removed from the fermentation broth by a centrifugation at 5,000 × g for 20 minutes at 4 °C and the supernatant was collected. Then, 100% saturated ammonium sulfate solution was slowly added to the cooled supernatant to 20% saturation. This procedure was done to both supernatants containing *Nc*IIA_*Af*GDH and *Nc*IIA_*Gc*GDH, respectively. The clear supernatants containing the chimeric enzymes were purified according to the purification scheme described by Sygmund et al. (2012) using two chromatographic steps.

3.5.2. Hydrophobic interaction chromatography (HIC)

The initial purification step for both chimeric enzymes was hydrophobic interaction chromatography and was performed using an Äkta Prime system (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA).

In the first step of the purification the clear supernatant containing the chimeric enzyme and 20% $(NH_4)_2SO_4$ was loaded onto a Phenyl Sepharose column. Employed buffers and specifications of the utilized column are listed in table 1. Before loading the supernatant onto the column, it was once more centrifuged at 4 °C and 5,000 × g for 10 minutes to remove remaining particles which may clog the column. After loading the supernatant, the column was washed with buffer A until all unbound protein was eluted from the column and the conductivity reading was constant. The elution of bound protein was performed with a linear gradient from 0 to 100% buffer B in two column volumes and collected in 6 mL fractions. Selected fractions that showed absorption at 280 nm, together with the flow-through, were subjected to SDS-PAGE and pooled together according to their molecular weight and the presence of impurities. The pooled enzyme-containing fractions were ultrafiltrated using Amicon Ultra-15 Centrifugal Filters (Millipore, Burlington, Massachusetts, USA) with a molecular weight cut-off (MWCO) of 30 kDa. After ultrafiltration, the protein concentration was around 10 mg mL^{-1}. The partially pure enzyme was subjected to standard activity assay. The same procedure was carried out for both chimeric enzymes – *Nc*IIA_*Af*GDH and *Nc*IIA_*Gc*GDH.

Table 1. Conditions and specification for HIC step

Column material	Phenyl Sepharose High Performance (GE Healthcare, USA)
Column volume	50 mL
Buffer A	50 mM sodium citrate buffer, pH 5.5 with 22% $(NH_4)_2SO_4$
Buffer B	50 mM sodium citrate buffer, pH 5.5
Gradient	0-100% buffer B

3.5.3. Anion exchange chromatography (AEX)

The second purification step was anion exchange chromatography and was performed using an Äkta Pure system (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA).

The sample was loaded onto a column and was washed with buffer A eluting all unbound protein. Employed buffers and specifications of the utilized column are listed in table 2. The elution of bound protein was performed with a linear gradient from 0 to 100% buffer B in 30 column volumes and collected in 1 mL fractions. Selected fractions that showed absorption at 280 nm, together with the flow-through, were subjected to SDS-PAGE and strictly pooled together according to their molecular weight and the presence of impurities. The pooled enzyme-containing fractions were ultrafiltrated using Amicon Ultra-2 Centrifugal Filters for DNA and Protein Purification and Concentration (Millipore, Burlington, Massachusetts, USA) with a molecular weight cut-off (MWCO) of 30 kDa. Concentrated enzyme solutions were subjected to the standard activity assay.

Table 2. Conditions and specification for AEX chromatography step

Column material	Resource Q anion exchange (GE Healthcare, USA)
Column volume	1 mL
Buffer A	25 mM potassium phosphate buffer, pH 7.0
Buffer B	25 mM potassium phosphate buffer, pH 7.0 with 1 M NaCl
Gradient	0-100% buffer B

3.5.4. Centrifugal ultrafiltration

For concentrating of the protein solution between the purification steps, for finally concentrating homogenous enzyme solution as well as for buffer exchanges, Amicon Ultra-15 Centrifugal Filter Units or Amicon Ultra-2 Centrifugal Filters for DNA and Protein Purification and Concentration (Millipore, Burlington, Massachusetts, USA) with a molecular weight cut-off (MWCO) of 30 kDa were used. The solution to be concentrated was loaded into the ultrafiltration device and centrifuged at 4,000 × g. The flow-through was discarded and the procedure repeated until the protein solution was concentrated to the desired concentration.

3.6. PURIFICATION OF GMC OXIDOREDUCTASES

3.6.1. Cell removal

The cells were removed from the fermentation broth by centrifugation at 5,000 × g for 20 minutes at 4 °C and the supernatant was harvested.

3.6.2. Immobilized metal affinity chromatography (IMAC)

Since both flavoenzymes were his-tagged, logically, the first step of purification was Immobilized metal affinity chromatography and was performed using an Äkta Prime system (GE Healthcare Bio-sciences, Pittsburgh, Pennsylvania, USA). To the clear supernatant imidazole was added to a final concentration of 10 mM as well as NaCl to a final concentration of 0.5 M.

Figure 6. Äkta Prime system with IMAC column

The sample was loaded onto a column and was washed with buffer A eluting all unbound protein. Employed buffers and specifications of the utilized column are listed in table 3. Column was prepared in the laboratory and as a resin Chelating Sepharose Fast Flow (GE Healthcare) was used. The column was saturated with nickel (Ni^{2+}) ions which have been generally proven to be the most successful for the purification of his-tagged protein. The elution of bound protein was performed with a linear gradient from 0 to 100% buffer B in 5 column

volumes and collected in 7 mL fractions. Chosen fractions that showed absorption at 280 nm, together with the flow-through, were subjected to SDS-PAGE and were pooled together according to their molecular weight and the presence of impurities.

Table 3. Conditions and specification for IMAC step

Column material	Chelating Sepharose Fast Flow (GE Healthcare, USA) saturated with nickel (Ni^{2+}) ions
Column volume	100 mL
Buffer A	20 mM sodium phosphate buffer, pH 7.0 with 0.5 M NaCl and 10 mM imidazole
Buffer B	20 mM sodium phosphate buffer, pH 7.0 with 0.5 M NaCl and 0.5 M imidazole
Gradient	0-100% buffer B

The pooled enzyme-containing fractions were diafiltrated in order to remove imidazole using Amicon Ultra-15 Centrifugal Filters (Millipore, Burlington, Massachusetts, USA) with a molecular weight cut-off (MWCO) of 30 kDa. After diafiltration and concentration to ≈ 10 mg mL^{-1}, pooled fractions were subjected to standard activity assay (DCIP assay for *Gc*GDH and ABTS assay for *An*GOX). For diafiltration, 50 mM sodium phosphate buffer, pH 7 was used.

3.6.3. Hydrophobic interaction chromatography (HIC)

The *An*GOX pool 1 obtained after IMAC step was subjected to the second purification step which was performed using an Äkta Pure system (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA).

Table 4. Conditions and specification for HIC step

Column material	Phenyl Source (GE Healthcare, USA)
Column volume	22 mL
Buffer A	50 mM sodium phosphate buffer, pH 7.0 with 25% $(NH_4)_2SO_4$
Buffer B	50 mM sodium phosphate buffer, pH 7.0
Gradient	0-100% buffer B

Prior to the second purification step, 100% saturated ammonium sulfate solution was slowly added to the cooled sample to 25% saturation. The sample was loaded onto a column and was washed with buffer A eluting all unbound protein. Employed buffers and specifications of the utilized column are listed in table 4. The elution of bound protein was performed with a linear gradient from 0 to 100% buffer B in 15 column volumes and collected in 3.5 mL fractions. Selected fractions that showed absorption at 280 nm, together with the flow-through, were subjected to SDS-PAGE. Flow-through and all enzyme-containing fractions were pooled together and diafiltrated using VivaFlow 40 filtration module with a molecular weight cut-off value of 30 kDa (Sartorius, Göttingen, Germany) in order to remove $(NH_4)_2SO_4$. Diafiltration was carried out by continuous concentrating to a certain volume and refilling with fresh buffer containing low salt concentration. For diafiltration, 50 mM sodium phosphate buffer, pH 7 was used.

3.7. DEFLAVINATION METHOD BY SWOBODA

For the determination of dissociation constant of flavoenzymes, deflavination had to be executed. In that purpose, conventional method developed by Swoboda in 1968 was used with slight modifications. The procedure was done according to a scheme described by Garajová et al. (2017). A saturated solution of ammonium sulfate was prepared with pH adjusted with sulfuric acid to pH 1.4. 20 mL of ammonium sulfate solution was cooled to 4 °C. 1 mL of the enzyme solution was added dropwise to the cooled solution of ammonium sulfate while constantly mixing with a magnetic stirrer. Precipitated protein was separated from the solvent by centrifugation at 13,500 rpm for 30 minutes at -5 °C. The pellet was resuspended in 2 ml of 2.5 M sodium acetate, pH 8.5. The neutralized solution was added into 20 ml of cooled ammonium sulfate solution pH 1.4. This cycle has been repeated twice, on the third repetition the neutralized solution was added into 20 ml of cooled 90% saturated ammonium sulfate solution, pH 7.0, instead. The mix was centrifuged, and the pellet was resuspended in 1 ml of 100 mM phosphate buffer, pH 6.5. Prepared deflavinated enzyme (apo-form) was dialyzed against 10 mM phosphate buffer, pH 6.5 at 4 °C for 24 hours. Dialysis solution was changed after 12 hours. Dialysis was followed by centrifugation at 10,000 rpm and 4 °C for 30 minutes to remove aggregates present in the protein solution.

3.8. CHARACTERIZATION

3.8.1. Protein concentration

In order to monitor the cultivation process and the course of the purification the protein concentration was determined using the rapid protein quantification method developed by Bradford (1976).

The protein concentration of the purified homogenous protein solutions was determined spectrophotometrically at 280 nm using a molar absorption coefficient calculated from the amino acid sequence of the protein.

3.8.1.1. *Bradford*

The reagent for the method was purchased from Bio-Rad Laboratories (Hercules, California, USA) and prepared according to the manufacturers' guidelines. After incubating 15 µL of the samples in 600 µL Bradford reagent for 15 minutes, the measurement was performed against a blank containing only the reagent using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Brea, California, USA). The protein concentration was automatically calculated from a standard curve generated with bovine serum albumin.

3.8.1.2. *Spectrophotometrically*

Homogeneous enzyme concentration was determined in a 1 cm or 0.3 cm quartz cuvette from the absorption at 280 nm using the calculated molar extinction coefficient for each protein. To convert between absorption and protein concentration Lambert-Beer law was used.

$$A = \varepsilon \cdot c \cdot l$$

A – absorption

ε – molar extinction coefficient [M^{-1} cm^{-1}]

c – molar concentration [M]

l – pathlength [cm]

3.8.2. UV-Vis spectroscopy

To record the absorption spectra of pure enzyme solutions Agilent 8453 UV-visible Spectroscopy System (Agilent Technologies, Santa Clara, California, USA) was used. The whole spectrum from 200 nm to 900 nm was recorded against a blank (buffer). The samples were diluted with the buffer used in the blank to OD_{280} of ≈ 1.

3.8.3. Dehydrogenase activity assay using DCIP

This method is a standard method for determining glucose dehydrogenase activity in crude extracts or partially purified samples. The activity is detected by the reduction of DCIP (2,6-dichloroindophenol) which decolorizes the initially blue assay mixture (Bao et al., 1993; Baminger et al., 2001). The activity of GDH towards DCIP does not depend on the heme-containing domain, so this assay can be employed for both chimeric enzymes, which have a cytochrome domain with heme *b*, as well for GDHs without that domain such as *Gc*GDH. The reaction happens if the FAD cofactor is present. The measurements were carried in 1 mL reaction volume which was prepared according to the pipetting scheme shown in table 5. The measurements were performed using the Perkin Elmer Lambda 35 UV-VIS Spectrometer photometer (Waltham, Massachusetts, USA). All the solutions except the sample were pipetted in a cuvette and the mixture was incubated at 30 °C in a water bath for a minimum of 20 minutes and then transferred to the sample holder. The reaction began when 20 µL of the sample was added to the cuvette, which was already placed in the sample holder. The absorbance was recorded at 520 nm for 180 seconds. The software calculates the volumetric activity from the negative slope (decrease) of the absorption. One unit of enzyme activity was defined as the amount of enzyme that reduces 1 mmol of DCIP per minute under the selected assay conditions.

Table 5. Pipetting scheme for dehydrogenase activity assay using DCIP

Component	**Concentration**	**Volume**
DCIP (in 10% (v/v) ethanol)	3 mM	100 µL
Glucose solution	1 M	100 µL
McIlvaine buffer, pH 5.5; Sodium phosphate buffer, pH 5.5	100 mM 50 mM	780 µL
Sample	0.5-5.0 U mL^{-1}	20 µL

3.8.4. Dehydrogenase activity assay using cytochrome c

Enzymatic activity of partially purified chimeric enzymes was determined by monitoring the reduction of the one-electron acceptor cytochrome *c* in the presence of glucose. The activity of chimeric enzymes towards cytochrome *c* depends on the heme *b*-containing domain, whereas no reaction is observed if only the catalytic dehydrogenase domain, containing FAD as a cofactor, is present. Therefore, this assay procedure was used as a reference method to measure direct electron transfer ability of the *Nc*IIA_*Gc*GDH and *Nc*IIA_*Af*GDH that contain cytochrome domain with heme *b* from *Neurospora crassa* CDH IIA. Upon measurement, cytochrome c is reduced, which causes the color to change from orange to a more pinkish tone (Baminger et al., 2001). The measurements were performed with the same spectrophotometer as for the DCIP activity assay. The measurements were performed in 1 mL reaction volume which was prepared according to the pipetting scheme shown in table 6. Cytochrome c solution, substrate solution and, McIlvaine buffer were pipetted in a cuvette and the mixture was incubated at 30 °C in a water bath for a minimum of 20 minutes and then transferred to the sample holder. The reaction started when 20 µL of the sample was added to the solution. The absorbance was measured at 550 nm for 180 seconds. Again, volumetric activity was automatically calculated by the software from the positive slope (increase) of the absorption. One unit of enzyme activity is defined as the amount of enzyme reducing 1 µmole of cytochrome *c* per minute at the specified conditions.

Table 6. Pipetting scheme for dehydrogenase activity assay using cytochrome c

Component	Concentration	Volume
Cytochrome c (in distilled water)	1 mM	20 µL
Glucose solution	1 M	100 µL
McIlvaine buffer, pH 5.5	100 mM	860 µL
Sample	0-0.3 U mL^{-1}	20 µL

3.8.5. Oxidase activity assay using ABTS

This method is a standard method used for determining glucose oxidase activity in crude extracts or partially purified samples. During this oxygen-dependent reaction ABTS is oxidized to the ABTS radical wherein colorless reaction mixture becomes green (Michal et al., 1983). All measurements were performed with the Lambda 35 UV/Vis spectrometer and its accompanying software. The measurements were carried in 1 mL reaction volume which was

prepared according to the pipetting scheme shown in table 7. All the solutions except the sample were pipetted in a cuvette and the mixture was incubated at 30 °C in a water bath for a minimum of 15 minutes and then transferred to the sample holder. The reaction began when 20 µL of the sample was added to the cuvette, which was already placed in the sample holder. The absorbance was recorded at 420 nm for 180 seconds. The volumetric activity was calculated automatically by the software. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 µmole of D-glucose per minute at the specified conditions.

Table 7. Pipetting scheme for oxidase activity assay using ABTS

Component	Concentration	Volume
ABTS reagent	-	20 µL
D-glucose solution	1 M	20 µL
Potassium phosphate buffer, pH 5.5	100 mM	860 µL
Sample	0-0.3 U mL^{-1}	20 µL

ABTS reagent was prepared by dissolving 14.7 mg of ABTS in 2.5 mL distilled water and addition of 100 µL of peroxidase solution. Peroxidase solution is prepared by dissolving lyophilized peroxidase to the final concentration of 1,428 U mL^{-1} in 50 mM potassium phosphate buffer, pH 6.5 with 1 M $(NH_4)_2SO_4$.

3.8.6. SDS-PAGE

To the enzyme solution, Laemmli-Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added (the enzyme solution and the Laemmli-Buffer were mixed in 1:1 ratio) and kept in a heating block at 99 ºC for 3 minutes for the complete denaturation of proteins. Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad Laboratories, Hercules, California, USA) were used for SDS-PAGE. Depending on the size of the wells 10 or 15 µL was loaded onto the gel together with 5 µL of Precision Plus Protein Unstained Standard (Bio-Rad Laboratories, Hercules, California, USA). Electrophoresis was performed at 90 V for 10 minutes and then the voltage was increased to 150 V until complete separation (30-40 minutes). When the samples reached the end of the gel, the gel was analyzed using a Gel Doc XR and Image Lab software (Bio-Rad, USA).

3.8.7. Native-PAGE and heme staining

In the native-PAGE, protein solutions are prepared in a non-denaturating sample buffer, which maintains native structure and mass to charge ratio. Utilized gel for native-PAGE was the same as for the SDS-PAGE, but samples were prepared by mixing equal volumes of sample and native sample buffer from Bio-Rad Laboratories (Hercules, California, USA). Electrophoresis was performed at a constant voltage of 200 V for approximately 90 minutes. This procedure was carried out for chimeric enzyme *Nc*IIA_*Af*GDH in order to verify if the enzyme had cytochrome domain with heme *b*. For a standard, 5 µL of Serva Native Marker liquid mix (SERVA Electrophoresis GmbH, Heidelberg, Germany) was used.

The produced gel was then stained to detect bands that contain enzymes with cytochrome domain with heme. The staining solution was prepared by mixing 30 mL of 6.3 mM TMBZ (3,3',5,5'-tetramethylbenzidine) in methanol with 70 mL 0.25 M sodium acetate, pH 5.0 buffer. The gel was immersed in the solution above and put in the dark for 2 hours with occasional mixing. Then 500 µL of 30% hydrogen peroxide was added and bands with heme became visible. The gel was analyzed using a Gel Doc XR and Image Lab software (Bio-Rad, USA).

3.8.8. Dissociation constant (K_d) determination

The dissociation constant K_d was determined for *An*GOX and *Gc*GDH expressed in different hosts. Prior to the determination, FAD was removed from enzymes by the Swoboda method described in paragraph 3.7. Deflavinated enzyme was diluted and mixed with FAD solution to a concentration of 1 µM of enzyme and different concentrations of FAD. The apo-form enzyme is not very stable, so this procedure was done quickly after deflavination. The initial FAD concentrations used to measure enzyme activity and determine K_d were as follows:

- *An*GOX produced in *A. niger* – 0; 50; 100; 200; 400; 600; 800; 1,000; 1,250; 1,500; 2,000; 2,500 nM
- *Gc*GDH produced in *Pichia pastoris* X-33 – 0; 0.5; 1; 2.5; 5; 7.5; 10; 25; 50; 75; 100; 150; 200 nM
- *An*GOX produced in *Pichia pastoris* SuperMan5 – 0; 10; 25; 50; 75; 100; 150; 200; 400; 600; 800; 1,000; 1,250; 1,500; 2,000; 2,500; 5,000; 10,000; 100,000 nM
- *Gc*GDH produced in *Pichia pastoris* SuperMan5 – 0; 1; 2.5; 5; 7.5; 10; 25; 50; 75; 100; 150; 200; 400; 600; 800; 1,000; 1,250; 1,500; 2,000; 2,500 nM

The change in activity was measured with standard assays and all measurements were carried out in at least triplicates. The residual activity of apo-form was subtracted from all of the other values. A nonlinear minimal squares regression model, based on the Michaelis-Menten equation, was used to calculate the K_d and v_{max} values applying SigmaPlot 12.5 with enzyme kinetics add-on (Systat Software, San Jose, California, USA).

3.8.9. Deglycosylation

The endoglycosidase H_f, which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins, was used for deglycosylation (all chemicals and enzyme from New England Biolabs). The enzyme was first denatured by heat treatment to make glycan structures accessible for enzymatic treatment. The enzyme was mixed with 10 × glycoprotein denaturating buffer (5% SDS, 400 mM DTT) and then was denaturated by heating reaction at 100 °C for 10 minutes. After cooling down, to the mixture were added 10 × glycobuffer 3 (50 mM sodium acetate buffer, pH 6), H_20, and Endo H_f to make a total reaction volume of 20 µL. The reaction was incubated at 37 °C for 1 hour. The endoglycosidase H_f was inactivated by heating the mixture one more time to 75 °C for 10 minutes. Afterward, the sample mix was subjected to SDS-PAGE.

4. RESULTS AND DISCUSSION

This thesis can be divided into two parts. The first part investigates the function of the cytochrome domain of cellobiose dehydrogenase as an electron transferring domain for oxidoreductases. The aim of this thesis was the production of two different chimeric enzymes in shaking flasks using *Pichia pastoris* KM71H strain and optimization of their purification. As already stated, both enzymes had a cytochrome domain and linker from *Neurospora crassa* CDH IIA to which glucose dehydrogenases were added. One glucose dehydrogenase was from *Glomerella cingulata* and the other was from *Aspergillus flavus*.

The second part of this thesis aims to improve the performance of enzymes used in glucose biosensors by the functionalization of FAD. The first step in this process is establishing a successful deflavination method which was the goal of this thesis. Two different enzymes were tested – glucose oxidase from *Aspergillus niger* and glucose dehydrogenase from *Glomerella cingulata*. Both enzymes were produced in *Pichia pastoris* GlycoSwitch strain and then purified.

All data is shown in the form of tables, figures, and pictures with a detailed explanation of their contents. After collection of all the data, it was analyzed and organized using the Microsoft Office 365 package (Microsoft, Redmond, Washington, USA) and SigmaPlot 12.5 (Systat Software, San Jose, California, USA).

4.1. PRODUCTION OF CHIMERIC ENZYMES

After insertion of the gene for cellobiose dehydrogenase IIA from *Neurospora crassa* in vector pPICZ A, the sequence for dehydrogenase domain was “cut out” and replaced by the gene for glucose dehydrogenase. Two different plasmids were prepared of which one had inserted the gene for glucose dehydrogenase from *Glomerella cingulata* (*Nc*IIA_*Gc*GDH) and the other one had it from *Aspergillus flavus* (*Nc*IIA_*Af*GDH).

Assembled plasmids were used to transform chemically competent *E. coli* NEB 5-alpha cells in order to produce larger quantities of plasmids. Transformed cells were cultivated and plasmids were extracted according to the procedure in paragraph 3.3.4. “Confirmed” plasmids were linearized with restriction enzyme PmeI and then used to transform previously prepared electrocompetent *Pichia pastoris* KM71H cells. By other members of the lab screening in 96-

well plate for best producers was carried out. For future use, glycerol stocks of transformed *E. coli* NEB5-alpha were prepared, as well as best *Pichia pastoris* producers.

The pre-culture was used to inoculate the BMGY medium in order to generate more biomass since *Pichia pastoris* KM71H strain is Mut^{s}. Growth of biomass in BMGY was conducted like it was suggested by Invitrogen (2010), but because OD_{600} reached a higher value than suggested only part of the centrifuged cells were used for resuspension in BMMY medium. *Nc*IIA_*Af*GDH was produced in three 1 litre baffled flasks of which one was the PreSens flask and *Nc*IIA_*Gc*GDH was produced in two 1 litre baffled flasks, respectively. The PreSens flask allowed monitoring of the process by receiving online data for relative biomass, oxygen saturation, and pH. After induction, samples were taken every 24 hours to check the protein concentration and activity using the DCIP assay. At that time, methanol was added to a final concentration of 1% or 1.5% to maintain inducing protein expression as can be seen in figure 7.

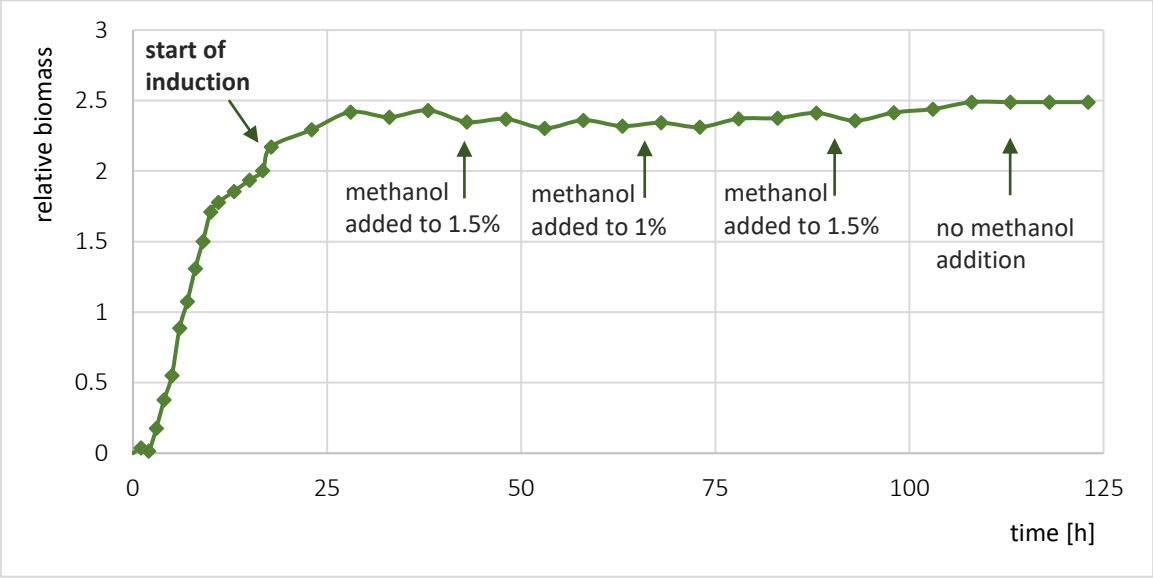

Figure 7. Relative biomass in PreSens flask

Additionally, one can see that cells grow only in the glycerol medium. After the induction with methanol changes in biomass concentration are minimal. That's the case because Mut^{s} strain is used for the production which has disrupted *AOX1* gene what results in a reduction in the cells' ability to metabolize methanol. The cells, therefore, exhibit poor growth on methanol medium.

When volumetric activity started to drop, it was decided that expression should be stopped and at that time cells were removed by centrifugation (Figure 8). The volume of the supernatant containing *Nc*IIA_*Af*GDH was 353 mL with a protein concentration of 0.204 mg mL^{-1} and a volumetric activity of 55.72 U mL^{-1}. The volume of the supernatant containing *Nc*IIA_*Gc*GDH was 253 mL with a protein concentration of 0.083 mg mL^{-1} and a volumetric activity of only 1.97 U mL^{-1}. Saturated ammonium sulfate solution was added to the final concertation of 22% to each supernatant.

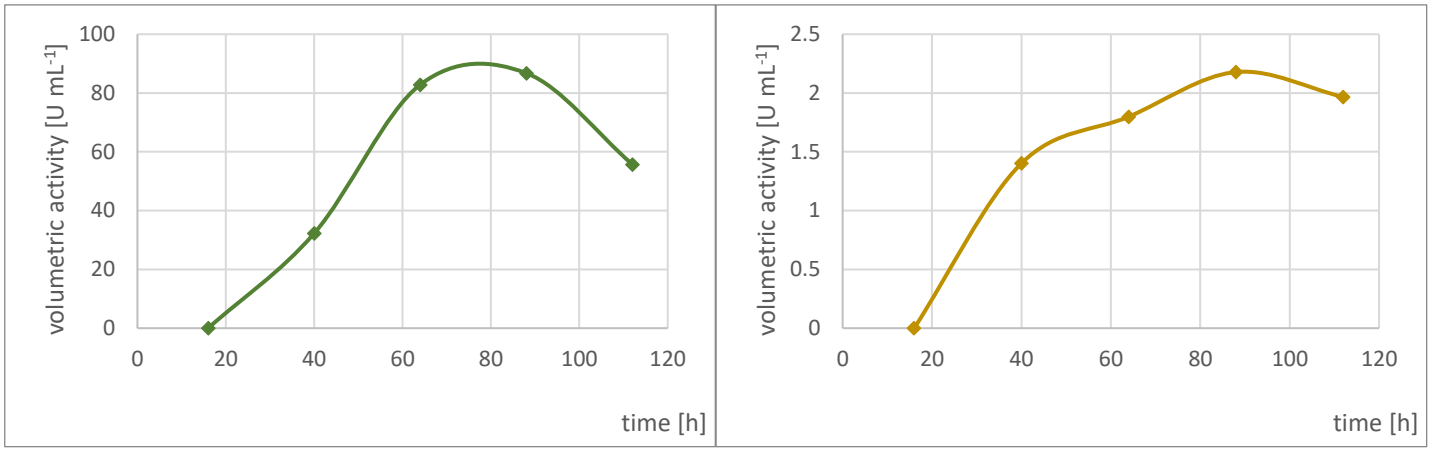

Figure 8. Volumetric activity of *Nc*IIA_*Af*GDH (left) and *Nc*IIA_*Gc*GDH (right)

Although the production of *Nc*IIA_*Gc*GDH was not very successful, what can be seen in the low volumetric enzyme activity, it was decided to purify the protein anyway in order to gain more information about optimizing the downstream process. The reason why the production of *Nc*IIA_*Af*GDH was much better than the production of *Nc*IIA_*Gc*GDH may be due to the lack of online data about dissolved oxygen and biomass. It is possible that the fed methanol wasn't used up by the cells and accumulated to the concentration above 2% (v/v) what is toxic for *Pichia* cells (Invitrogen, 2010).

4.2. PURIFICATION OF CHIMERIC ENZYMES

For both enzymes, the same two-step purification was applied. The first step of purification was hydrophobic interaction chromatography. Selected fractions that showed absorption at 280 nm, together with the flow-through, were subjected to SDS-PAGE to check if the enzyme of interest is in those selected fractions. Fractions that had the protein with the same molecular weight as the enzyme of interest were pooled together, diafiltrated and

concentrated. Protein concentration and activity were determined using the Bradford and DCIP assay, respectively. The second step of purification was anion exchange chromatography. Once again, selected fractions that showed absorption at 280 nm, together with the flow-through, were analyzed by SDS-PAGE and fractions that contained enzyme of interest were pooled together and ultrafiltrated.

4.2.1. Purification of *Nc*IIA_*Af*GDH

After the first step of purification, fractions 20 to 50 were pooled together, then diafiltrated to remove ammonium sulfate and concentrated. The molecular weight of the chimeric enzyme is 86.985 kDa calculated from the amino acid sequence. Due to the glycosylation in *Pichia pastoris*, the enzyme of interest has a molecular weight of approximately 125 kDa what can be seen on SDS-PAGE shown in figure 9. In the same figure, one can see that a lot of the enzyme of interest ended in the flow-through (blue rectangle) what means that a high amount of the protein has not bound to the column. It was calculated that the yield of this purification step was only 10.2%. For future purifications, it is advisable to use higher ammonium sulfate concentration in the sample and buffer A to improve yield.

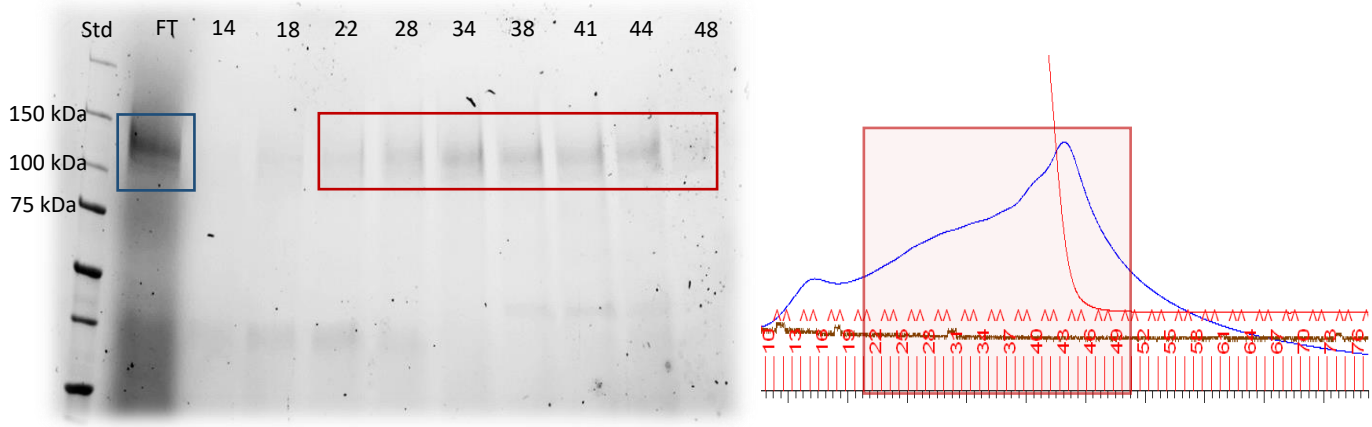

Figure 9. Results of hydrophobic interaction chromatography purification step for *Nc*IIA_*Af*GDH

Pooled fractions were concentrated to 1 mL with a protein concentration of 5.74 mg mL^{-1} and volumetric activity measured with DCIP assay was 2,005.5 U mL^{-1}. A detailed purification scheme is shown in table 8.

Table 8. Purification scheme of *Nc*IIA_*Af*GDH

Step	Total protein according to Bradford [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
Supernatant	72.0	19,669.2	273.14	1	100
HIC + diafiltration	5.74	2,005.5	349.33	1.28	10.2
AEX + diafiltration	0.89	804.3	899.56	3.29	4.1

The second step of purification was anion exchange chromatography. SDS-PAGE, shown in figure 10, indicates that the protein of interest was in fractions 6, 7 and 8 (red rectangle) so those fractions were pooled together and then diafiltrated in order to remove NaCl. It seemed that chimeric enzyme could be in the flow-through too, so flow-through was diafiltrated as well. In both diafiltrated pooled fractions and flow-through, the protein concentration and activity were measured by DCIP and cytochrome c assay, respectively. In addition, both samples had their spectrum measured.

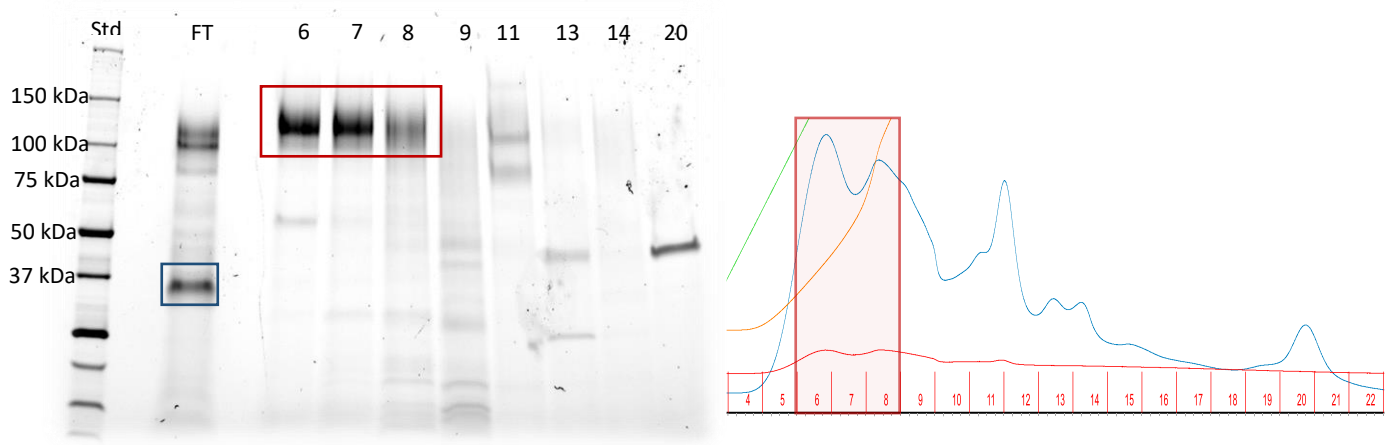

Figure 10. Results of anion exchange chromatography purification step for *Nc*IIA_*Af*GDH

Heme *b* in cytochrome domain absorbs at 420 nm, therefore, a peak at that wavelength is an indicator of a functional cytochrome domain. Spectra of, both, fractions 6-8 and flow-through are shown in figure 11 and one can see that there is a much higher Soret peak at 420 nm in flow-through than in fractions 6-8.

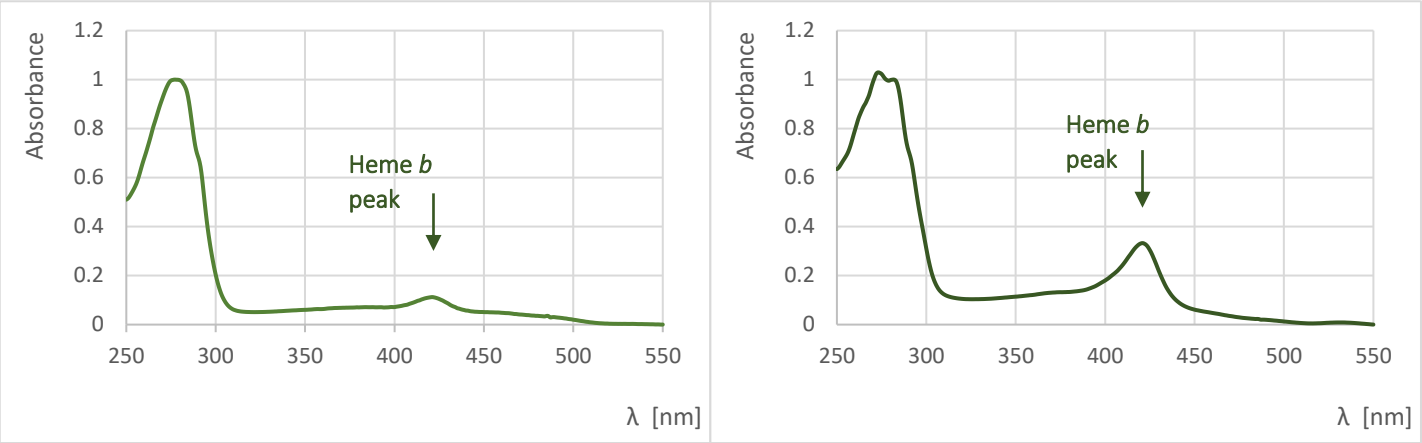

Figure 11. Spectra of diafiltrated fractions 6-8 (left) and flow-through (right)

The A_{420}/A_{280} ratio provides information about the presence of heme *b* in samples. When absorption at 420 nm and absorption at 280 nm were put in ratio, the results showed that there was 3 times more heme *b* in the flow-through than in the pooled fractions. Precisely, the A_{420}/A_{280} ratio for the pooled fractions was 0.12 and for the flow-through was 0.36 as one can see in table 9. The A_{420}/A_{280} ratio for cellobiose dehydrogenase produced in different expression systems, including *Pichia pastoris*, is around 0.6 (Ma et al., 2017). From this information can be concluded that there was a problem with heme *b* loading of the cytochrome domain. For future productions, the addition of hemin, which is a precursor of heme, in medium should be considered in order to increase the yield of active protein what was demonstrated to be a successful supplement in the production of recombinant heme peroxidase in *Pichia pastoris* (Krainer et al., 2015).

Table 9. Activity measurements of purified enzyme

Samples	A_{420}/A_{280}	Specific DCIP activity [U mg^{-1}]	Specific Cytochrome c activity [U mg^{-1}]
AEX flow-through	0.36	n/a	0.04
ultrafiltrated fractions 6-8	0.12	899.6	0.16

Since the A_{420}/A_{280} ratio was higher in the flow-through, it was expected that cytochrome c specific activity will be higher as well, but it was 4 times lower than in the pooled fractions. Furthermore, for CDH from *Neurospora crassa*, DCIP specific activity is around 2.5 times higher than cytochrome c specific activity (Sygmund et al., 2012) and for this chimeric enzyme the difference is more substantial. It may be that there was a problem with the correct expression of the chimeric enzyme or its folding. Additionally, it is possible that the cofactor loading of the cytochrome domain was very low or the protein has undergone proteolysis leading to a separation of the cytochrome and dehydrogenase domain.

In order to discover if cytochrome domain is part of the enzyme in flow-through or if it was disengaged, heme staining was performed. It was thought that the cytochrome domain might be separated from the rest of the enzyme because of the band at 35 kDa in flow-through on SDS-PAGE shown in figure 10 (blue rectangle). Additionally, pooled fractions were checked for heme as well. When staining for heme, native-PAGE should take place so that heme cannot break loose from the cytochrome domain due to the harsh conditions of SDS-PAGE. Hence, Native-PAGE was run, but, unfortunately, the running time of the native PAGE was too short and, therefore, only fragments with a molecular weight below 70 kDa can be detected.

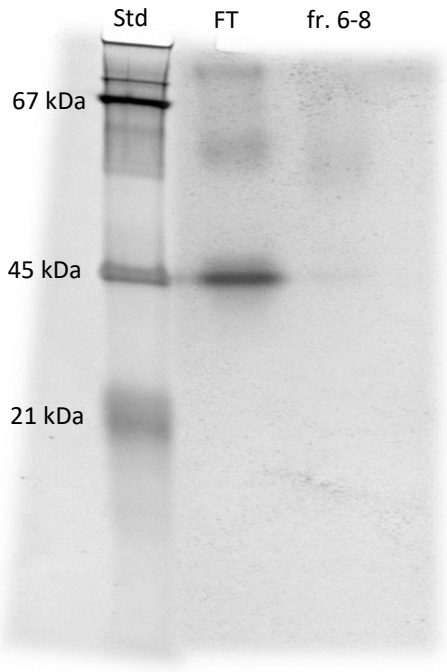

Figure 12. Native-PAGE after staining for heme

Gel after the staining for heme is shown in figure 12 and there is an intense band with heme at 43 kDa in flow-through. That band is, probably, the same as the band at 37 kDa in flow-through on SDS-PAGE, shown in figure 10 (blue rectangle), but due to different conditions of native-PAGE, it is at a different position on the gel. In native-PAGE, samples are prepared in the non-denaturing buffer, which maintains native structure of the proteins and mass to charge ratio. Though native-PAGE uses the same moving boundary as SDS-PAGE, protein mobility depends on several factors other than molecular weight, including the shape and charge of the protein.

It appears that some of the cytochrome domain, containing heme *b*, is not part of the chimeric enzyme. It could be that there was proteolysis in the linker region which led to the disjunction of cytochrome and dehydrogenase domain and that cytochrome domain ended in the flow-through during anion exchange chromatography. That would explain the high Soret peak at 420 nm in figure 11 and low cytochrome c specific activity in the flow-through.

Moreover, on SDS-PAGE shown in figure 10 can be seen that there are bands at 120 kDa in fractions 6-8, which corresponds to a glycosylated chimeric enzyme with both dehydrogenase and cytochrome domain. In addition, there is a peak at 420 nm for fractions 6-8 which is characteristic for heme *b* as mentioned before. This suggests that “whole” chimeric enzyme, with both dehydrogenase and cytochrome domain, was produced, but with very low heme loading.

Due to time restrictions, it was not possible to do some further measurements (e.g. repeat heme staining, but with longer running of native-PAGE) which would be necessary do draw a more exact conclusion.

4.2.2. Purification of *Nc*IIA_*Gc*GDH

As outlined above, the production of *Nc*IIA_*Gc*GDH was not as successful as the production of *Nc*IIA_*Af*GDH. The obtained supernatant after removing cells contained only 21 mg of proteins and total activity was 497.5 U as stated in the purification scheme in table 10.

Table 10. Purification scheme of *Nc*IIA_*Gc*GDH

Step	Total protein according to Bradford [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
Supernatant	21	497.5	23.7	1	100
HIC + diafiltration	2.5	186.1	74.4	3.1	37.4
AEX + diafiltration	0.08	0.4	5.0	0.2	0.08

After the first step of purification, fractions 16 to 32 were pooled together, then diafiltrated to remove ammonium sulfate and concentrated. The molecular weight of *Nc*IIA_*Gc*GDH, calculated from its amino acid sequence, is 86.988 kDa. Due to the glycosylation in *Pichia pastoris*, the chimeric enzyme of interest has a molecular weight of approximately 120 kDa what can be seen on SDS-PAGE shown in figure 13. The activity of pooled fractions was measured and it was calculated that the yield of this purification step was 37.4% and the purification factor was 3.1.

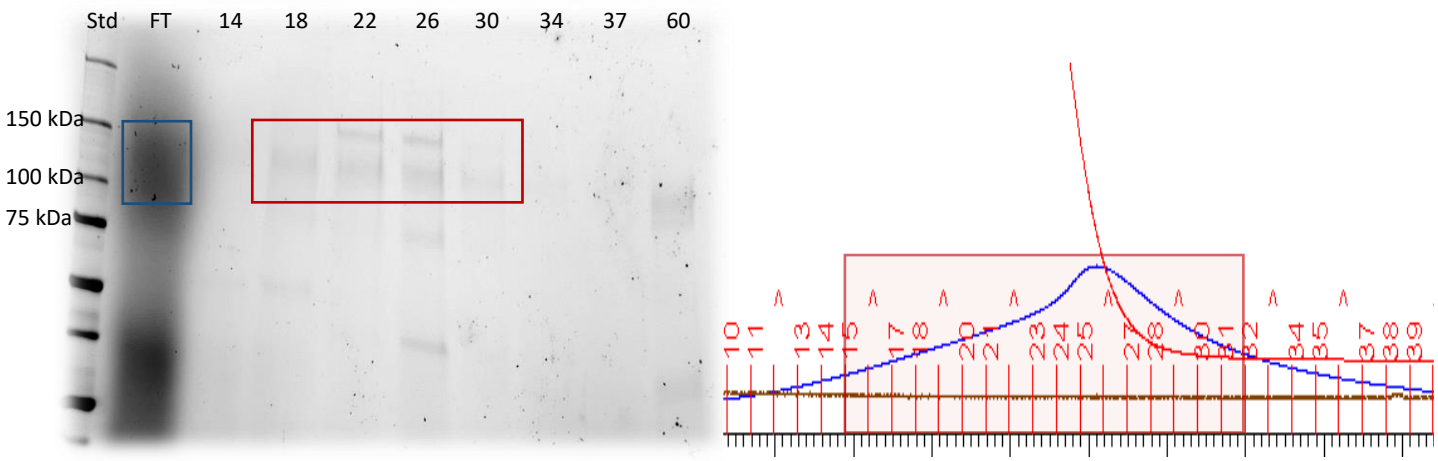

Figure 13. Results of the hydrophobic interaction chromatography purification step for *Nc*IIA_*Gc*GDH

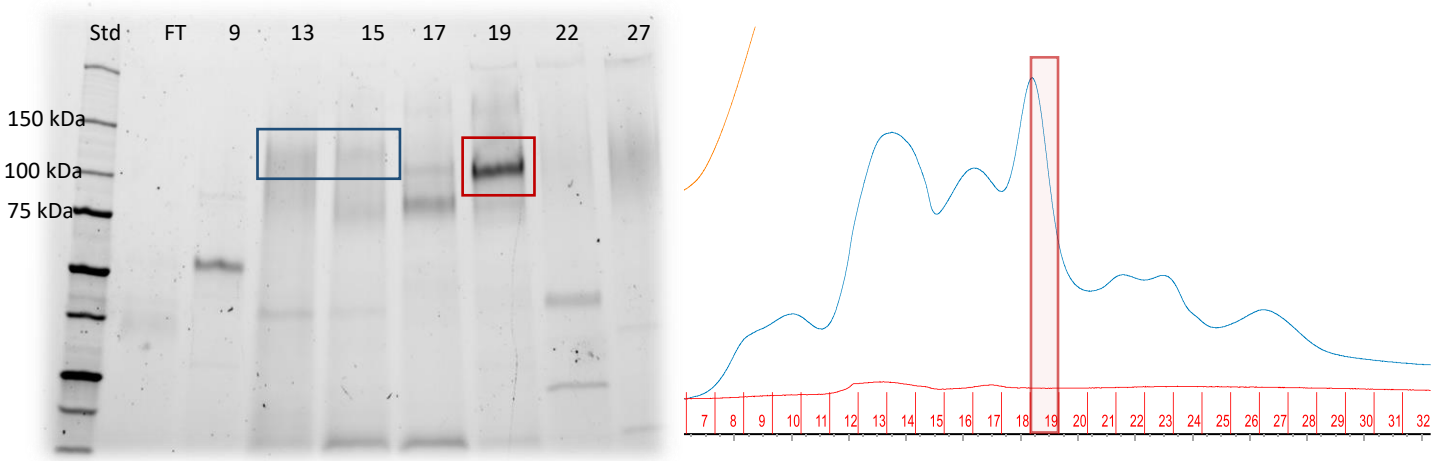

Figure 14. Results of the anion exchange chromatography purification step for *Nc*IIA_*Gc*GDH

Pooled fractions were subjected to the second step of the purification and the results are shown in figure 14. It was thought that the enzyme of interest was in fraction 19 (red rectangle) which was diafiltrated in order to remove sodium chloride and measure activity. It was determined that the specific activity in that sample was only 5 U mg^{-1} which was less than in the supernatant. Nevertheless, spectrum, which is shown in figure 15, was measured and it can be seen that there is no peak at 420 nm which is characteristic for cytochrome domain with heme. Additionally, there was no detectible cytochrome c activity.

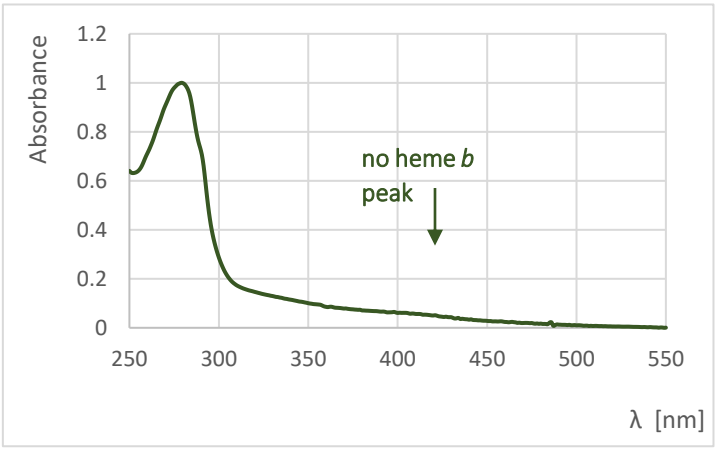

Figure 15. Spectrum of diafiltrated fraction 19

Later on, it was thought that the protein of interest might be a weak band in fractions 13 and 15 on SDS-PAGE shown in figure 14 (blue rectangle), but further actions were not made due to low protein mass.

4.3. DEFLAVINATION OF *An*GOX and *Gc*GDH

As previously mentioned, the second part of the thesis is about deflavination and reconstitution of two different GMC oxidoreductases – glucose oxidase from *Aspergillus niger* and glucose dehydrogenase from *Glomerella cingulata.* By other members in the lab, *An*GOX and *Gc*GDH were produced in *Pichia pastoris* X-33 and then purified. It was attempted deflavination of those two enzymes according to the procedure by Swoboda described in paragraph 3.7. Due to the high level of glycosylation in *Pichia pastoris* X-33, it was impossible to precipitate protein because it was too stable although harsh conditions were applied. Referring to the Swoboda deflavination method, for precipitation of proteins saturated ammonium sulfate solution with pH 1.4 is used. It was decided to try deglycosylation of proteins prior to the deflavination procedure. On one hand, that resolved the problem for deflavination of *Gc*GDH for which then dissociation constant was determined. On the other hand, deflavinated *An*GOX (apo-form) was not stable enough to restore its structure when mixed with FAD. In other words, coarse conditions of deflavination denaturated protein to the point that it was impossible to fully restore original, active conformation. At this point, it was resolved to try deflavination of *An*GOX produced in *Aspergillus niger* which has a lower amount of glycosylation than the one produced in *Pichia pastoris* X-33. That was successful. After production of apo-forms of both enzymes (*An*GOX and deglycosylated *Gc*GDH), they were mixed with FAD solution to make different concentrations as aforementioned in paragraph 3.8.8. The mixture was left overnight to restore structure and then activity was measured with ABTS assay for *An*GOX and DCIP assay for *Gc*GDH. The curves used for the determination of dissociation constant are shown in figure 16 and the results are accounted in paragraph 4.6.

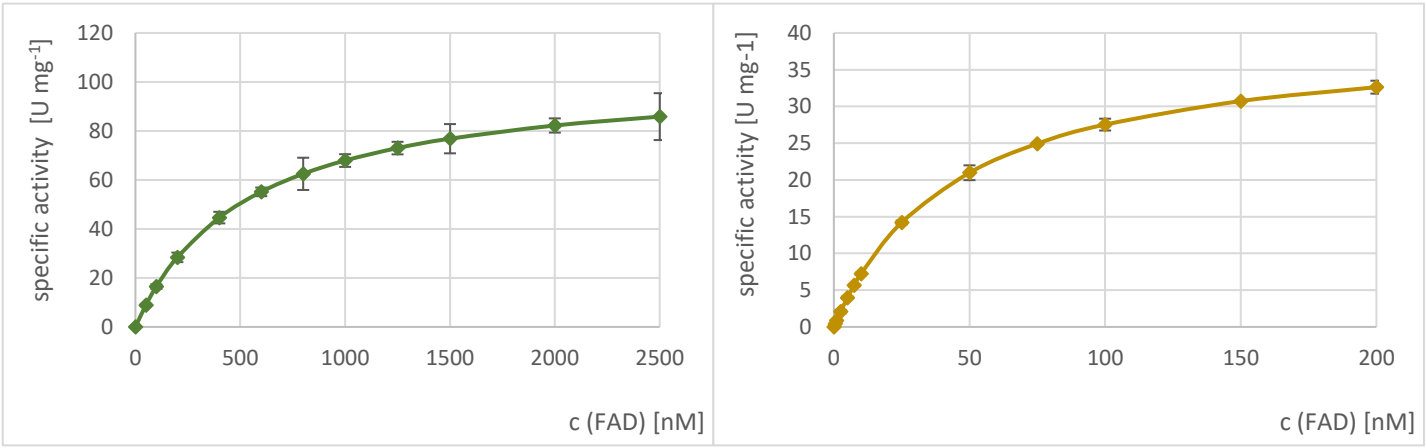

Figure 16. Curves used for determination of dissociation constants of *An*GOX from Sigma (left) and *Gc*GDH from *Pichia pastoris* X-33 (right)

In order to make future deflavination process easier, it was decided to produce both flavoenzymes in *Pichia pastoris* GlycoSwitch SuperMan5. As mentioned earlier, that strain of *Pichia* has genetically modified glycosylation pathways in order to produce more human-like glycoproteins which are less glycosylated.

4.4. PRODUCTION OF GMC OXIDOREDUCTASES IN *Pichia pastoris* SuperMan5

Transformed *Escherichia coli* NEB 5-alpha cells with plasmids pPICZ A with genes of interest were already prepared by other members of the laboratory. Therefore, the first step was the cultivation of transformed *E. coli* cells and extracting plasmids which were then linearized using restriction enzyme PmeI. Linearized plasmids were used for the transformation of previously prepared electrocompetent *Pichia pastoris* GlycoSwitch SuperMan5 cells. Screening of enzyme producers was carried out in a 96 deep well plate according to the procedure described in paragraph 3.3.8. The colony that expressed enzyme with the highest volumetric activity was used to produce larger amounts of enzyme in shaking flasks as it is shown in figure 17.

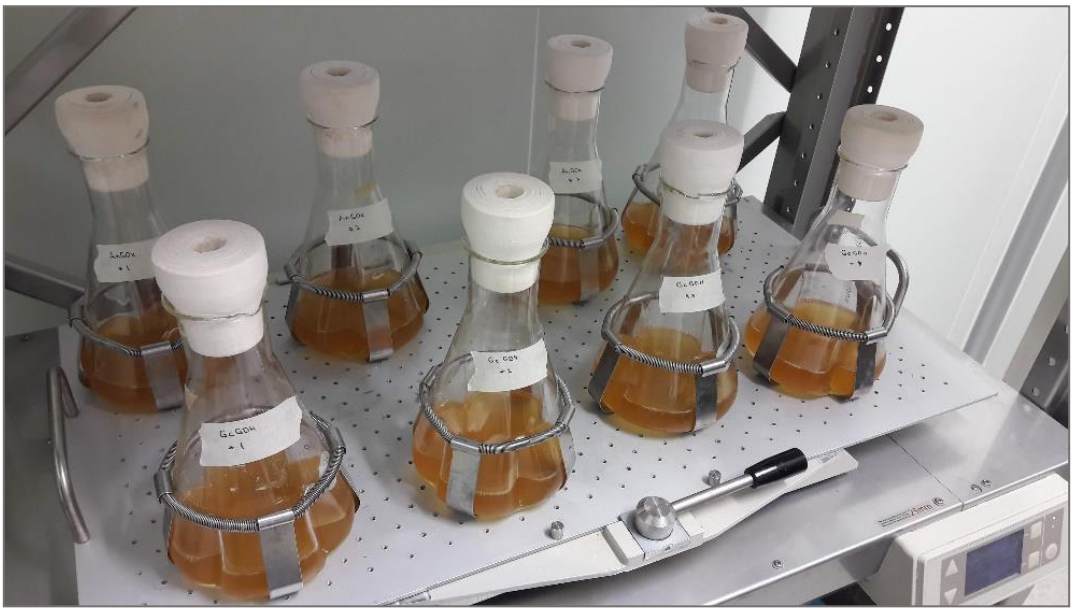

Figure 17. Production of *An*GOX and *Gc*GDH in shaking flasks

25 mL of the pre-culture produced in the YPD medium was used to inoculate 250 mL of BMY medium. Since SuperMan5 is Mut^+, there was no glycerol phase to generate biomass which was in this case produced during the methanol phase. Growth curves for both productions are shown in figure 18.

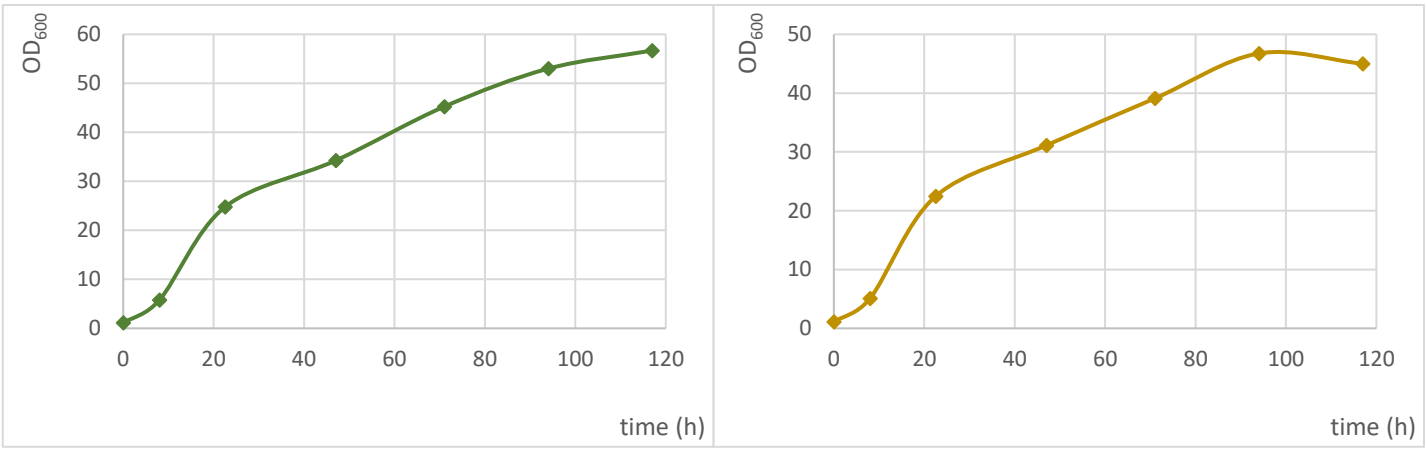

Figure 18. Growth curves of SuperMan5 cells producing *An*GOX (left) and *Gc*GDH (right)

As a carbon source and inducer, methanol was added twice a day – in the morning to a final concentration of 0.5% and in the evening to a final concentration of 0.8% in the cultures. Each day samples were taken to check protein concentration and activity. To decide when harvesting should take place, changes in specific activity were observed as shown in figure 19. When specific activity stopped increasing it was decided that expression should be stopped and at that time fermented broth was centrifuged in order to remove cells.

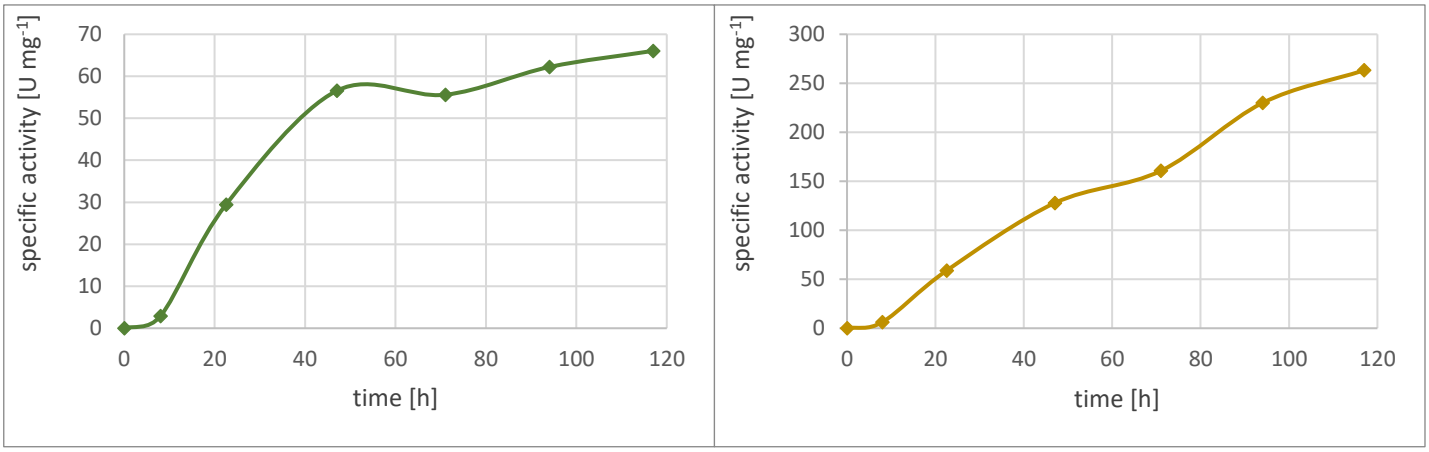

Figure 19. Changes in specific activity during production of *An*GOX (left) and *Gc*GDH (right)

The volume of supernatant containing *An*GOX was 815 mL with the protein concentration of 0.2533 mg mL^{-1} and the volumetric activity of 13.65 U mL^{-1}. On the other hand, 1,030 mL of the supernatant containing *Gc*GDH was obtained with the protein concentration of 0.1653 mg mL^{-1} and the volumetric activity of 43.79 U mL^{-1}.

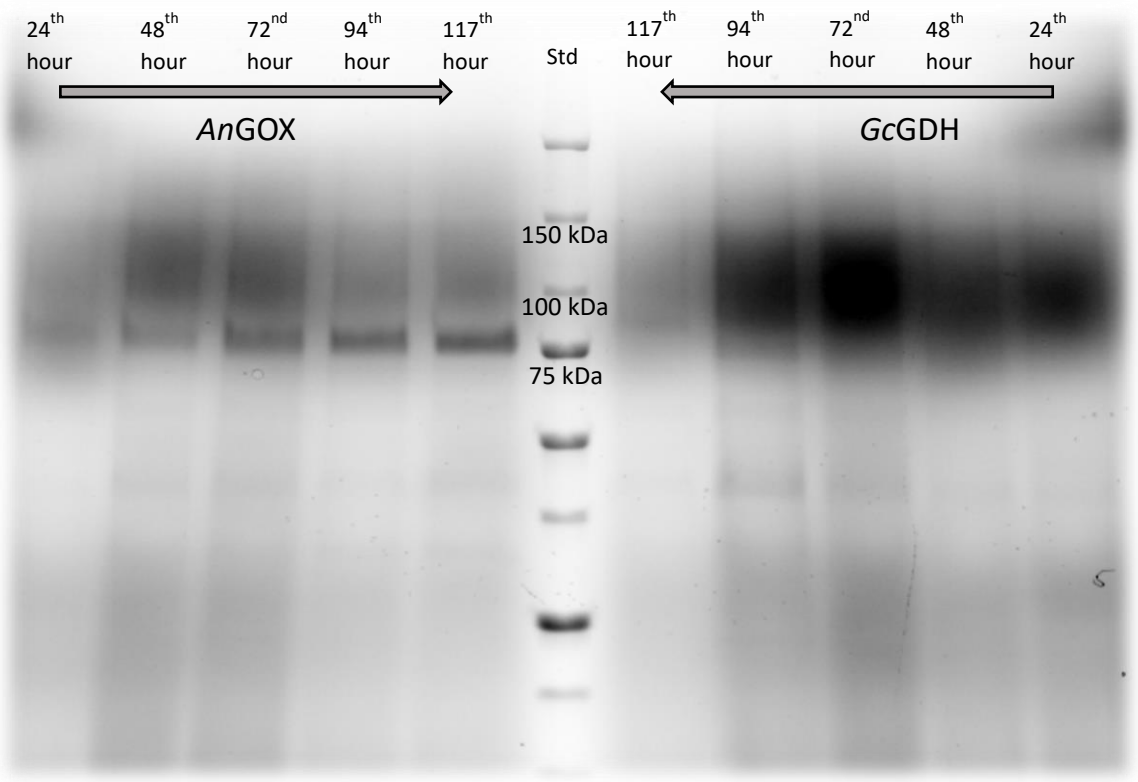

Figure 20. Samples from production of *An*GOX (left) and *Gc*GDH (right) on SDS-PAGE

In figure 20 is shown SDS-PAGE with samples from the production of *An*GOX and *Gc*GDH. There was a decrease in band strength in a sample from the last day of production of *Gc*GDH what suggests that expression lasted too long, but on the other hand the specific activity was still growing as noted in figure 19. According to the left part of SDS-PAGE, two different proteins were produced or one in two different glycoforms what can be deduced from two separated bands on the gel. The molecular weight of *An*GOX, calculated from its amino acid sequence, is 64.053 kDa and bands on the gel are at approximately 80 kDa and at 100 kDa. On the other hand, the molecular weight of *Gc*GDH, calculated from its amino acid sequence, is 62.292 kDa and the band on the gel is broad and at approximately 100 kDa.

4.5. PURIFICATION OF GMC OXIDOREDUCTASES

Both enzymes were his-tagged so, naturally, the first step of purification was immobilized metal affinity chromatography (IMAC). Before starting the purification to both supernatants imidazole was added to a concentration of 10 mM and NaCl to 0.5 M.

4.5.1. Purification of *An*GOX

After the IMAC step of purification, selected fractions that absorbed at 280 nm, together with the flow-through, were checked on SDS-PAGE and developed gel is shown in figure 21. Fractions that contained 2 bands (red rectangle) were combined in pool 1 and fractions that contained 1 band (blue rectangle) were combined in pool 2. Both pools were diafiltrated in order to remove imidazole and NaCl and then concentrated. The full purification scheme is shown in table 11.

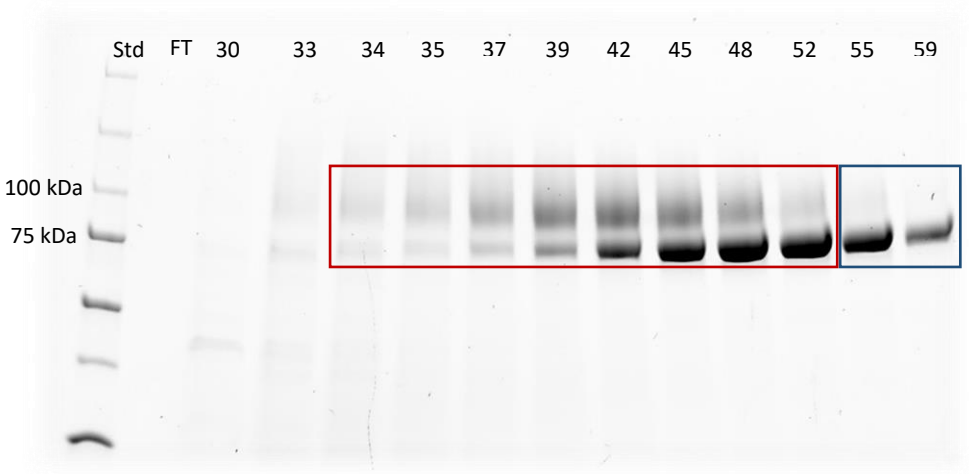

Figure 21. SDS-PAGE after IMAC step of *An*GOX purification – pool 1 (red rectangle) and pool 2 (blue rectangle)

It was assumed that both bands are the same protein (*An*GOX) but in two different glycoforms. That assumption was supported by the fact that pool 1 and pool 2 have almost the same specific activity as one can see in table 11. Afterward, deglycosylation of both pools was carried out and then samples were checked with SDS-PAGE. Both samples had the same molecular weight at around 65 kDa what corresponds to determined molecular weight from the amino acid sequence.

Table 11. Purification scheme of *An*GOX

Step		Total protein according to Bradford [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
Supernatant		206.44	11,130.1	53.0	1	100
Pool 1	IMAC	49.66	6,212.3	125.1	2.32	55.82
	ultrafiltration	46.75	5,325.5	113.9	2.11	47.85
Pool 2	IMAC	16.91	1,815.1	107.3	1.99	16.31
	ultrafiltration	11.35	1,155.1	101.8	1.89	10.38

Nevertheless, the pool 1 was subjected to the second step of purification – hydrophobic interaction chromatography in order to separate two different glycoforms. SDS-PAGE of the fractions and the flow-through is shown in figure 22. It can be observed that a lot of protein didn't bind to the column and that fractions from elution mostly contain both glycoforms. It was decided to combine all the fractions and diafiltrate them to remove ammonium sulfate. In addition, flow-through was diafiltrated as well and kept because it contained the enzyme of interest.

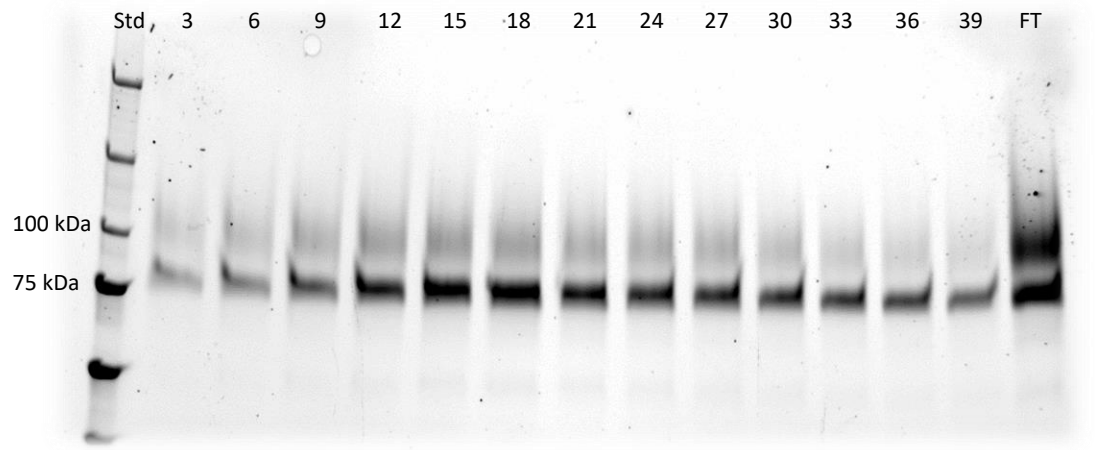

Figure 22. SDS-PAGE after HIC step of *An*GOX pool 1

The purification scheme of this step is shown in table 12. It is important to say that protein concentrations were determined by absorption at 280 nm since the samples were pure. The molar extinction coefficient for recalculating absorption at 280 nm to concentration was determined from the amino acid sequence of the protein. After diafiltration, the specific activity of the flow-through was determined as well and the value was 87.5 U mg^{-1}. The specific activity of diafiltrated fractions was 89.4 U mg^{-1} which is very similar to specific activity of the flow-

through. It can be concluded that two glycoforms cannot be separated by employed HIC column and applied conditions of purification step.

Table 12. Purification scheme of *An*GOX pool 1

Step	Total protein according to UV_{280} [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
***An*GOX pool 1**	32.5	3,170.0	97.5	1	100
Diafiltrated fractions	16.4	1,466.0	89.4	0.92	46.25

4.5.2. Purification of *Gc*GDH

After the IMAC step of purification, selected fractions that absorbed at 280 nm, together with the flow-through, were checked on SDS-PAGE and developed gel is shown in figure 23. Although it seems that there are 2 different glycoforms of *Gc*GDH in the fractions, all fractions that contained both glycoforms (red rectangle) were pooled together and then diafiltrated in order to remove imidazole and sodium chloride.

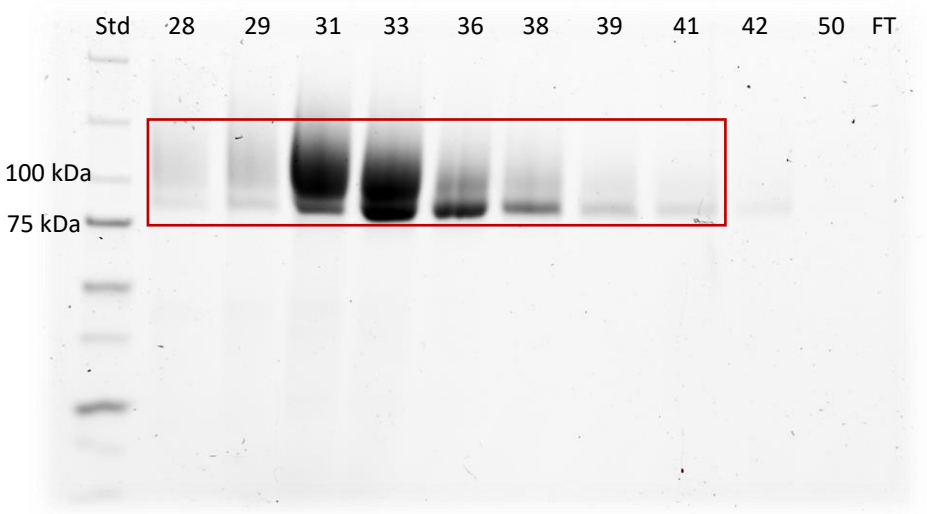

Figure 23. SDS-PAGE after IMAC step of *Gc*GDH

The yield of the purification was only 86.12% because some of the protein precipitated during diafiltration due to imidazole in the sample. The full purification scheme is shown in table 13.

Table 13. Purification scheme of *Gc*GDH

Step	Total protein according to Bradford [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Purification factor	Yield [%]
Supernatant	170.26	45,098.6	264.9	1	100
IMAC	27.05	44,509.0	1,645.4	6.21	98.69
Ultrafiltration	26.58	38,839.1	1,461.2	5.52	86.12

4.6. COMPARISON OF *An*GOX AND *Gc*GDH PRODUCED IN DIFFERENT EXPRESSION SYSTEMS

In figure 24 can be seen that both enzymes produced in *Pichia pastoris* X-33 have a molecular weight of around 100 kDa, although their nominal molecular weights are 64.053 kDa for *An*GOX and 62.292 kDa for *Gc*GDH as mentioned earlier. *An*GOX from *A. niger* has the molecular weight of approximately 80 kDa and it was noted above that *An*GOX produced in X-33 is too glycosylated to successfully deflavinate enzyme by Swoboda method, but one from *A. niger* can be deflavinated by that sane method. Hence, the goal was to produce *An*GOX in genetically modified *P. pastoris* GlycoSwitch SuperMan5 in order to obtain less glycosylated enzyme. One can see that SuperMan5 produced two glycoforms of *An*GOX of which one has the same molecular weight as one produced in X-33 and the other one has the same molecular weight as one produced in *A. niger*. In order to confirm that both bands are the same enzyme, but in different glycoforms, deglycosylation of the enzyme solution was carried out and one can see on SDS-PAGE that only one band appeared with a molecular weight of approximately 65 kDa what corresponds to *An*GOX. A similar case was with *Gc*GDH as well. Two different glycoforms of the same enzyme were produced and with deglycosylation was confirmed that indeed it is the same protein.

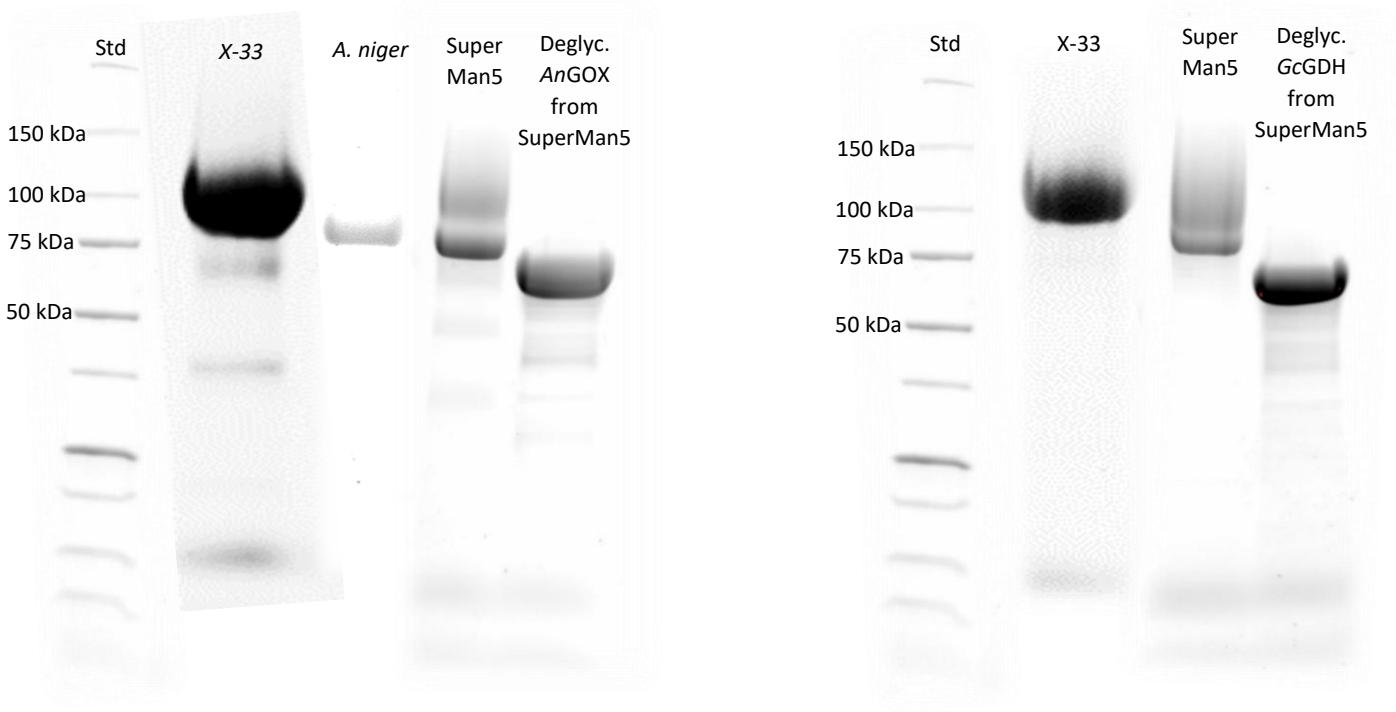

Figure 24. Different glycoforms of *An*GOX (left) and *Gc*GDH (right)

Enzymes produced in SuperMan5 were deflavinated successfully by the Swoboda method and then produced apo-form was mixed with FAD solutions in order to determine the dissociation constant as described in paragraph 3.8.8. Obtained data from activity measurements are shown in figure 25.

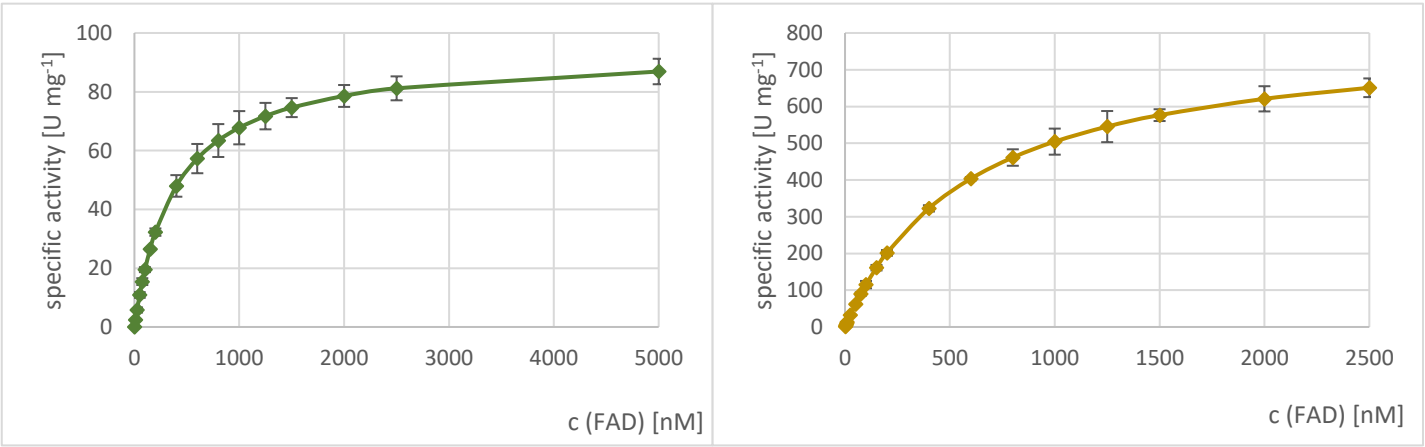

Figure 25. Curves used for determination of dissociation constants of *An*GOX (left) and *Gc*GDH (right) from *Pichia pastoris* SuperMan5

The spectra of the native enzyme, deflavinated enzyme and then reconstituted enzyme was measured for both *An*GOX and *Gc*GDH produced in *Pichia pastoris* SuperMan5. Results are shown in figure 26 and it can be seen that deflavination procedure by Swoboda was successful because there is no characteristic peak for FAD at 375 and 450 nm. Reconstituted enzymes were acquired by mixing prepared apo-form with FAD solution and then diafiltrated to remove the excess of FAD. It can be observed that reconstituted enzymes reacquired FAD and peaks at characteristic values.

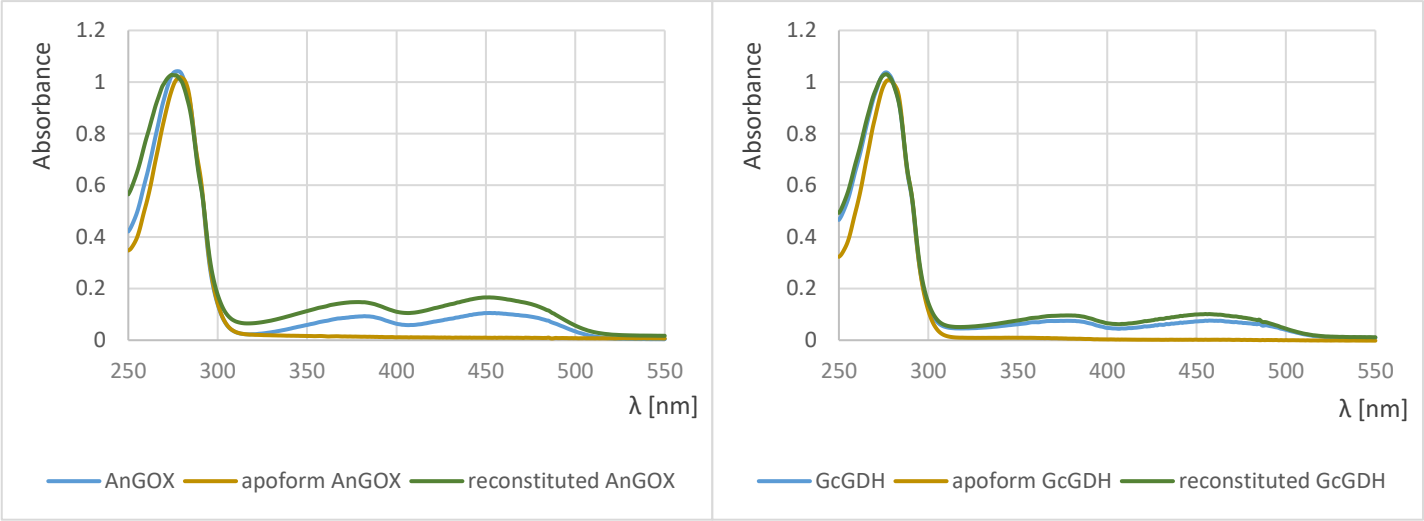

Figure 26. Spectra of native, deflavinated and reconstituted *An*GOX (left) and *Gc*GDH (right) produced in SuperMan5

Efficacy of *An*GOX deflavination and reconstitution is shown in table 14. *An*GOX produced in *A. niger* has a higher specific activity than the one produced in *P. pastoris* SuperMan5. The same deflavination procedure by Swoboda was applied to both enzymes and *An*GOX from *A. niger* had a residual activity of only 4.75% and *An*GOX produced in SuperMan5 had a residual activity of 24.88%. That means that enzyme from SuperMan5 is more stable and that harsh deflavination conditions did not affect the structure of the enzyme as much as the enzyme from *A. niger*. That is the reason why reconstituted *An*GOX from *A. niger* reacquired only 69.45% of native activity, and reconstituted *An*GOX produced in SuperMan5 had higher activity than native enzyme for 8.3%.

Table 14. Deflavination efficacy of different *An*GOX glycoforms

***An*GOX**	**produced in…**	**specific activity [U mg^{-1}]**	**% of native activity**
native	*A. niger*	150.01	100.00
apo-form		7.12	4.75
reconstituted		104.18	69.45
native	*P. pastoris* SuperMan5	86.34	100.00
apo-form		21.48	24.88
reconstituted		93.50	108.29

The efficacy of *Gc*GDH deflavination and reconstitution is shown in table 15. Since *Gc*GDH produced in *P. pastoris* X-33 was too stable to deflavinate it, deglycosylation was carried out which reduced the activity of the enzyme for 5%. The deglycosylated enzyme was successfully deflavinated and only 0.45% of residual activity remained. Harsh conditions of deflavination were too extreme for enzyme without any protection from sugars on protein surface so only 10.4% activity was restored by reconstitution. On the other hand, *Gc*GDH produced in SuperMan5 had native activity almost double than the activity of *Gc*GDH produced in X-33. Apo-form GcGDH from SuperMan5 had only 3% residual activity and by reconstitution, all the activity was restored and even exceeded native activity for 12.2%.

Table 15. Deflavination efficacy of different *Gc*GDH glycoforms

***Gc*GDH**	**produced in…**	**specific activity [U mg^{-1}]**	**% of native activity**
native	*P. pastoris* X-33	385.61	100.00
deglycosylated		366.20	94.97
apo-form		1.74	0.45
reconstituted		40.03	10.38
native	*P. pastoris* SuperMan5	719.51	100.00
apo-form		21.64	3.01
reconstituted		807.38	112.21

Results of K_d determination tests are shown in table 16. Dissociation constant values are shown as well as the maximum specific activity of reconstituted enzymes. *An*GOX from *A. niger* has similar maximum specific activity as *An*GOX produced in SuperMan5, but dissociation constants are different. *An*GOX from *A. niger* has a dissociation constant of 533.72 nM and *An*GOX from SuperMan5 has a K_d value of 379.49 nM which means that enzyme from SuperMan5 has a higher affinity for FAD. It should be taken into the consideration the fact that during deflavination procedure structure of *An*GOX from *A. niger* was affected more than the structure of *An*GOX produced in SuperMan5. On the other hand, *Gc*GDH produced in *P. pastoris* X-33 has much lower both maximum specific activity and dissociation constant than the *Gc*GDH produced in SuperMan5, but the reason for that lies in the fact that deglycosylated enzyme from X-33 was denaturated during deflavination to a high degree. The dissociation constant for *Gc*GDH produced in SuperMan5 is 600.78 nM.

Table 16. Results of K_d determination

enzyme	**produced in…**	**maximum specific activity [U mg^{-1}]**	**dissociation constant [nM]**
***An*GOX**	*A. niger*	104.18 ± 5.76	533.72 ± 87.11
***Gc*GDH**	*P. pastoris* X-33	40.03 ± 0.91	45.41 ± 2.95
***An*GOX**	*P. pastoris* SuperMan 5	93.50 ± 2.07	379.49 ± 33.93
***Gc*GDH**	*P. pastoris* SuperMan 5	807.38 ± 41.78	600.78 ± 72.64

As a part of this thesis method for deflavination of flavoenzymes was successfully established. A successful method for deflavination and subsequent reconstitution of flavoenzymes is crucial for follow-up experiments in order to improve their binding of FAD cofactor and, therefore, increase their stability and activity.

In addition, one of the aims of this thesis was the production of chimeric enzymes, capable of DET, and optimization of their purification. During this research, valuable data were obtained and conclusions made, which can be utilized in future experiments in order to develop new third-generation biosensors.

5. CONCLUSIONS

1. Chimeric enzyme *Nc*IIA_*Af*GDH was successfully produced in shaking flasks, but *Nc*IIA_*Gc*GDH was not due to methanol toxicity. Expression of chimeric enzymes must be monitored and controlled in order to successfully produce enzymes since methanol is used as an inducer which is toxic for *Pichia* cells in the concentrations higher than 2% (v/v).

2. Addition of hemin, which is a precursor of heme, in medium should be considered in order to increase the yield of active chimeric enzymes what was demonstrated to be successful supplement in the production of recombinant heme peroxidase in *Pichia pastoris*.

3. Further optimization of the purification of chimeric enzymes is required. For example, higher ammonium sulfate concentration in supernatant should be set up in order to achieve better protein binding to the Phenyl Sepharose column.

4. Flavoenzymes produced in *Pichia pastoris* X-33 are too stable to be deflavinated by the Swoboda method due to high glycosylation level. On the other hand, deglycosylated enzymes are too “fragile” and during deflavination procedure are mostly denaturated to the point that it was impossible to restore their active structure.

5. Both *An*GOX and *Gc*GDH were successfully produced in *Pichia pastoris* GlycoSwitch SuperMan5 and purified. Enzymes were expressed in two different glycoforms of which one has the same molecular weight as one produced in *Pichia pastoris* X-33 and the other one is less glycosylated.

6. Enzymes produced in *Pichia pastoris* GlycoSwitch SuperMan5 were successfully deflavinated by the Swoboda method and then reconstituted when mixed with FAD. Dissociation constants were determined and it turned out that *An*GOX has a higher affinity for FAD than *Gc*GDH.

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APPENDIX

List of abbreviations	
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AEX	Anion exchange chromatography
***An*GOX**	Glucose oxidase from *Aspergillus niger*
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BMY	Buffered complex medium
BYPD	Buffered yeast extract peptone dextrose
CDH	Cellobiose dehydrogenase
CBM	Cellobiose binding molecule
DCIP	2,6-dichloroindophenol
DET	Direct electron transfer
FAD	Flavin adenine dinucleotide
***Gc*GDH**	Glucose dehydrogenase from *Glomerella cingulata*
GDH	Glucose dehydrogenase
GMC	Glucose-methanol-choline
GOX	Glucose oxidase
HIC	Hydrophobic interaction chromatography
HQ-H_2O	High quality purified water
IET	Interdomain electron transfer
IMAC	Immobilized metal affinity chromatography
LB	Luria broth
LS-LB	Low salt Luria broth
MET	Mediated electron transfer
MWCO	Molecular weight cut-off
***Nc*IIA_*Af*GDH**	Chimeric enzyme CDH IIA from *Neurospora crassa* which has replaced dehydrogenase domain with glucose dehydrogenase from *Aspergillus flavus*
***Nc*IIA_*Gc*GDH**	Chimeric enzyme CDH IIA from *Neurospora crassa* which has replaced dehydrogenase domain with glucose dehydrogenase from *Glomerella cingulata*
NEB	New England Biolabs
OD_{600}	Optical density at 600 nm
RO-H_2O	Reverse osmosis water
SDS_PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sh ble	*Streptoalloteichus hindustanus* bleomycin gene
SOC	Super optimal broth with catabolite repression
TMBZ	3,3’,5,5’-tetramethylbenzidine
UV-VIS	Ultraviolet-visible spectroscopy
YPD	Yeast extract peptone dextrose

STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

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Karlo Koraj