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GALAB Laboratories

Master Thesis
Pharmaceutical Biotechnologie
Master of Science

Development and Optimization of Purification Processes and Protein Stabilization Methods for Enzymes Applied for Biocatalytical N-Acetylneuraminic Acid Synthesis

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Abstract

The production of N-acetylneuraminic acid with a sequential biocatalysis using two enzymes has a high potential for high yield synthesis. However, the efficiency of enzymatic catalysis depends on the purity, yield and activity of the enzymes. This thesis focuses on the optimization of the purification of his-tagged N-acetylneuraminic acid lyase (Neu5AC lyase) and N-acetyl-D-glucosamine 2-epimerase (GlcNAc 2-epimerase) using immobilized metal affinity chromatography. Additionally, a method for the stabilization of both enzymes during lyophilization was developed. Binding experiments with pure enzymes reveals higher binding capacities on column materials than stated by the manufacturer on Ni^{2+} (up to 380% higher) and Co^{2+} (33 to 400% higher) columns. Co^{2+} columns showed 30 to 50% higher binding capacities compared to Ni^{2+} resins. Reduced protein binding arises from metal ion binding properties. Metal ion transfer due to high binding affinity coordination sites on the protein is a main cause. A comparison of different column materials and buffers for GlcNAc 2-epimerase purification showed reduced yields when using Tris buffers. Samples disrupted and purified with Tris generated 36 and 13% lower yields than sodium phosphate buffered samples. Protein elution is promoted by competitive binding of amines in Tris buffers which leads to a reduced enzyme yield. The stability of purified GlcNAc 2-epimerase in solution could be maintained over a period of 49 days by applying sterile storage and handling conditions. Additionally, the time of the filtration process could be reduced by 50% by changing the filter system from cellulose acetate membrane filters to polyethersulfone filter capsules. Studies on maintaining enzyme stability during lyophilization showed enzyme-specific results. Mannose proved to be an effective lyoprotectant for GlcNAc 2-epimerase at a concentration of 20 mM in 20 mM Tris buffer. Due to the structural similarity of mannose and the substrate of GlcNAc 2-epimerase, the native form is stabilized by binding the active site. All Neu5Ac lyase samples showed low recovery after lyophilization in Tris buffer. However, the absence of buffer salts prevents pH shifts arising from freeze concentration in eutectic mixtures. Thus, high recoveries could be achieved by freeze-drying Neu5Ac lyase in distilled water.

Contents

List of Abbreviations and Symbols

ΔG	Change in Gibbs free energy
ΔG°	Standard free energy change
APS	Ammoniumperoxide Sulfate
ATP	Adenosine Triphosphate
AU	Absorption Units
BSA	Bovine Serum Albumin
CM-ASP	Carboxymethyl Aspartarte
CV	Column Volume
Da	Dalton
E.coli	*Escherichia coli*
EFA	Effective Filtration Area
EL	Elution Buffer
EQ	Equilibration Buffer
GlcNAc	N-Acetyl-D-Glucosamine
His_6	Recombinant polyhistidine tag with six residues
HCP	Host Cell Proteins
IMAC	Immobilized Metal Ion Affinity Chromatography
ManNAc	N-Acety-D-Mannosamine
ManDH	NAacetyl-D-Mannosamine Dehydrogenase
MIT	Metal Ion Transfer
MWCO	Molecular Weight Cut-Off
NAD	Nicotinamide Adenine Dinucleotide
Neu5Ac	N-Acetylneuraminic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PES	Polyethersulfone
pH	Negative of the base 10 logarithm of the 'proton concentration'
pK_a	Negative of the base 10 logarithm of the acid constant
PMSF	Phenylmethylsulfonyl Fluoride
RT	Room Temperature
RPM	Rounds Per Minute
SDS	Sodium Dodecyl Sulfate
TBA	Thiobarbituric Acid

T_c	Collapse Temperature
TEMED	Tetramethylethylediamine
T'_g	Glass transition temperature of the maximal concentrated solute
T_m	Melting Temperature
U	Unit of enzyme activity that converts one mole of substrate per minute
WCW	Wet Cell Weight

1 Introduction and Objectives

This section gives an overview of the subject of this thesis. In addition, the objectives for answering the underlying challenges are presented.

1.1 Introduction

N-acetylneuraminic acid (Neu5Ac), one of the most common sialic acids, is present in a variety of organisms as a terminal molecule of glycoconjugates. The role as an important component of receptor interactions with hormones, viruses and the regulation of the immune system makes this molecule to a highly interesting target structure for many research studies. Selective biocatalytical synthesis offers the possibility to produce Neu5Ac with high product yield and a reduced consumption of chemicals. However, biocatalysis reveals also some challenges like the limit through the equilibrium position of the reaction or low enzyme stability. A variety of processes has been described which solves some of the issues of biocatalysis. Mahmoudian et al. used immobilized Neu5Ac lyase and base catalysis of ManNAc for Neu5Ac production, but inhibition of lyase required a large excess of pyruvate. In 1995, Kragl et al. increased the yield of Neu5Ac with the use of a membrane reactor with Neu5Ac lyase and base catalysed ManNAc synthesis. By the development of a two-enzyme system, Maru et al. was able to produce high amounts of Neu5Ac using GlcNAc 2-epimerase for the synthesis of ManNac in combination with a Neu5Ac lyase converting ManNac with pyruvate to Neu5Ac.
With the goal of Neu5Ac production, the BioPharma department of GALAB Laboratories also works on two-enzyme biocatalysis using recombinantly produced Neu5Ac lyase and GlcNAc 2-epimerase. In order to obtain high product yields, the purity, quantity and stability of the two enzymes play a major role.

1.2 Objectives

The aim of this thesis is to improve the purification of the two target enzymes GlcNAc 2-epimerase and Neu5Ac lyase in terms of efficiency, yield and purity and to establish a lyophilization method of both enzymes. There are many different factors influencing the quality and quantity of the purification process. Important factors are the choice of chromatography material, conditions of the purification and the selected enzyme preparation. The questions which arise are: (1) Can a higher purity and yield of target enzymes be achieved by using other chromatography column materials and buffers? (2) How does the stability of these enzymes depend on the chromatographic conditions? (3) Which additives in which concentration achieve a high recovery after lyophilization of the enzymes? (4) How can time consuming purification steps be reduced? To answer these questions, the following objectives were defined:

1. Increase purity and storage stability of GlcNac 2-epimerase by direct comparison of different chromatography materials and buffer types. By determination of product yield, purity and enzyme activity, a suitable purification strategy is selected.

2. Comparison of two different chromatography column types to determine binding capacity of both enzymes and column material life time. Purified His-tagged enzymes are applied on different columns. The binding and elution of enzymes are repeated to observe a potential reduction in binding capacity.

3. Evaluation of different excipients and solvents for lyophilization to achieve high enzymatic recovery. Based on various stress factors during freeze-drying, additives are selected that provide stabilisation in all phases.

4. Implementation of filter capsules as substitute for cellulose filters to reduce filtration time. In order to exclude product adsorption induced by the filter material, enzyme solutions with GlcNAc 2-epimerase and Neu5Ac lyase are filtered and quantified thereafter.

2 Theoretical Background

The following sections describe the scientifically relevant backgrounds for sialic acids and the production of the most frequently occurring derivative N-acetylneuraminic acid. It also addresses the scientific principles and methods applied in this thesis for the purification and stabilization of the enzymes used for its production.

2.1 Sialic Acids

The group of sialic acids belongs to the family of monosaccharides and is typically found as terminal group on oligosaccharide chains on glycoproteins and glycolipids [Varki and Schauer, 2009]. Their structure is different compared to other monosaccharides with the presence of a typically ionized carboxylate group at C-1 and different substitutions at the hydroxyl groups on C-4, C-7, C-8 and C-9. One of the most common sialic acid derivates is Neu5Ac named by the N-acetyl group positioned at C-5 [Jones and De Meo, 2018]. Due to their unique structure and terminal positions in glycoproteins, sialic acids and especially Neu5Ac are involved in many cell-cell interactions, recognition and pathogenic infection processes. Some viruses, as the influenza viruses, use Neu5Ac-specific lectins to mediate host-cell specific attachment or have Neu5Ac bound to their surface as not to be identified by the humane immune system. Due to the many biologic properties of Neu5Ac, it has become the focus of many research areas in recent years and has stimulated the development of new types of therapeutics [Maru et al., 1998]. One of the currently most frequently studied methods for the production of Neu5Ac and other derivatives is enzymatic catalysis.

2.1.1 Biocatalysis of N-Acetylneuraminic Acid

To date, Neu5Ac has been obtained by hydrolysis of colominic acid compounds of a Neu5Ac homopolymer or by extraction from natural resources such as bovine milk or eggs [Maru et al., 1998]. Biocatalysis offers the advantage that large scale production of Neu5Ac is possible and subsequent purification steps of NeuAc and its derivatives could be avoided [Bednarski, 1991].

Until now, the production of Neu5Ac trough biosynthesis was described with the catalytic reaction of Neu5Ac lyase and pyruvate from ManNAc to Neu5Ac. Due to difficulties of the large scale production of ManNAc, a method for the production of ManNAc from N-acetyl-D-glucosamine with GlcNAc 2-epimerase was developed [Maru et al., 1998; Kragl et al., 1991]. Adenosine triphosphate (ATP) is an allosteric activator and does not function as a co-substrate for Neu5Ac lyase. The whole pathway of the cataylsis of Neu5Ac by GlcNAc 2-epimerase and Neu5Ac lyase is shown in Figure 1.

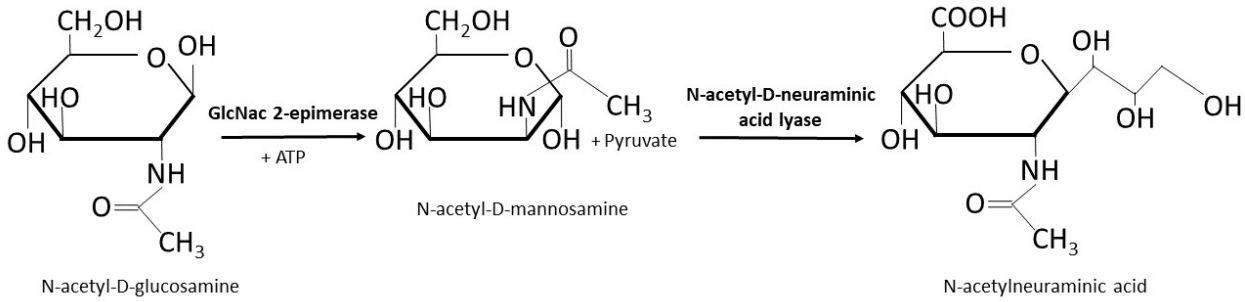

Figure 1: Pathway of biocatalysis of N-acetylneuraminic acid from N-acetyl-D-glucosamines with the two catalytic enzymes N-acetyl-D-glucosamine 2-epimerase and N-acetylneuraminic acid lyase.

In order to achieve Neu5Ac production on a large scale, methods for the efficient purification and effective stabilization of the two enzymes, which are described in chapters 2.1.2 and 2.1.3, are necessary.

2.1.2 N-Acetyl-D-Glucosamine 2-Epimerase

Epimerases belong to the subgroup of isomerases and catalyse the inversion of stereoisomers in eukaryotes as well as in prokaryotes. In the human organism, GlcNAc 2-epimerase functions as renin inhibitor by forming dimers [Kragl et al., 1991]. The GlcNAc 2-epimerase from the gram negative bacterium *Pedobacter heparinus* is a class 5 isomerase that catalyses the reaction of GlcNAc to ManNAc. By protonation and deprotonation of the C-2 atom by Arg63 and Glu314, this GlcNAc 2-epimerase is also able to catalyse the reaction from ManNAc to GlcNAc. Despite the ability of GlcNac 2-epimerase to catalyse the reaction in both directions, the equilibrium favours the production of ManNAc [Wang et al., 2016; Kragl et al., 1991]. The gene of the GlcNAc 2-epimerase used here is transferred into *E.coli* BL21 (DE3) and expressed with a (His_6)-tag at the N-terminus. As a homodimer, it has

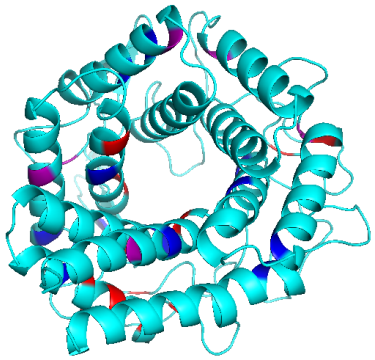

Figure 2: Predicted 3-dimensional structure of GlcNAc 2-epimerase (EC = 5.1.3.8) subunit from *Pedobacter heparinus*. With colored amino acid residues: histidine = red, tryptophane = blue, cysteine = purple. (3-dimensional structure predicted by Phyre2 (URL: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Image created and edited with PyMol based on the amino acid sequence)

two subunits with 46.8 kDa each and thus a total molecular weight of 93.6 kDa. Studies of the enzyme by Wang et al. showed that GlcNAc 2-epimerase has the reaction optimum at 37 °C and is stable in a range of pH 7 - 10 [Wang et al., 2016]. It shows a high stability at ambient temperature.
Figure 2 shows a prediction of the three-dimensional structure of a subunit of the homodimer. Due to lack of structural analysis of the enzyme, no accurate 3D models are available. This prediction was created using the AA sequence by Phyre2 (summary in appendix Figure B5) based on the research of *Kelley et al.* from 2015 [Kelley et al., 2015]. The predicted structure was illustrated using the open source program PyMol (listed in section 3.1.4).

2.1.3 N-Acetylneuraminic Acid Lyase

Aldolases belong to enzyme class 1 of lyases and are present in mammalian tissue as well as in many bacteria. Aldolases catalyse the formation or cleavage of carbohydrate-carbohydrate bonds [Daniels et al., 2014]. Neu5Ac lyase (or aldolase) originates from the bacterium *E.coli* K12 and is expressed in *E.coli* BL21 (DE3) with an N-terminal (His_6)-tag. It is a homotetramer with a total molecular mass of 137.3 kDa and 34.4 kDa per subunit. As described by *Daniels et al.*, Neu5Ac lyase follows an ordered Bi-Uni aldol condensation kinetic mechanism. By enamine formation between pyruvate and Lys615, a pyruvate-Schiff base is produced. Neu5Ac-Schiff base is formed by protonation of the

aldehyde oxygen which is converted after hydrolysis into Neu5Ac [Daniels et al., 2014].

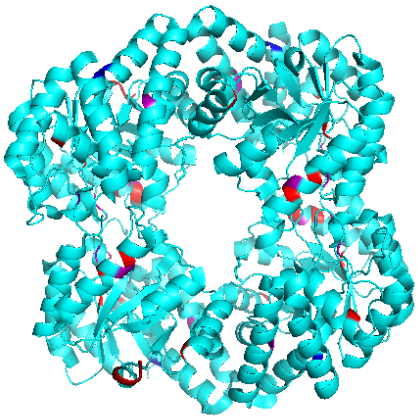

Figure 3: 3-dimensional structure of the homotetramer Neu5Ac lyase (EC = 4.1.3.3) from *Escherichia coli* K12. With colored amino acid residues: histidine = red, tryptophane = blue, cysteine = purple. (Image created and edited with PyMol based on the amino acid sequence, PDB ID: 4bwl)

2.2 IMAC

Immobilized metal affinity chromatography (IMAC) is a separation method based on different binding affinities of metal ions to soluble proteins in solution. In environments with a neutral pH, amino acids like histidine, tryptophan and cystein are able to form complexes with metal ions chelated to a solid matrix.
IMAC was first described by *Porath et al.* in 1975 as "metal chelat affinity chromatography" [Porath et al., 1975]. Over the years, IMAC became an important topic for many researches and plenty of scientists have developed new materials and optimized methods. Today, IMAC is an important tool in biochemistry and is often used for the purification of a wide range of proteins or peptides. Besides the purification purposes, IMAC offers a variety of possible applications. This method is used e.g. for proteomic or kinetic approaches. Also the binding of IMAC materials to different surfaces than chromatography materials, such as surface plasmone resonance (SPR), is employed [Block et al., 2009; Kågedal, 2011].
One of the main subjects for IMAC is the purification of proteins with polyhistidine-tag encoded on the N- or C-terminal end. Due to the high binding affinity of bivalent metal ions, such as Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}, an increasing number of available histidine-residues

causes a stronger retention [Kågedal, 2011; Wong et al., 1991].
Despite the highly affine binding of proteins through specific binding of amino acid residues to metal ions, this method is influenced by a variety of factors. The structure of the chromatography column, as well as the choice of the metal ion, buffer, pH value and the molecular structure of the proteins, have a significant influence on the result.

2.2.1 IMAC Resins

The matrix of the support have a significant influence on the stability of the complex formed between the metal ion and the protein. In general, properties such as uniform particle size and shape are preferred, as these lead to easier column packing, a sufficient surface area and stability [Wong et al., 1991]. A small particle size leads to increased stability against high flow rates, but tends to block. Large particle sizes are suitable for crude cell extracts, but they have a lower flow rate resistance and a smaller surface area and consequently a reduced capacity [Bornhorst and Falke, 2000]. Those matrices are often silica gels or polymer-based. Beaded agarose is predominantly used for IMAC purposes [Kågedal, 2011]. The chelating groups for binding metal ions are often multidentate chelates and coupled via a spacer to the matrix. A common linker is imminodiactetic acid (IDA), which chelates metals on three coordinating sides [Block et al., 2009]. Also the tetradentate nitrilotriacetic acid (NTA), which was first described as Ni^{2+} chelator by *Hochuli et al.* in 1987, is frequently used [Hochuli et al., 1987]. An disadvantage of chelating metals with IDA is that the metal ion is bound weakly and leakage can occur. Leaking of metal ions leads to a decreased binding capacity and to metal ions in the eluate. Pentadentate or, also commonly used, tetradentate ligands offer stable coordinated metal ions [Bornhorst and Falke, 2000].
Within the scope of this work the TALON Superflow and Profinity IMAC materials are investigated for IMAC regarding their different characteristics..

TALON Superflow
The IMAC resin TALON is a Co^{2+}-based material for the purification of polyhistidine-tagged proteins and is a commercially available product of *Clontech Laboratories*. This resins can be used in batch mode, gravity flow and as spin columns for small and large scale purifications. The Superflow columns are specially designed for high flow

Table 2: Data of TALON Superflow resins. ([1]determination of binding capacity with His-tagged GFPuv (29 kDa), column volume = 1 ml, flow rate = 1 ml/min, 5 ml washing with HisTALON equilibration buffer pH 7.4, elution with 3 ml HisTALON elution buffer pH 7.2 [Takara Bio, 2020])

	Value
Matrix	Superflow-6 (6% cross-linked agarose)
Bead size [µm]	60 – 160
Binding Capacity[1] [mg/ml resin]	5 – 20
Metal Ion Capacity [µmol Co^{2+}/ml]	≥ 12

rates with medium pressure and are suitable for FPLC systems [Clontech Laboratories, 2019b].

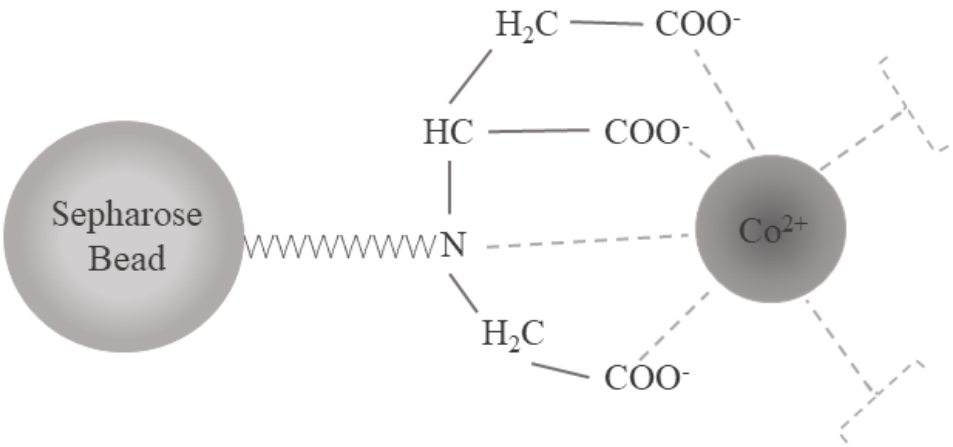

Figure 4: Schematic illustration of the TALON Superflow material structure. (Based on illustrations from *Clontech* [Clontech Laboratories, 2019a].)

Figure 4 shows a schematic illustration of the material structure. The matrix consists of 6% cross-linked agarose (Superflow-6), which is chemically stabilized to withstand biological degradation. The chelating ligand is a carboxymethyl aspartarte (CM-ASP) material which forms an octahedral binding pocket structure and bind to four of the six coordination sites [Clontech Laboratories, 2019b]. Four binding sites leads to a stable bond between the chelator and metal and therefore prevents leaking. The stability of this metal bond allows denaturing agents and the regeneration of the resin [Bornhorst and Falke, 2000].

Profinity IMAC

Profinity IMAC is also a material for the purification of His-tagged proteins on a Ni^{2+}-base, produced by *Bio-Rad Laboratories*.

The matrix is formed out of UNOsphere Beads, a patented polymer-based material (patent number: 6.423.666 [Bio-Rad Laboratories Inc., 2019]). This material is designed to withstand high flow rates and offers high loading capacities for ions. It can be used under native and denaturing conditions as well as for spin, gravity flow and chromatographic columns [Bio-Rad Laboratories Inc., 2019]. The tridentate IDA serves as chelating ligand for the binding of Ni^{2+} ions. IDA binds in three metal coordination sides and thus leaves three for the binding of histidine-residues [Kågedal, 2011]. The structure of the Profinity IMAC material is shown in Figure 5.

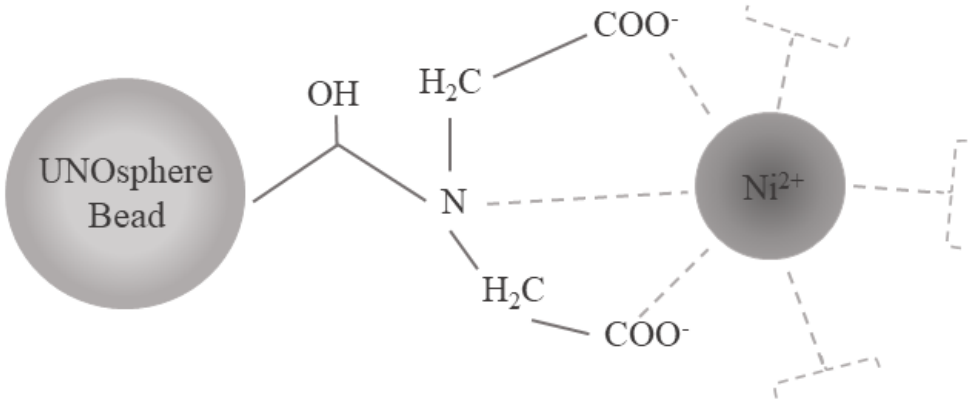

Figure 5: Schematic illustration of the Profinity IMAC material structure. (Based on illustrations from *Bio-Rad* [Bio-Rad Laboratories Inc., 2019])

Table 3: Data of Profinity IMAC resins. ([1]determination of binding capacity with 1 mg/ml protein (32 kDa), column volume = 1 ml, 1 ml/min loading, 2 ml/min washing and elution, 50 mM sodium phosphate with 0.3 M NaCl and 5 mM imidazole, pH 8 [Bio-Rad Laboratories Inc., 2019].)

	Value
Matrix	UNOsphere (polymer-based)
Bead size [µm]	45 – 90
Binding Capacity[1] [mg/ml resin]	10 – 15
Metal Ion Capacity [µmol Cu^{2+}/ml]	12 – 30

2.2.2 Adsorption and Desorption Mechanisms

Adsorption and desorption in IMAC can be modified by the choice of linker, metal ion, buffers, pH and protein structure.

Resin Properties
As previously described, the matrix type, its bead size and structure can affect the protein binding. A smaller particle pore size results in increased surface and thus offers a larger area for binding metal ions and thereby to an increased number of binding sites for proteins. Also the choice of chelating ligand influences the strength of affinity. A higher number of bound coordination sites by multidentates leads to a more stable immobilization of the metal ions to the matrix, but also to a reduction of the freely available binding sites for proteins. In general, it can be said that with a decreasing number of binding sites the number of possible bound histidines is reduced [Wong et al., 1991; Merck, 2020]. Another influencing parameter for the optimal operating of the IMAC is the choice of an appropriate metal ion. The commonly used metal ions have the following order for their affinity to histidine: $Ni^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+}$ [Block et al., 2009]. Metals with a high binding affinity like Ni^{2+} offers a higher yield of protein, but tend to bind more contaminants than e.g. Co^{2+} resins.

Mobile Phase
By increasing the salt concentration of IMAC buffers, protein-protein interactions or ionic interactions between proteins and the matrix can be suppressed. The choice of buffer type can affect the retention e.g. by competitive binding of amines on the metal ions when using high Tris concentrations [Kågedal, 2011].
Different approaches can be followed for elution. By reducing the pH value, the bonds between the coordinating sides and the proteins are weakened by protonation of the nitrogen atoms on the histidine imidazole. Due to the similar structure of imidazole and the histidine side chain, the addition of imidazole in the elution buffer displaces proteins by competitive occupation of the metal ions [Clontech Laboratories, 2019b; Bornhorst and Falke, 2000].

Protein Structure
The application of recombinant proteins with additional polyhistidines (often (His_6)-tags) plays an essential role [Kågedal, 2011]. For the purification of His-tagged proteins, bivalent

metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} are used. A high binding affinity between the protein chains and the ion leads to a better purification of the target protein. The use of (His_6)-tags favours an easier isolation of the proteins from a variety of polypeptides [Bornhorst and Falke, 2000]. The binding is most effective when the His-tag is easily accessible. Therefore, if the binding is insufficient, the tag should be exchanged between the C- and N-terminus. For critical separations, the His-tag can be extended up to ten residues [Kågedal, 2011]. However, a too strong binding affinity of the protein to the metal chelate can also have disadvantages. If the binding site of the protein has better stereochemical properties than the linker, the metal ion can be transferred to the protein. This effect is called metal ion transfer (MIT) and was already described by *Sulkowski* in 1989 [Sulkowski, 1989].

2.3 Storage of Enzymes

The storage of proteins and enzymes for a certain period of time is common practice. The challenge of storage is to preserve the stability of the protein or enzyme and avoid denaturation and aggregation [Ó'Fágáin and Colliton, 2017]. For the preservation of protein and enzyme characteristics, two mechanisms have to be considered: thermodynamic stability and kinetic stability. The thermodynamic stability has an equilibrium of the native (active) state N and partially unfolded (inactive) protein state U.

$$N \overset{K}{\longleftrightarrow} U \tag{2.1}$$

K is the unfolding equilibrium constant which is related to the standard unfolding free-energy change ΔG°. Enzymes tend to unfold with increasing temperatures due to the shift of the equilibrium to the right side. The temperature at which N = U is called melting temperature T_m [Iyer and Ananthanarayan, 2008; Ó'Fágáin and Colliton, 2017]. Kinetic stability is concerned by storage conditions. It is essential for long time storage and describes the loss of enzymatic activity with time.

$$N \overset{k}{\longleftrightarrow} I \tag{2.2}$$

N represents the native (active) form of the protein and I the irreversibly inactivated state [Miklos et al., 2009; Sanchez-Ruiz, 2010]. The rate constant for irreversible protein denaturation is described by k, which is related to the free energy ΔG, also called Gibbs energy. If the free energy is large enough, the equilibrium is shifted to the left side and protein remains in its active state. However, the unfolded state U can undergo alteration processes such as proteolysis and aggregation that inhibit the folding back to the native state and promotes inactivation [Iyer and Ananthanarayan, 2008].

For the use of Neu5Ac-lyase and GlcNAc-2-epimerase as catalysts, the conservation of activity for short storage times in solution or for longer storage time periods as lyophilisate is essential for the success of biocatalysis and thus the production of Neu5Ac.

2.3.1 Aqueous Protein Solutions

Due to their complex three-dimensional structure and partially conformation into subunits, proteins require specific conditions to achieve stability. Unfavourable conditions or changes in the environment can induce protein degradation, precipitation and denaturation and thus loss in activity. To store proteins in solution, it is essential to ensure optimal pH conditions and to avoid destabilizing interactions with buffer components. The buffer salts should also be selected considering suitable pK_a values at storage temperature to ensure a stable pH [Ó'Fágáin and Colliton, 2017; Sanchez-Ruiz, 2010]. To prevent proteolysis arising from microbial contamination, the use of sterile vessels and potentially the addition of biozides should be considered. Particularly with enzymes, it should be tested whether the addition of such biozides has an influence on enzyme activity. As describes by Gloger in 2002, the use of excipients can increase protein stability by increasing the proteins T_m. A higher barrier of free energy favours the proteins native state. This can be achieved by using two mechanism for protein stabilization in aqueous solutions: preferential exclusion or hydration and preferential binding as seen in Figure 6 [Gloger, 2002; Lee, 2000].

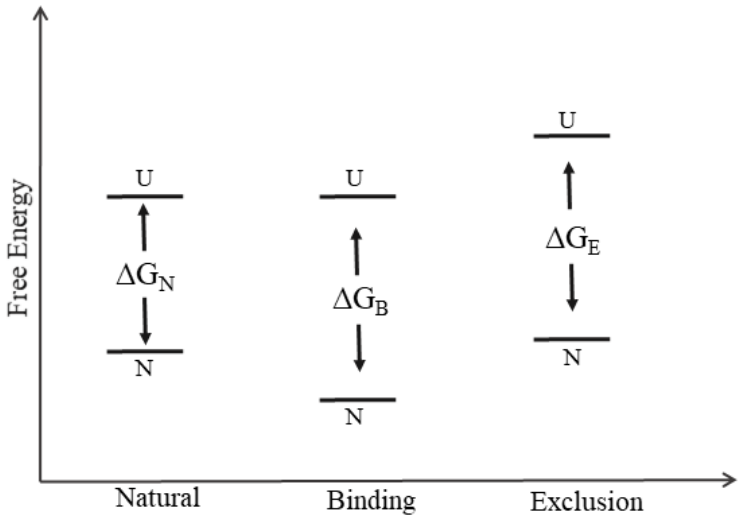

Figure 6: Change of free energy of a protein in aqueous solution. Three different states are shown: protein in natural state, change after binding of co-solvents and change after exclusion of co-solvents. (N = native/ active state, U = unfolded/inactive state. Based on the illustration by *Lee* [Lee, 2000].)

Preferential Exclusion/ Hydration

The effect of preferential exclusion/ hydration for protein stabilization is based on the unfavourable binding of the co-solvent to the protein. The co-solvent (here also called excipient) is excluded from the proteins surface which leads to the formation of a hydrate shell around the protein as seen in Figure 7. This effect arises from an unfavourable binding between the excipient and the protein backbone. This results in the stabilization of the native protein state [Ó'Fágáin and Colliton, 2017; Iyer and Ananthanarayan, 2008]. Additionally, a polyol-induced increase in the surface tension strengthens the stabilizing effect. The loss of the proteins structure would cause a larger protein surface. This results in a higher free energy for the denaturated state $\Delta G_E > \Delta G_N$ (see Figure 6). Thus the native state is stabilized [Gloger, 2002]. The strength of this exclusion/ hydration effect depends on the type of excipient and protein characteristics [Lee, 2000]. Common excipients for the stabilization of proteins, due to preferential exclusion, are large non-interacting polymers like glycerol, sucrose or polyethylene glycol (PEG).

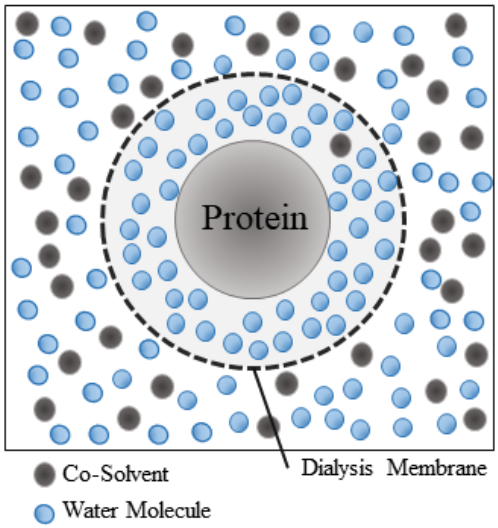

Figure 7: Dialysis equilibrium for a protein in aqueous solution with excluded co-solvent.(Based on the illustration by *Gloger, 2002* [Gloger, 2002].)

Preferential Binding

When the co-solvent binds favourable to the proteins native state, the formed complex has a lower free energy at the native side. Due to $\Delta G_B > \Delta G_N$, this results in stabilization of the folded state. Thus the native state is energetically more favourable than the denaturated state. Figure 8 shows the dialysis equilibrium. If the affinity for the additive is greater than for water, there is an excess of co-solvent in the near areas around the protein surface [Gloger, 2002].The binding and the exclusion mechanisms are similar effects. Both are defined by the affinity for the co-solvent and water which results in exclusion of one [Timasheff, 1998].

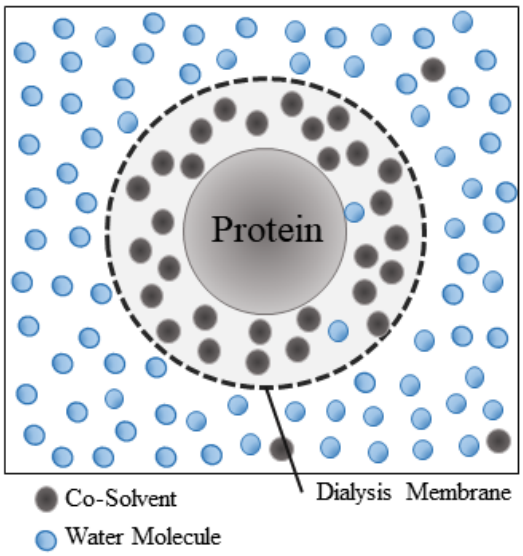

Figure 8: Dialysis equilibrium for a protein in aqueous solution with favourable bound co-solvent. (Based on the illustration by *Gloger* [Gloger, 2002].)

2.3.2 Lyophilization

Lyophilization, also called freeze-drying, is a desiccation process where the solvent is first frozen and then removed by sublimation in vacuum. It is an often used tool to increase the stability of proteins, for better transportation conditions and due to easier handling compared to protein solution [Iyer and Ananthanarayan, 2008]. Lyophilization is a multi step process:

- Freezing:

  If excipients are necessary, they are added beforehand. Typically, the sample is dialysed into a suitable buffer before freezing. The aqueous sample is frozen until it is completely solid. The sample temperature should be below the eutectic temperature to ensure crystallization of the solute. For amorphous components, the temperature should be below T'_g to achieve a completely solid sample [Pikal, 1994]. The freezing rate is an important process value since it influences the product quality. According to *Jiang and Nail*, intermediate cooling rates results in the highest recovery. Due to a small degree of supercooling, the sample crystallizes homogeneously [Jiang and Nail, 1998]. High freezing rates causes a high degree of supercooling and thus an increase in surface area and small pore sizes. This favours degradation of the protein and slower primary drying [Pikal, 1994].

- Primary Drying:

  This step is carried out under vacuum with condenser temperature of -60°C. Sublimation of the bulk ice from the sample to the colder condenser takes place. The duration of the primary drying depends on the chamber pressure and shelf temperature. Primary drying over 24 hours is not unusual. However, the goal is to minimize the process time to reach a good product quality. Based on the discussion by *Pikal* from 1994, it is recommended to choose a product temperature as high as possible, since the process time is reduced by a factor of two when increasing the temperature by 5°C [Pikal, 1994]. To avoid product degradation and collapse of the cake, the product temperature should be two to five degree under the collapse temperature T_c. A cake is the porous structure formed during the lyophilization of proteins and excipients. A collapse can lead to a heterogeneous structure and a higher degree of moist after lyophilization. In general, T_c lies some degree over the glass transition temperature of the system T'_g [Ó'Fágáin and Colliton, 2017; Pikal, 1994]

- Secondary Drying:

  This step is also performed under vacuum. After the bulk water is removed, unfrozen bound water is desorpted from the dried cake surface while slowly increasing the temperature. To avoid collapse, the temperature is not increased until the whole bulk water is removed. Secondary drying is performed until a target moisture content is reached. The exact determination of the collapse and glass transition temperatures can be done by differential scanning calorimetry [Pikal, 1994].

Due to 'stress' during the freezing and drying process, proteins may not recover their full activity. It is important to determine optimal lyophilization conditions for every protein, since the degree of stabilization varies with protein characteristics [Ó'Fágáin and Colliton, 2017; Carpenter et al., 1992]. A way to increase protein stability during lyophilization is the addition of stabilizers. In 1993, *Carpenter et al* considered the freezing and drying as separate processes in order to identify the 'stress' effects and to use suitable stabilizers [Carpenter et al., 1992].

Effects During Freezing

Although lower temperatures are generally associated with increased stability of proteins, freezing can induce denaturation of the proteins and is essentially involved in the loss of activity after the lyophilization process. To increase the stability of proteins while freezing, excipients, so-called cryoprotectans, are added. It has been shown that the same excipients can be used as for the stabilization of proteins in solution [Gloger, 2002]. Water and many other substances are eutectic-forming material with a sharp transition between the solid and the liquid state [Ó'Fágáin and Colliton, 2017]. As water is converted into ice, solutes are concentrated as they do not crystallize. 'Freeze concentration' has to be considered as solutes like NaCl or buffer salts could damage the protein before they crystallize. Especially phosphate buffers tend to high pH shifts due to salts with different freezing temperatures [Pikal, 1994]. Non-crystalline materials, like proteins and most of the cryoprotectans, undergo a glass transformation. Below the glass transformation temperature of the maximally concentrated solute T'_g, the system becomes rigid and molecular movement is effectively decreased [Ó'Fágáin and Colliton, 2017; Gloger, 2002]. In order to prevent denaturation during the freezing process, a buffer system with stable pH must be selected. Due to the two different effects of preferential binding or exclusion, the stabilizing effect of cryoprotectans must be determined for every protein. Generally, co-solvents which causes preferential exclusion effects may not stabilize proteins sufficiently during freezing [Ó'Fágáin and Colliton, 2017]. As discussed by *Carpenter et al.*, PEG is one of the most effective cryoprotectans tested [Carpenter et al., 1992].

Effects During Drying

Solutes that are able to stabilize proteins in aqueous solutions or during freezing, cannot stabilize proteins during drying. *Carpenter et al.* showed in 1993, that PEG is an excellent cryoprotectant but fails by stabilizing proteins during drying without the addition of sugars [Carpenter et al., 1992]. Due to sublimation of bulk water and bound water during desiccation processes, no preferential hydration of the protein is possible. This explains why cryoprotectans like PEG do not provide sufficient stability during drying. The protecting hydration shell of the protein is no longer existent [Carpenter and Crowe, 1989; Gloger, 2002]. Stabilization on the dry state is depending on direct interactions between the protein and the excipient. One requirement is that the proteins as well as the excipient are both amorphous [Gloger, 2002]. Stabilization is present when the excipient is able to form hydrogen bonds with the protein and serves as water substitute. This effect was first described by *Carpenter and Crowe* as 'water replacement'. Experiments have shown that under these conditions, every sugar can serve as lyoprotectant in combination with PEG [Carpenter et al., 1992; Carpenter and Crowe, 1989].

3 Material and Methods

Within this chapter, all materials are listed and methods are explained that were used or applied to answer the objectives of this thesis.

3.1 Material

The used materials are listed as chemicals, proteins and enzymes, chromatography resins as well as consumables, devices and software.

3.1.1 Chemicals

The following chemicals were used in this thesis.

Table 4: List of used chemicals

Description	Manufacturer
Acrylamide (30%)	Carl Roth Newbury, New England
Acetonitrile	Sigma Aldrich Missouri, US
Acetic Acid	Merck Darmstadt, Germany
N-Acetyl-D-Mannosamine	Carbosynth Karlsruhe, Germany
N-Acetyl-D-Neuraminic Acid	Sigma Aldrich Missouri, US
Ammonium Persulfate	Carl Roth Newbury, New England
Bisacrylamide (2%)	Carl Roth Newbury, New England
Bis-Tris Hydrochloride	Sigma Aldrich Missouri, US

Description	Manufacturer
Brilliant Blue G	Sigma Aldrich Missouri, US
Bradford Reagent	Thermo Fisher Scientific Massachusetts, US
Bromphenole Blue	Sigma Aldrich Missouri, US
Ethanol (96 %)	Carl Roth Newbury, New England
Glycerine	Carl Roth Newbury, New England
Hydrochloric Acid (37 %)	Carl Roth Newbury, New England
Imidazole	Sigma Aldrich Missouri, US
Magnesium Chloride	Sigma Aldrich Missouri, US
D-Mannose	Carbosynth Newbury, New England
β-Mercaptoethanol	Sigma Aldrich Missouri, US
Nicotinamide Adenine Dinucleotide	Carl Roth Newbury, New England
Phenylmethylsulfonyl Fluoride	Sigma Aldrich Missouri, US
Polyethylenglycol (1000 g/mol)	Carl Roth Newbury, New England
Sodium Arsenite	Santa Cruz Biotechnology California, US
Sodium Chloride	VWR Darmstadt, Germany
Disodium Phosphate Dihydrate	Carl Roth Newbury, New England

Description	Manufacturer
Sodium Hydroxide	Carl Roth Newbury, New England
Sodium Diphosphate Dihydrate	Carl Roth Newbury, New England
Sodium Dodecyl Sulfate	Carl Roth Newbury, New England
Sodium Pyruvate	Carbosynth Newbury, New England
Sodium Peroidate	Fluka North Carolina, US
Thiobarbituric Acid	Tokyo Chemical Industry Tokyo, Japan
Tetramethylethylenediamide	Carl Roth Newbury, New England

3.1.2 Proteins and Enzymes

For experimental purposes, the following enzymes were produced in the BioPharma department of GALAB laboratories and the following proteins were used

Table 5: List of used enzymes produced in the GALAB BioPharma laboratory

Description	Origin	Host Cell
N-Acylglucosamine 2-Epimerase	Pedobacter Heparinus	*E.coli* BL21 (DE 3)
N-Acylmannosamin-Dehydrogennase	Flavobacterium	*E.coli* BL21 (DE 3)
N-Acetylneuraminic Acid Lyase	*E.coli* K12	*E.coli* BL21 (DE 3)

Table 6: List of used proteins and protein mixtures

Description	Origin	Manufacturer
Ovalbumine	Egg White	Sigma Aldrich Missouri, US
Serum Albumine	Bovine	Sigma Aldrich Missouri, US
Transferrin	Human	Sigma Aldrich Missouri, US
Precision Blue Protein Standards (Prestained)	-	BioRad California, US

3.1.3 Resins

For the determination of optimal purification conditions, the following chromatographic resins were used.

Table 7: List of used chromatographic resins

Description	Properties	Manufacturer
Talon Superflow	Co^{2+}-charged IMAC resin	Takara Bio California, US
Profinity	Ni^{2+}-charged IMAC resin	BioRad California, US

3.1.4 Consumables, Devices and Software

The consumables, devices and software listed here were used for experimental work and analysis.

Table 8: List of used consumables and devices

Name	Description	Manufacturer
Äkta	FPLC system	GE Healthcare Buckinghamshire, England

Name	Description	Manufacturer
Alpha 1-2 LD plus	Freeze-Dryer	Christ Osterode, Germany
BioSpectrometer basic	Spectrometer	Eppendorf Hamburg, Germany
Cellulose Acetate Filter	Filters 0.8, 0.45 and 0.2 µm	Sartorius Göttingen, Germany
Microplate	96er Well Plate	Greiner Bio-One Kremsmünster, Austria
Rotary Vane Pump RZ6	Vacuum Pump	Vacuubrand Wertheim, Germany
Sonotrode S26d14	Coin Sonotrode	Hielscher Teltow, Germany
Tecan GENios	Microplate Reader	Tecan Group Männedorf, Swiss
UP200St	Ultrasonic Device	Hielscher Teltow, Germany
Masterflex Console Drive L/S	Peristaltic Pump	Cole-Parmer Wertheim, Germany
MiniStar Silverline	Microcentrifuge	VWR Radnor, US
Vivaflow 200	Ultrafiltration Module	Sartorius Göttingen, Germany
Safe-Lock Tubes 1.5 ml	1.5 ml Reaction Tubes	Eppendorf Hamburg, Germany
T25 Ultra-Turaxx	Homogenizer	Kia Staufen, Germany
Whatman Polycap TC 36	Filter Capsules 0.6 + 0.45 and 0.2 µm	GE Healthcare Buckinghamshire, England

Table 9: List of used software

Description	Version	Manufacturer
Unicorn	5.31	GE Healthcare Buckinghamshire, England
PyMol	2.3.4	Schrödinger New York, US
XFluor 4	4.51	Tecan Group Männedorf, Swiss

3.2 Methods

In the following section, all methods applied for the purification of target enzymes from cell mass, the freeze-drying process as well as the analytical methods for the evaluation of the processes are presented. The information concerning all chemicals, enzymes, devices, etc. listed in this chapter can be found in chapter 3.1 .

3.2.1 Protein Purification

The purification of both target enzymes from *E. coli* BL21 (DE 3) was carried out in several steps, starting with cell disruption using ultrasound, subsequent centrifugation and several filtration steps up to the purification of the (His_6)-tagged enzymes via IMAC. After fermentation, the culture broth was centrifuged for 30 min at 17,000 g at 4 °C. The supernatant was discarded and the wet cell weight determined. The pellet was stored at -80 °C until purification. The fermentation as well as these described steps were carried out by employees of the BioPharma department.

3.2.1.1 Homogenization and Cell Disruption

In order to isolate the target enzymes from the expression organism, the cells were first homogenised and then disrupted using ultrasound.
The frozen cell pellet was weighted and diluted 20% (w/v) according to the wet cell

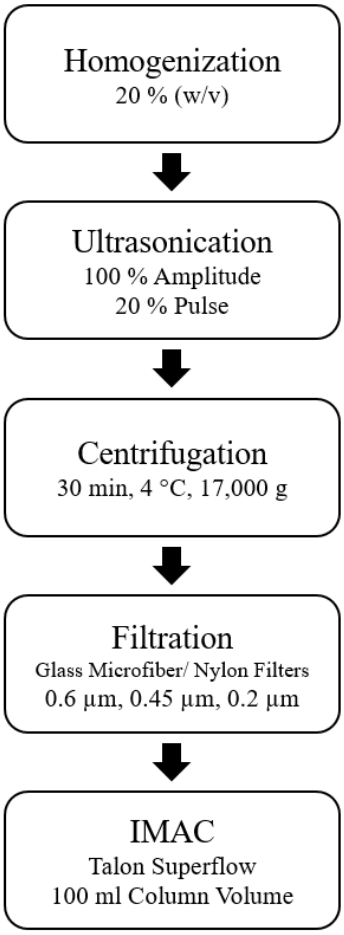

Figure 9: Workflow of the general purification process

weight (WCW) with IMAC equilibriation buffer (EQ, prepared according to Table 10). Further steps were performed in an ice bath. The thawed pellet was homogenized at 10,000 rpm in 3x 30 seconds intervals using a homogeniser. Prior to lysis, 1 mM Phenylmethylsulfonyl Fluoride (PMSF) was added to the homogenate to protect the proteins from enzymatic degradation by proteases. The cell disruption by ultrasonication with a coin sonotrode was performed in 10 minute intervals with an amplitude of 100% and a pulse of 20%. Before and after each interval, samples were taken and the total protein concentration was determined using the Bradford method (according to the instructions in chapter 3.2.3) to ensure a successful cell disruption. In general, the cell disruption was completed after 30 to 40 minutes with the total protein concentration remaining constant. The lysate was centrifuged for 30 minutes at 17,000 g and 4 °C to separate the proteins from cell fragments. The centrifuged cell homogenate was stored overnight at 4 °C if necessary.

3.2.1.2 Filtration

In order to separate remaining cell components, protein aggregates and other impurities, which could lead to blocking of the column, the sample was filtered in several steps. Since conventional cellulose acetate filters quickly form a filter cake, the filter capacity drops rapidly and thus makes multiple filter changes necessary. Pre-filtrations with 0.8, 0.6 and 0.45 µm pore size are time-consuming and do not always allow a filtration with 0.2 µm. In order to improve and simplify this process, new alternatives for cellulose acetate filters were tested. One of the tested Whatman TC filter capsules has two pore sizes in one capsule and serves as a pre-filter for larger impurities, thus ensuring a longer service life of the 0.2 µm filter capsule. The filter capsules were installed according to Figure

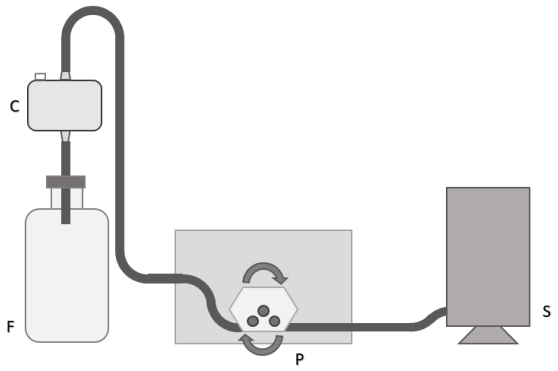

Figure 10: Construction of the filtration system for clarification and steril filtration of the homogenate with filter capsules. (F = filtrate, C = filter capsule, P = peristaltic pump, S = sample reservoir)

10. After 30 minutes centrifugation at 17,000 g and 4 °C, the supernatant was separated from the pellet. Both filters were washed before use with at least 300 ml distilled water and subsequently with 300 ml EQ. To avoid dilution of the sample, the sample tube was removed from the sample reservoir and the complete liquid is pumped out of the filter. This is performed at a low pumping rate. As the peristaltic pump used here does not have any exact setting options for the pump rates, these cannot be specified. Before applying the sample, the ventilation valve of the filter capsules was opened. The sample was pumped slowly into the filter capsule at a low pump rate until the capsule was completely filled. After closing the valve, the pump rate was increased and the sample was filtered completely. After filtration of the sample through the 0.6/0.45 and 0.2 µm filter, the filter capsules were first cleaned with EQ and afterwards with distilled water in and against the flow direction. For storage, the filter capsules were washed with 20% ethanol.

Implementation of Filter Capsules

To reduce filtration time, vacuum filtration through cellulose membrane filters was replaced by Whatman TC filter capsules with pore sizes of 0.6/0.45 µm and 0.2 µm. Before using these filters for the purification of large amounts of protein, they were tested with already purified target enzymes to verify possible enzyme losses or structural changes during filtration. Filtration was carried out as described above. Enzyme solutions of GlcNAc 2-epimerase and Neu5Ac lyase were filled to a volume of 120 ml and filtered first using the 0.6/ 0.45 µm and afterwards using the 0.2 µm filter. Before filtration and of each filtrate, the protein concentration was determined using Bradford protein assay and the specific enzyme activity (according to section 3.2.3.3).

3.2.1.3 IMAC

The immobilized metal ion affinity chromatography (IMAC) is a variant of affinity chromatography where proteins are separated due to their different binding affinities to metal ions. The target enzymes used in this thesis have six additional histidine residues and can be isolated from a large number of *E.coli* host cell proteins (HCP) trough their high binding affinity to cobalt and nickel charged IMAC resins. Depending on the sample volume and thus the amount of target protein, the column volume (CV) reached from 0.6 ml up to 100 ml.

Purification of Large Fermentation Pellets Quantities

For further experiments, mostly large quantities of proteins were required. For the purification of pellets of about 80 to 100 g, a column with a volume of 100 ml was used. The equilibration as well as the elution buffer (EL) were prepared according to table 10. Prior to every chromatographic run, the system including the pumps and the column were washed with distilled water before starting the equilibration. According to the steps from table 11, the column was equilibrated with 3 CV of EQ. Due to the large volume, the sample was applied via the P-960 pump of the Äkta system. To reduce unspecific binding on the column surface, 10 mM imidazole was added to the sample before loading the column. Unbound and slightly bound proteins were washed with EQ and a gradient of 6.6% EL for 3 CV. Elution of the bound and thus target proteins was carried out with 100%

Table 10: Composition of equilibration and elution buffer for purification of His-tagged proteins trough IMAC. Both buffers contain 50 mM sodium phosphate and 300 mM sodium chloride. For the elution buffer, 50 mM imidazole is added.

Component	Equilibration Buffer	Elution Buffer
Disodium Phosphate Dihydrate	5.41 g/l	5.41 g/l
Sodium Diphosphate Dihydrate	2.71 g/l	2.71 g/l
Sodium Chloride	17.53 g/l	17.53 g/l
Imidazole	-	10.2 g/l
pH	7.50	7.50

Table 11: Steps for protein purification using IMAC with 100 ml column volume.

Step	Medium	Gradient EL [%]	CV	Flow Rate [ml/min]
System Wash	Distilled Water	-	3	2.5
Equilibration	EQ	-	3	2.5
Sample Application	-	-	-	2
Wash after Application	EQ	6.6	3	2.5
Elution	-	100	2	2
Reequilibration	EQ	-	3	2.5
System Wash	Distilled Water	-	3	2.5

EL for 2 CV while the column output was collected in 10 ml fractions. After elution, the column was reequilibrated with 3 CV of EQ and 3 CV of distilled water. The column was washed with 3 CV of 20% ethanol for storage.

Comparison of IMAC Resin and Buffer Variations for GlcNAc 2-Epimerase Purification

One subject area of the optimization of the purification process was increasing the purity of GlcNAc 2-epimerase after IMAC. Some previous purifications have shown fractions of the target enzyme containing a significant amount of impurities from HCPs. To minimize these, the use of a Ni^{2+} charged column material for the purification of GlcNAc 2-epimerase by IMAC was investigated. Therefore, a direct comparison of the GlcNAc 2-epimerase purification using Profinity Ni^{2+} and TALON Co^{2+} IMAC resin was performed. In addition to both materials, the purification in sodium phosphate and Tris Buffer was compared to investigate the enzyme stability and an increased purity after IMAC. The purifications were performed according to the steps in Table 12.

Table 12: IMAC steps for the comparison of different buffers and of Co^{2+} and Ni^{2+} IMAC resin. Sample application with P960.(Co^{2+} resin = Talon Superflow, Ni^{2+} resin = Profinity IMAC, CV = 0.6 ml, BL = equilibration until stable baseline is reached)

Step	Medium	Gradient EL [%]	CV	Flow Rate [ml/min]
System Wash	Distilled Water	-	3	1
Equilibration	EQ	-	BL	1
Sample Application	-	-	P960	0.5
Wash after Application	EQ	-	4	1
Elution	-	100	7	1
Reequilibration	EQ	-	3	1

The sodium phosphate EQ as well as the sodium phosphate EL were prepared according to table 10 and the Tris buffers according to Table 13. Table 14 shows the used WCW, resupension volumes and protein concentrations of the used GlcNAc 2-epimerase fermentation.

Table 13: Composition of Tris equilibration and elution buffer for purification of GlcNAc 2-epimerase trough IMAC. pH is adjusted with 2M HCl.

Component	Equilibration Buffer	Elution Buffer
Tris Base	6.06 g/l	6.06 g/l
Sodium Chloride	17.53 g/l	17.53 g/l
Imidazole	-	10.2 g/l
pH	7.5	7.5

Table 14: Resuspended pellet mass, resuspension volume and protein concentration of the samples for the comparison of GlcNAc 2-epimerase purification with different buffers and column materials.

Resuspension Buffer	50 mM Sodium Phosphate 0.3 M NaCl pH 7.5	50 mM Tris 0.3 M NaCl pH 7.5
Wet Cell Weight [g]	5.8	6.3
Resuspension Volume [ml]	25	25
IMAC Sample Volume [ml]	6.8	5.3
Sample Protein Concentration [mg/ml]	13.3	8.39

The samples were applied to the column using the P960 sample pump. The peak area of each purification was determined with the Unicorn software. Bradford protein assay was used to calculate the protein concentrations of the elution fractions and determine their specific GlcNAc 2-epimerase activity (see section 3.2.3.3).

Column Performance of Profinity Ni^{2+} and TALON Co^{2+} IMAC Resins

To determine the binding capacity and column performance after several chromatographies, 0.6 ml columns were used for TALON Co^{2+} and Profinity Ni^{2+} resins. Before each test series, the volume to be applied was determined experimentally until the capacity was exhausted and a breakthrough curve appeared in the chromatogram. Afterwards the columns were regenerated according to the manufacturer manuals (see Table 15). The

Table 15: Steps for the regeneration of Co^{2+} and Ni^{2+} IMAC resin. (Co^{2+} resin = Talon Superflow, Ni^{2+} resin = Profinity IMAC, column volume= 0.6 ml, flow rate = 1 ml/min)

	Talon Superflow		Profinity IMAC	
Step	Medium	CV	Medium	CV
1	0.2M EDTA pH 7.0	10	50 mM Sodium Phosphate	10
2	Distilled Water	10	300 mM Sodium Chloride	10
3	50 mM Cobalt Chloride	10	0.2 M EDTA pH 7.0	10
4	Distilled Water	7	70% Ethanol	15
5	300M Sodium Chloride	3	50 mM Sodium Acetate 0.3 M Sodium Chloride pH 4	5
6	Distilled Water	3	0.2 M Nickel Chloride	5
7	-	-	50 mM Sodium Acetate 0.3 M Sodium Chloride pH 4	5
8	-	-	Distilled Water	10

chromatographies were performed as described in Table 16. The sample volume applied varies from 1.5 ml to 2.5 ml, depending on the column material and enzyme used. The equilibration as well as the EL were prepared according to table 10. For all experiments, except GlcNAc 2-epimerase and Profinity Ni^{2+} resin, a sample loop with a volume of 2 ml was used. For the experiments with GlcNAc 2-epimerase and Ni^{2+} resin, a volume of 2.5 ml was applied in a 5 ml sample loop. The chromatographies were repeated until a loss of capacity was detected or the enzyme solution was exhausted. The peak area distribution was used for the calculations of the binding capacities of the columns and the respective enzymes. The peak area was determined by using the Unicorn software. All peaks were assigned to either breakthrough or elution, their size was determined and the proportion in relation to the total peak area was calculated. Due to a constant applied sample volume and sample concentration within a test series, a fixed amount of protein was used in each chro-

Table 16: IMAC steps for determination of the column performance with Co^{2+} and Ni^{2+} IMAC resin.(Co^{2+} resin = Talon Superflow, Ni^{2+} resin = Profinity IMAC, CV = 0.6 ml, SV= sample volume, BL= equilibration until stable baseline is reached)

Step	Medium	Gradient EL [%]	CV	Flow Rate [ml/min]
System Wash	Distilled Water	-	3	0.5
Equilibration	EQ	-	BL	0.5
Sample Application	-	-	SV	0.2
Wash after Application	EQ	-	2x SV	0.5
Elution	-	100	7	0.5
Reequilibration	EQ	-	3	0.5

matography. The fraction of peak areas was used to calculate the amount of enzyme that bound to the column and the amount in the breakthrough.

3.2.1.4 Diafiltration and Dialysis

Diafiltration or dialysis were performed to exchange sample buffers in order to determine sample stability for storage, for the use of a suitable lyophilization solvent or for further experiments. The choice of the right buffer exchange process was based on the sample volume.

Diafiltration

The fractions of the elution peak were filtrated with an ultrafiltration module in diafiltration mode, in order to exchange the buffer. The choice of the target buffer depended on further experiments. Diafiltration with ultrafiltration modules is suitable for sample volumes >20 ml. The filtration module was installed as shown in Figure 11 and all steps were performed in an ice bath. Before use, the system was washed with at least 200 ml distilled water and afterwards equilibrated with 200 ml of the target buffer and a maximum transmembrane pressure of 2 bar. After washing, excess buffer in the reservoir and the filtrate was discarded. In order to achieve an almost complete buffer exchange (>99% according to the manufacturer [Sartorius, 2016]), a target buffer volume at least 5 times greater than the sample volume, was filled into the buffer reservoir. As the sample from the sample reservoir passed trough the membrane, the volume of buffer removed by the filtrate was refilled from the buffer reservoir until it is emptied. During diafiltration the flow rate of the peristaltic pump was adjusted in order not to exceed a transmembrane

pressure >1 bar. After diafiltration the system was washed with 200 ml distilled water and for storage with 200 ml of 20% ethanol.

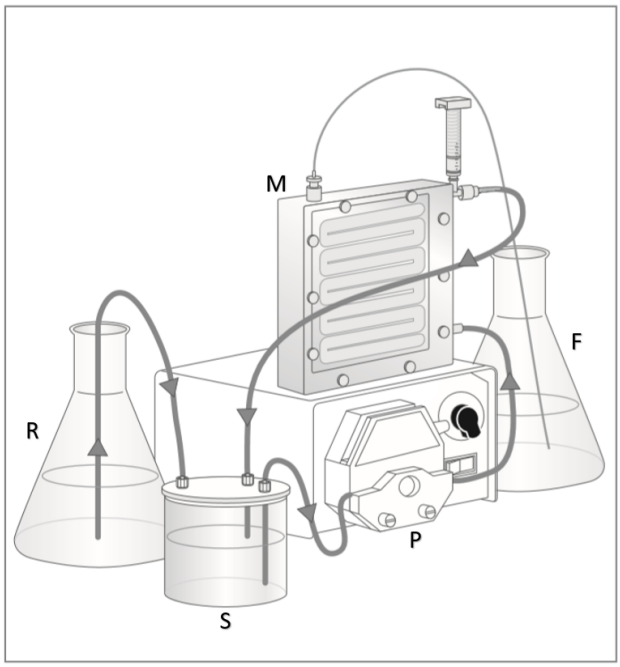

Figure 11: Construction of the ultrafiltration module Vivaflow 200 MWCO PES from Sartorius for diafiltration [Sartorius, 2016]. (R = buffer reservoir, S = sample reservoir, M = ultrafiltration module, F = filtrate, P = peristaltic Pump)

Dialysis

Dialysis of samples was recommended for sample volumes up to 20 ml.

For a dialysis, dialysis tube was cut with a length suitable for the used sample volume and soaked in distilled water for 30 minutes. A dialysis tube with 3500 molecular weight cut-off (MWCO) was used. One end of the tube was knotted or closed using a clamp. The sample was filled into the tube and the second end was closed. The buffer volume used was be at least 200 times the sample volume. The tube was placed in the buffer and stirred overnight in the refrigerator.

3.2.2 Lyophilization

In order to stabilize the produced and purified enzymes for long time storage, they were lyophilized. Lyophilization was divided into two separate processes: freezing and drying of the sample.

3.2.2.1 Determination of Optimal Lyophilization Conditions

To determine the optimal lyophilization conditions during freezing and drying for Neu5Ac lyase and GlcNAc 2-epimerase and their individual characteristics, conditions such as freezing rate, buffer type, buffer strength and the addition of excipients were investigated. The examined parameters were based on the effects discussed in the theoretical part for the lyophilization process in section 2.3.2. The influence of the excipients on the stabilisation of the enzymes was determined by investigating the freezing and effects during freeze-drying of the samples. A 20 mM Tris buffer with pH 7 and a freezing temperature of -20 °C were used. Table 17 lists all sample types with the tested excipient concentrations and combinations.

Table 17: List of samples with different concentrations of excipients for the determination of optimal lyophilization conditions for Neu5Ac-lyase and GlcNAc-2-epimerase. All samples contain 20 mM Tris buffer, pH 7. PEG was weighted in % (w/v).

Sample Name	excipients
w/o	-
1P	1% PEG
2P	2% PEG
10M	10 mM Mannose
20M	20 mM Mannose
1P10M	1% PEG + 10 mM Mannose
1P20M	1% PEG + 20 mM Mannose
2P10M	2% PEG + 10 mM Mannose
2P20M	2% PEG + 20 mM Mannose

Bulk solutions with 20% (w/v) PEG and 200 mM mannose were used for the preparation of the samples according to the pipetting scheme in Table 18. Each sample was prepared twice with a total volume of 500 µl in a 1.5 ml tube. Every sample was centrifuged to remove liquid from the lid. The freezing process was performed in a regular laboratory freezer with a temperature of -20 °C for at least 2 hours. The freeze-dryer as well as the pump were started on 30 minutes before use. All inlet and outlet air paths of the freeze-dryer were closed, except the connection ventile between freeze-dryer and pump. The pumps aeration ventile was open. After freezing, the sample tube was opened and a

Table 18: Pipetting scheme for the preparation of enzyme samples with different excipient concentrations. Using a 20% (w/v) PEG stock and a 200 mM mannose stock. Buffer = 20 mM Tris, pH 7)

Sample Name	PEG stock [µl]	Mannose stock [µl]	Buffer [µl]	Sample [µl]
w/o	0	0	0	500
1P	25	0	75	400
2P	50	0	50	400
1M	0	25	75	400
2M	0	50	50	400
1P1M	25	25	50	400
1P2M	25	50	25	400
2P1M	50	25	25	400
2P2M	50	50	0	400

2 x 2 cm piece of tissue was wrapped around the tube. The tissue prevented product loss during sublimation. Vacuum from the freeze-dryer was removed and all samples were placed immediately into the chamber. Drying was performed with a chamber temperature of -55 °C and a vacuum of 0.021 mbar. The freeze-drying process for 500 µl samples was finished after 24 hours. The frozen samples were thawed at room temperature and the lyophilized samples were resuspended in 500 µl distilled water. All samples were centrifuged for 5 minutes in a microcentrifuge to separate precipitate. Determination of the protein concentration and enzymatic activity, were carried out according to the methods discussed in chapter 3.2.3.3.

3.2.3 Analysis

In order to investigate the success of the previous methods, the samples were examined at different times for purity, total protein concentration or specific enzyme activity.

3.2.3.1 Bradford Protein Assay

The Bradford protein assay is a colorimetric method for determining the total protein concentration of a solution. The triphenylmethane dye Coomassie Brilliant Blue G-20 changes its adsorbance maximum and color from 465 nm (brown) to 595 nm (blue) by forming a protein-dye complex in the presence of the protein residues arginine and lysine.

Since the degree of colour reaction depends on the structure of the protein, it was mandatory to use a calibration curve with a standard protein, whereby the right choice of this standard protein was important for an accurate concentration determination. On this basis, the ratio of molecular weight to arginine/lysine content was calculated from the analysed proteins and compared with the ratios of some standard proteins. The closer these two ratios are to each other, the more accurate the measured protein concentration values were expected. Table 19 shows the calculated values of the proteins and the suitable standard proteins used for the Bradford assay. Bovine serum albumine (BSA) was used as standard for the quantification of the total protein concentration from a protein mixture like cell lysate.

Table 19: Target proteins, their calculated ratio of molecular mass to arginine/lysine content and the standard protein used for Bradford protein assay. (AA= amino acids (here: lysine and arginine))

Target Protein	Ratio Target Protein [kDa/AA content]	Standard Protein	Ratio Standard Protein [kDa/AA content]
GlcNAc 2-Epimerase	0.76	Transferrin	0.74
Neu5Ac Lyase	0.88	Ovalbumine	1.02
Cell Lysate	-	BSA	0.67

Standards were prepared in a concentration range from 0 µg/ml (blanc) up to 30 µg/ml. The samples were diluted to not exceed the protein concentration of the highest standard values. 100 µl of sample, sample buffer blanc and standard were pipetted into a 96 well microplate in quadruplicates and mixed with 100 µl Bradford reagent. After 5 minutes of incubation, the absorption at 595 nm was measured using a microplate reader.
For the determination of the protein concentration, the blank was subtracted from the standards as well as from the samples and a standard curve was generated. Using the obtained linear equation, the protein concentration of the measured samples could be calculated.

3.2.3.2 SDS-PAGE

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a variant of the gel electrophoresis for the separation of proteins due to their molecular size in an electric field. It is a basic method in biochemistry for a qualitative analysis of samples.

Using protein markers with known molecular masses, the presence of target protein and impurities from contamination or HCP can be monitored throughout the purification process. For qualitative analysis after purification of Neu5Ac lyase or GlcNAc-2 epimerase, 10% polyacrylamide gels were used. The preparation tables for the gels, electrophoresis buffers and sample buffers can be found in Table 20.

Table 20: Composition of electrophoresis and sample buffers used for SDS-PAGE. Before use, the electrophoresis buffer is diluted 1:10 with distilled water.

Component	Electrophoresis Buffer	Sample Buffer
Tris Base [g/l]	30.28	-
Glycine [g/l]	144.13	-
SDS 1% [g/l]	10.0	-
SDS [ml]	-	0.10
Tris [g/l]	-	157.6
Glycerole [g/l]	-	0.20
β-Mercaptoethanol [ml]	-	0.050
Distilled Water [ml]	-	0.397
Bromphenole Blue [ml]	-	0.040

If necessary, the samples were diluted to not exceed a total protein amount of 5 to 15 µ g in a sample volume of 20 µl. The samples were mixed with 1:1 sample buffer and heated at 95 ° for 10 minutes. Subsequently, 20 µl of each sample and 5 µl of prestained standard protein mix were pipetted into the gel pockets. The gel was placed into the electrophoresis chamber and filled with 250 ml of electrophoresis buffer. The electrophoresis is running 15 minutes with a constant current of 100 V and afterwards for 90 minutes at 120 V. For staining, the gel was placed in staining solution for 30 to 60 minutes. The gel was destained in destaining solution for at least 60 minutes while slewed slowly or overnight in distilled water. Both solutions were prepared according to Table 21.

Table 21: Composition of staining and destaining solution used for SDS-PAGE.

Component	Staining Solution	Destaining Solution
Coomassie Brilliant Blue R250 [g/l]	0.25	-
Methanole [ml]	400	400
Acetic Acid [ml]	70.0	100
Distilled Water [ml]	1000	1000

3.2.3.3 Enzyme Activity Assay

The analysis of enzyme activity for Neu5Ac lyase and GlcNAc-2 epimerase was important for the determination of product quality after purification and as an indicator of stress resistance during the freeze-drying process. The analysis was carried out by measuring the reaction products catalysed by the enzymes. For the comparison of purification and product quality, the specific enzyme activity was calculated using the following equation:

$$A_{specific} = \frac{c_{product} / T_{reaction}}{c_{enzyme}} \tag{3.1}$$

With the parameters for the specific enzyme activity $A_{specific}$ in U per mg enzyme, calculated using the enzyme reaction product concentration $c_{product}$ in M, the reaction time $T_{reaction}$ in minutes and the used enzyme concentration c_{enzyme} in mg per l.
Substrate solutions for both enzyme assays were prepared according to the Table 22.

Table 22: Composition of substrate solutions for the determination of Neu5Ac lyase and GlcNAc 2 epimerase activity. All components are solved in 100 mM Tris buffer pH 8.

Component	Neu5Ac Lyase	GlcNAc 2 Epimerase
N-Acetylmannosamine [mM]	111.11	-
Sodiumpyruvate [mM]	277.77	-
N-Acetylglucosamine [mM]	-	111.11
Adenosinetriphosphate [mM]	-	1.11
Magnesiumchloride [mM]	-	1.11
Buffer	100 mM Tris, pH 8	

Determination of Neu5Ac Lyase Activity
Neu5Ac lyase catalyses the reversible condensation of N-acetylmannosamine (ManNAc) with pyruvate to Neu5Ac, as shown in Figure 12. The reaction product was detected by a modified thiobarbituric acid (TBA) assay for sialic acids according to *Warren* [Warren, 1959]. To start the reaction, a substrate solution containing ManNAc and pyruvate was prepared. The samples were diluted with 100 mM Tris buffer (pH 8) to reach a Neu5Ac

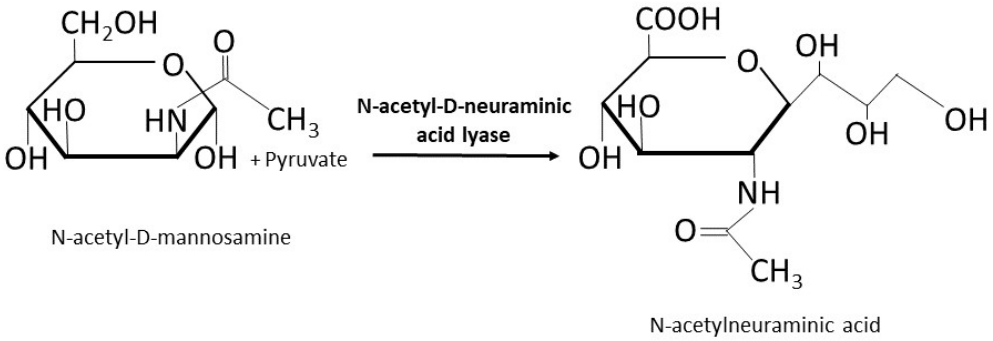

Figure 12: Substrate and product of reaction catalysed by N-Acetylneuraminic acid lyase.

lyase concentration of 1 mg/ml. Per sample, two 1.5 ml tubes with 0.9 ml substrate solution were heated at 40 °C for 5 minutes while shook at 10,000 rpm. The reaction started after adding 0.1 ml of sample and was performed in a shaker at 40 °C for 10 minutes at 10,000 rpm. Subsequently, 500 µl acetonitrile were added to stop the reaction. For following analysis, all samples were diluted 1:20 with 100 mM Tris buffer. For quantification of the produced Neu5Ac, the modified TBA assay was performed. Standard solutions as well as the oxidation and reduction solution were prepared (see Tables 24 and 23).

Table 23: Preparation of standard solutions for the modified version of the TBA assay according to Warren [Warren, 1959]

Standard	Neu5Ac [mM]	ManNAc [mM]	Pyruvate [mM]
Blank	0	1	2.5
1	0.2	0.8	2.0
2	0.4	0.6	1.5
3	0.6	0.4	1.0
4	0.8	0.2	0.5
5	1.0	0	0

Table 24: Composition of oxidation, reduction and thiobarbiutic acid solutions for the determination of Neu5Ac lyase activity.

Component	Oxidation Solution	Reduction Solution	TBA Solution
Sodium Peroidate [M]	0.067	-	-
Phosphoric Acid 85% [M]	3	-	-
Sodium Arsenite [M]	-	0.7	-
Sulfuric Acid [M]	-	0.05	-
Sodium Sulphate [M]	-	0.5	0.5
Thiobarbiutic Acid [M]	-	-	0.4

0.1 ml of standard or sample were mixed with 0.05 ml oxidation solution and incubated at room temperature (RT) for 20 minutes while shaken. Meanwhile, the TBA for the TBA solution was heated in a water bath at 85 °C. 25 ml reduction solution was added and the sample was incubated for another 15 minutes at RT. The dissolved TBA was mixed with sodium sulphate, 0.35 ml of this solution was added to the samples/standards and shaken for 15 minutes at 85 °C. The samples/standards were quenched in an ice bath and 0.75 ml dimethyl sulphoxide was added. Afterwards, all samples/standards were centrifuged at 14,000 rpm for 1 minute. Quadruplicates of 200 µl supernatant were pipetted into a 96 well microplate and placed into a plate reader for absorption measurement at 550 nm. By subtracting the blank and then creating a standard curve from the measured standards, the Neu5Ac amount of samples was calculated from the obtained linear equation.

Determination of GlcNAc 2-Epimerase Activity

As shown in Figure 13, the prokaryotic enzyme GlcNAc 2-epimerase catalyses the reaction from GlcNAc to ManNAc. Since the reaction product of the GlcNAc 2-epimerase ManNAc and the substrate GlcNAc have a similar structure, conventional analytical methods for the detection of saccharides such as HPLC are difficult to realize. Therefore, for the activity determination of GlcNAc 2-epimerase the N-acetyl-D-mannosamine dehydrogenase (ManDH) was used. ManDH catalyses the reaction of ManNAc and nicotinamide adenine dinucleotide (NAD) to N-acetamino-2-deoxy-gluconic acid and NADH [Horiuchi and Kurokawa, 1988]. By measuring the absorption at 340 nm the amount of NAD resulting from the reaction was calculated. First, the standard activity assay for epimerase was performed to obtain the product ManNAc. For this purpose, substrate solutions and

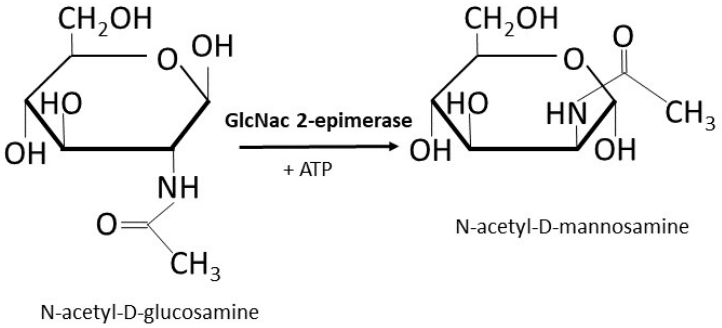

Figure 13: Substrate and product of reaction catalysed by GlcNAc 2-epimerase used for enzyme activity analysis

buffers were prepared according to the instructions in Table 22. The enzyme solution was diluted with 100 mM Tris to a concentration of 0,025 mg/ml GlcNAc 2-epimerase. Duplicates were prepared from each sample. For each sample, 0.9 ml of the substrate solution in 1.5 ml reaction tubes were heated for 5 minutes at 40 °C. Then 0.1 ml of the diluted enzyme solution was added and the reaction was carried out for 10 minutes at 40 °C in a thermoshaker at 10,000 rpm. At the end of the reaction time, 100 µl of the reaction solution were transferred into a new 1.5 ml reaction tube and the reaction was stopped for 10 minutes at 95 °C and shaken at 10,000 rpm. For further analysis the sample was diluted 50-fold with 100 mM Tris buffer.
Subsequently, the ManDH Assay was performed. All samples were prepared directly in 1.5 ml cuvettes. For the blank sample, 850 µl of 100 mM Tris buffer, 100 µl of NAD solution and 50 µl of ManDH solution were mixed. For the samples, 850 µl of the enzyme solution, 100 µl NAD solution and 50 µl ManDH solution were added and the mixture was incubated for 30 minutes at RT. At the end of the incubation period, first the blank value at 340 nm was determined and then the samples were measured. The content of NADH c_{NADH} in mol was calculated using the following formula

$$c_{NADH} = \frac{A_{340}}{\varepsilon(\lambda) * d} \tag{3.2}$$

with the absorption A at 340 nm, the cuvette thickness d (here d= 1 cm) and the molar extinction coefficient $\varepsilon(\lambda)$= 6270 for NADH in l/mol*cm.

4 Results

This chapter summarizes the results of the experiments to optimize the purification and development of lyophilization of GlcNAc 2-epimerase and Neu5Ac lyase.

4.1 Purification of Neu5Ac Lyase and GlcNAc 2-Epimerase

The optimization of the purification of GlcNAc 2-epimerase and Neu5Ac lyase ranges from the disruption of the cell pellet to the purification of the enzyme by IMAC. This workflow results in a large number of steps. The optimization of the purification process offers a wide range of possibilities. In this work, the needs of target enzymes were addressed, which involve different requirements. One general step for the optimization is the improvement of the supernatant filtration after centrifugation of the cell lysate. Due to different requirements addressed to GlcNAc 2-epimerase or Neu5Ac lyase, some optimization issues are more specific. The results are divided into topics related to the purification of GlcNAc 2-epimerase and Neu5Ac lyase (see subsection 3.2.1.3 and 4.1.1) as well as topics specifically related to GlcNAc 2-epimerase (subsection 3.2.1.3).

4.1.1 Implementation of Filter Capsules

Due to the high number of work steps as described in section 3.2.1, the simplification of time-consuming steps is an important factor. After homogenization and cell disruption, the lysate is centrifuged to remove cells and cell debris. Filtration of the supernatant before loading it to the IMAC column is one of these limiting factors to remove remained impurities. Thus column blocking during sample application can be avoided. Optimal is a filtration with a pore size of 0.2 µm. For efficient filtration of the supernatant, two Whatman TC filter capsules with pore sizes of 0.6/0.45 and 0.2 µm are examined. Experiments were performed with the pure target enzymes according to the instructions in subsection 3.2.1.2. Before and after each filtration, the protein concentration and the specific enzyme activity were determined. The tables 25 and 26 show the results of the filter experiments. The protein mass of the samples was determined using the Bradford Assay. The sample volume before and after the individual filtration steps were recorded. The calculated percentage of

Table 25: Calculated enzyme mass and activity loss of GlcNAc 2-epimerase (F190043) after filtration with 0.6/ 0.45 and 0.2 µm Whatman TC filter capsules. Calculated is the percentage protein mass and activity loss of the sample compared to the values before filtration.

GlcNAc 2-Epimerase (F190043)			
Pore Size [µm]	-	0.6/ 0.45	0.2
Protein Concentration [mg/ml]	11.65	11.54	11.69
Sample Volume [ml]	120.0	120.0	115.0
Protein Mass [mg]	1398	1384	1344
Protein Loss [%]	-	**0.945**	**3.863**
Spec. Enzyme Activity[U/mg]	277.1	274.8	271.8
Activity Loss [%]	-	**0.819**	**1.912**

protein loss was determined with respect to the protein mass before filtration. Therefore, a value of 0% corresponds to a constant protein mass and activity after filtration. The filtration of the GlcNAc 2-epimerase showed a loss of enzyme mass of 0.9% after the first step and a loss of 3.9% after the second filtration step as seen in Table 25. The samples were diluted according to their protein concentration and the enzyme assay for GlcNAc 2-epimerase was performed. The specific enzyme activity of GlcNAc 2-epimerase show losses of 0.8% from 277.1 U/mg to 274.8 U/mg after the first filtration step. After the second filtration the activity drops to 271.8 U/mg which is equal to 1.9% loss in activity. The values show a similar reduction of the enzyme activity after both filtration steps. With

Table 26: Calculated enzyme mass and activity loss of Neu5Ac lyase (F190040) after filtration with 0.6/ 0.45 and 0.2 µm Whatman TC filter capsules. Calculated is the percentage protein mass and activity loss of the sample compared to the values before filtration.

Neu5Ac Lyase (F190040)			
Pore Size [µm]	-	0.6/ 0.45	0.2
Protein Concentration [mg/ml]	14.04	13.85	13.74
Sample Volume [ml]	120.0	117.0	117.0
Protein Mass [mg]	1684	1620	1607
Protein Loss [%]	-	**3.816**	**4.572**
Spec. Enzyme Activity[U/mg]	16.99	16.25	16.17
Activity Loss [%]	-	**4.331**	**4.824**

Neu5Ac lyase the loss is larger compared to GlcNAc 2-epimerase. After the first filtration step 3.8% of the enzyme is lost, after the second step 4.6%. The activity decreases by 4.3% from 16.99 U/mg to 16.25 U/mg. After filtration with the 0.2 µm filter capsule, the enzyme activity is reduced by 4.8% with a value of 16.17 U/mg.

The results of the enzyme mass and enzyme activity determination show that losses can be determined for both enzymes. The total loss for mass and activity is greater for Neu5Ac lyase compared to GlcNAc 2-epimerase.

4.1.2 Column Performance of Ni and Co Charged IMAC Resins

One subject for the improvement of the purification process is the determination of binding capacities and column lifetime. For this purpose TALON Co^{2+} and Profinity Ni^{2+} column materials were used for chromatographies with GlcNAc 2-epimerase and Neu5Ac lyase . Purified enzymes were used for these experiments. To determine the column capacity, protein was applied until the capacity was exhausted and a breakthrough was visible in the chromatogram. To ensure comparability, the same batch of target enzyme was always used per column. The binding capacities were determined using the peak area distribution. Based on the total peak area, the percentage of elution peak area and breakthrough peak area was determined. The amount of protein in the eluate and breakthrough were calculated using the percentage distribution of the areas and the total protein amount. The data of the volume used and protein concentration applied are shown in the appendix Table A. To achieve a better comparability of the chromatograms, the UV values are corrected by the baseline value. For this purpose, the baseline value is subtracted from all absorption values. This is used only for the graphical illustration of the chromatography curves.

4.1.2.1 GlcNAc 2-Epimerase

GlcNAc 2-epimerase was applied on IMAC columns with TALON Co^{2+} and Profinity Ni^{2+} resins. These experiments were performed as described in the beginning of the section. For the columns experiments with TALON Co^{2+} charged IMAC resin, GlcNAc 2-epimerase from fermentation number F190046 was used. 2 ml of sample with a concentration of 23.5 mg GlcNAc 2-epimerase per ml were applied. 15 chromatography runs were performed in total. Table 27 gives an overview of the experimental data and measured values. The Figure 14A shows the comparison of the calculated binding capacities for each run. The binding capacities vary of about 25% between 22.7 mg/ml and 30.4 mg/ml. Despite the variations, no loss of capacity is apparent after 15 chromatography runs. The mean value of the capacities is 26.7 mg/ml and thus 33.5% higher than the value given by the manufacturer of 20 mg/ml.

Table 27: Experimental data for the determination of binding capacity and column life time for GlcNAc 2-epimerase with TALON Co^{2+} and Profinity Ni^{2+} resin.

GlcNAc 2-epimerase (F190046)		
Resin	Co^{2+}	Ni^{2+}
Protein Concentration [mg/ml]	23.5	3.95
Sample Volume [ml]	2	2.5
Number of Runs	15	16
Binding Capacity[mg/ml resin]	**26.7**	**11.6**
Loss of Capacity [%]	-	-

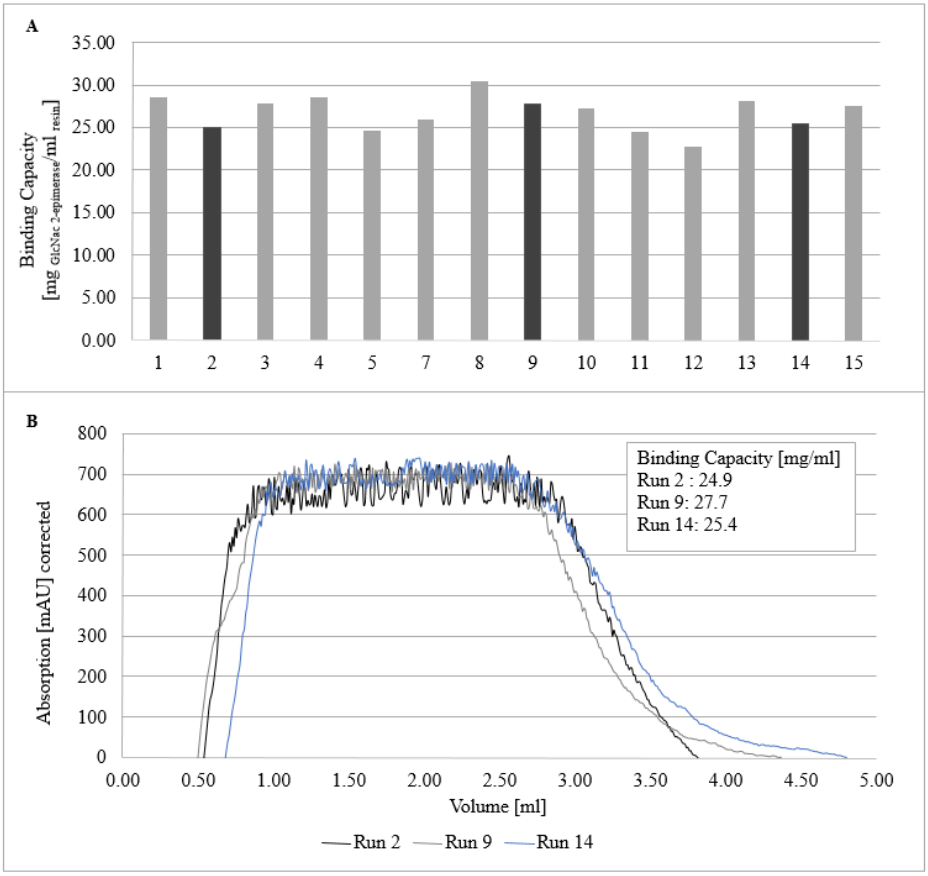

Figure 14: A Calculated binding capacities of TALON Co^{2+} IMAC resin for GlcNAc 2-epimerase for chromatography run 1 to 15. **B:** Curves of the breakthrough and binding capacities of the chromatography runs 2, 9 and 14 with TALON Co^{2+} IMAC resin for GlcNAc 2-epimerase.(Resin= Talon Superflow, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

The chromatograms of runs 2, 9 and 14 were illustrated to investigate changes in protein

binding. Run 2 has a binding capacity of 24.9 mg/ml, run 9 with 27.7 mg/ml and run 14 with 25.4 mg/ml. However, the respective curves in Figures 14B and A1 (in appendix) do not show significant differences. The curves have maximum absorption values of 650 to 700 mAU in the breakthrough.
Figure 15A shows the calculated binding capacities of the Profinity Ni^{2+} column material for GlcNAc 2-epimerase. GlcNAc 2-epimerase (fermentation number F190046) was used for these experiments. For each run 2.5 ml sample with a concentration of 3.95 mg/ml was applied. This corresponds to a total protein amount of 9.9 mg per run. 16 chromatography runs were performed in total.

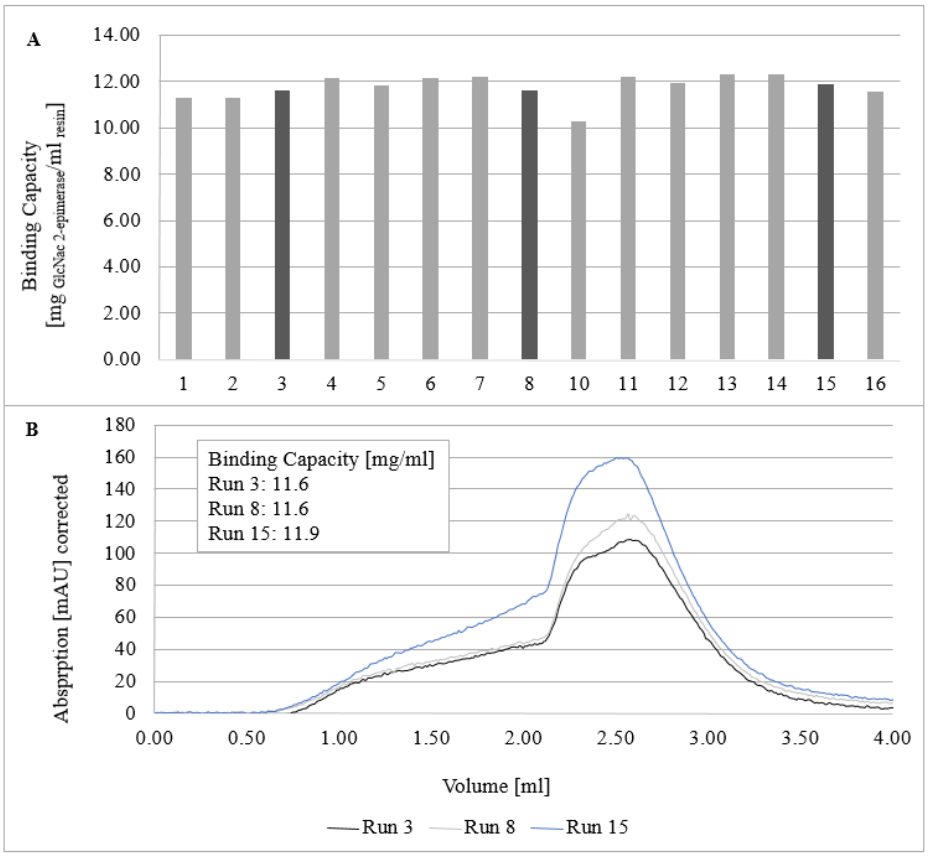

Figure 15: A Calculated binding capacities of Profnity Ni^{2+} IMAC resin for GlcNAc 2-epimerase for chromatography run 1 to 16. **B:** Curves of the breakthrough and binding capacities of the chromatography runs 3, 8 and 15 with Profinity Ni^{2+} IMAC resin for GlcNAc 2-epimerase.(Resin= Profinity IMAC, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 5 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

The capacities show no reduction with increasing number of runs. The average binding

capacity is 11.6 mg/ml and therefore 22.7% lower than the value given by the manufacturer (15 mg/ml). It is as well 56.5% lower than the average value of the TALON Co^{2+} resin. The breakthrough curves of runs 3, 8 and 15 are shown in Figures 15B. Elution curves are shown in A2 in the appendix. The breakthrough curves show different absorption maxima, which reaches 110 mAU to 160 mAU.

4.1.2.2 Neu5Ac Lyase

GlcNAc 2-epimerase was applied on IMAC columns with TALON Co^{2+} and Profinity Ni^{2+} resins. These experiments were performed as described in the beginning of the section. The determination of the binding capacity and lifetime of the TALON Co^{2+} column was performed with Neu5Ac lyase from batch F190040. 1.5 ml sample with a concentration of 37.3 mg/ml were applied for each chromatography. This corresponds to a protein amount of 55.8 mg/run. In total, 14 chromatographies were performed. Table 28 gives an overview of the experimental data and measured values. Figure 16A shows the calculated binding

Table 28: Experimental data for the determination of binding capacity and column life time for Neu5Ac lyase with TALON Co^{2+} and Profinity Ni^{2+} resin.

Neu5Ac lyase Fermentation	F190040	F190030
Resin	Co^{2+}	Ni^{2+}
Protein Concentration [mg/ml]	37.3	3.95
Sample Volume [ml]	1.5	21.9
Number of Runs	14	15
Binding Capacity [mg/ml resin]	**79.6**	**56.5**
Loss of Capacity [%]	12.2	17.5

capacities for each run. The reduction of the binding capacity increases with the number of runs on the column. The first 5 runs have an average capacity of 79.6 mg/ml. This is reduced by 12.2% to 69.9 mg/ml in the last five chromatography runs. Compared to the column capacity given by the manufacturer [Takara Bio, 2020], this is exceeded four times at the beginning. In this example the reduction in capacity is also clearly visible in the chromatograms of the breakthrough curves (see Figure 16B). The breaktrough curve in run 1 has an absorption maximum of 150 mAU with a capacity of 82.9 mg/ml. With decreasing capacity, the absorption values increases. Run 12 has maximum values of 410 mAU and thus the lowest binding capacity of 68.7 mg/ml.

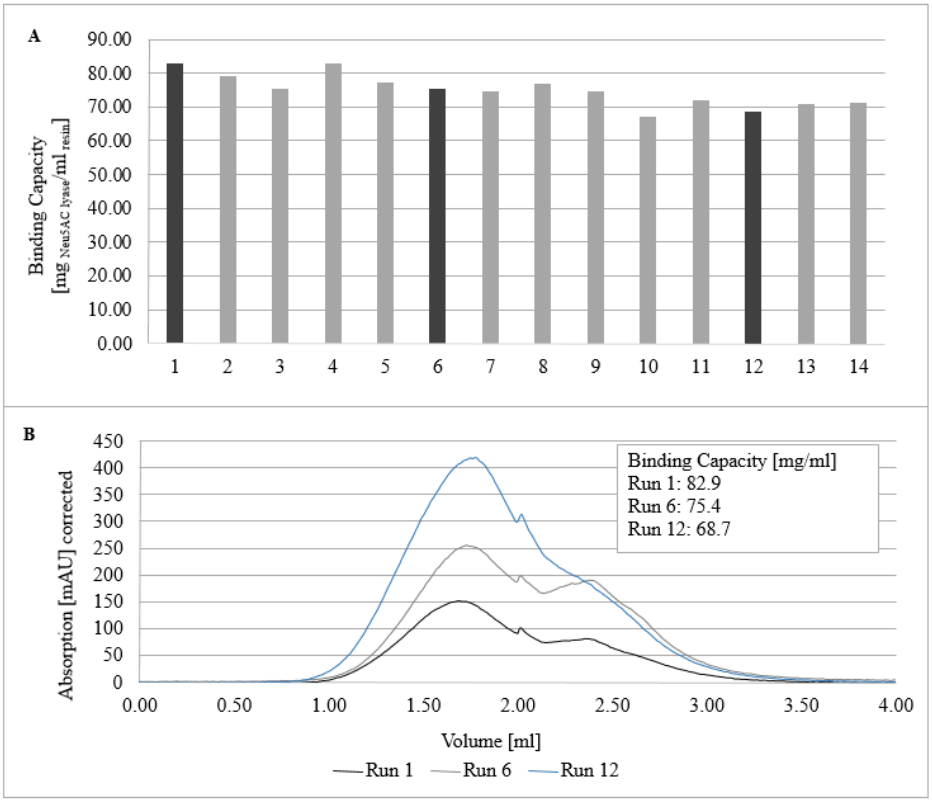

Figure 16: A Calculated binding capacities of TALON Co^{2+} IMAC resin for Neu5Ac lyase for chromatography run 1 to 14. **B:** Curves of the breakthrough and binding capacities of the chromatography runs 1, 6 and 12 with TALON Co^{2+} IMAC resin for Neu5Ac lyase.(Resin= Talon Superflow, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

Neu5Ac from fermentation number F190030 was used to determine the binding capacity and lifetime of the Profinity Ni^{2+} column. 15 chromatography runs with a sample volume of 2 ml and an enzyme concentration of 21.9 mg/ml were performed. This corresponds to a total enzyme mass of 43.8 mg per run. Figure A4A shows the calculated binding capacities for all 15 chromatography runs. It can be seen that the capacities decrease with increasing number of runs. In the first five runs the calculated binding capacity reaches 56.5 mg/ml and increases to 46.62 mg/ml (last five runs). The capacity of the first five runs is 3.7 times higher than the manufacturer's stated capacity of 15 mg/ml but it is 30% lower compared to the TALON Co^{2+} column. The breakthrough curves of the chromatography runs 2, 7 and 14 are shown in the Figure 17B. The elution curves are shown in the appendix Figure A4. The breakthrough curves show a significant increase in the maximum absorption values. Run 1 has a binding capacity of 58.1 mg/ml an an absorption maximum of 320 mAU.

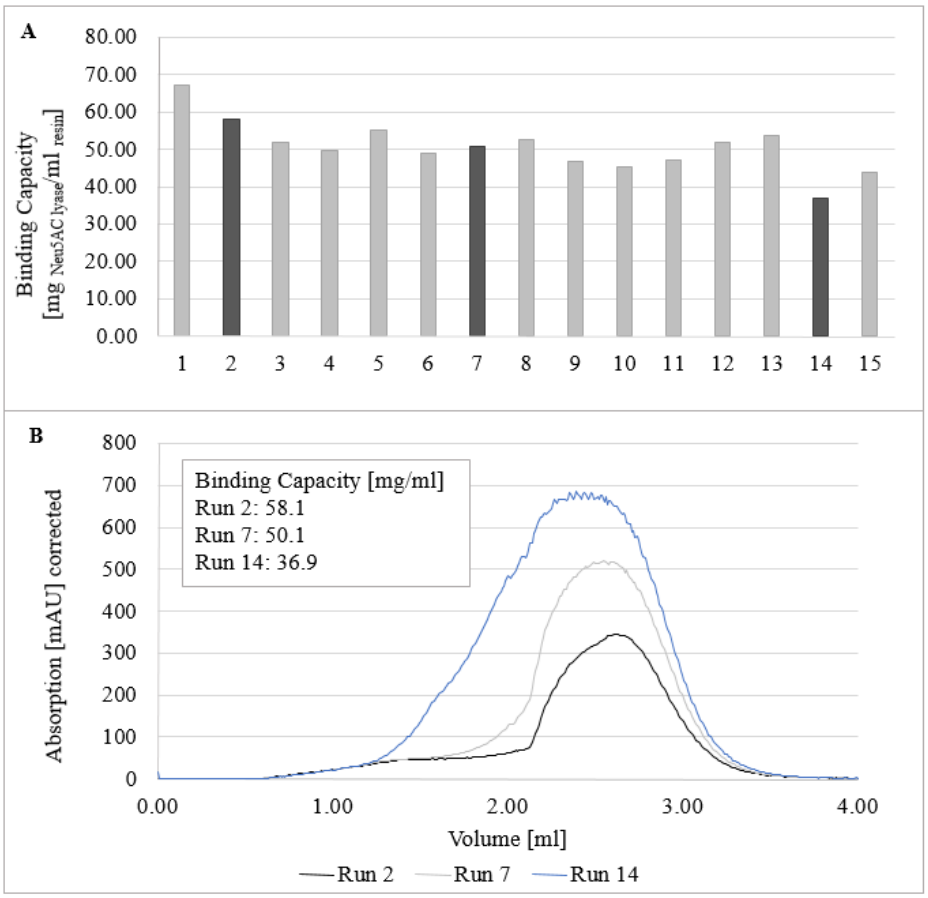

Figure 17: A Calculated binding capacities of Profinity Ni^{2+} IMAC resin for Neu5Ac lyase for chromatography run 1 to 15. **B:** Curves of the breakthrough and binding capacities of the chromatography runs 2, 7 and 14 with Profinity Ni^{2+} IMAC resin for Neu5Ac lyase.(Resin= Profinity IMAC, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

In run 14, the capacity decreases to 36.9 mg/ml with a maximum absorption value of 790 mAU. Parallel to the increase of the peak height in the breakthrough, a decrease can be seen in the elution peaks.

In summary, TALON Co^{2+} columns achieved higher binding capacities than Profinity Ni^{2+} columns. Compared to GlcNAc 2-epimerase, significantly more Neu5Ac lyase can be bound to both column materials. However, both columns used with Neu5Ac lyase show losses in binding capacity after nine to ten chromatography runs.

4.1.3 Comparison of IMAC Resin and Buffer Variations for GlcNAc 2-Epimerase Purification

To determine the purification success by comparing two column materials, *E.coli* cells from fermentation F190043 are used. The same amount of wet cell mass is disrupted in both buffers. The cell lysate is subsequently centrifuged and the supernatant filtered. In every experiment, the same amount of sample is applied to the IMAC as shown in the appendix Table 14. The peak areas of the elution peaks and the enzyme mass from the collected fractions are examined. Additionally, the specific enzyme activity of the elution fractions are measured. For the qualitative analysis, SDS-PAGE is performed.

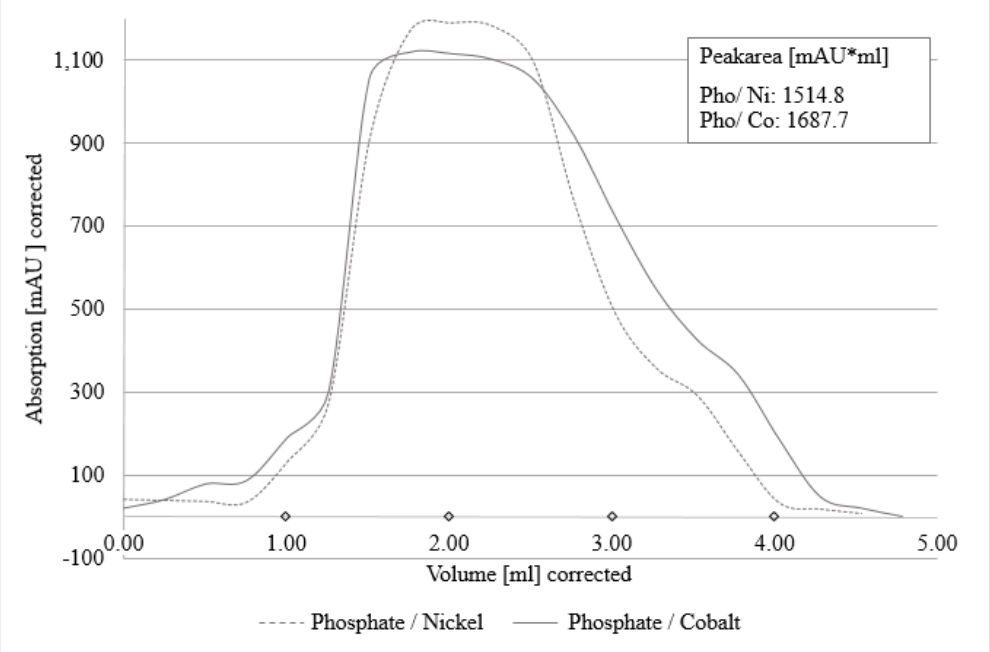

Figure 18: Comparison of IMAC elution curves for the purification of GlcNAc 2-epimerase (F190043) using 50 mM sodium phosphate buffer containing 50 mM imidazole and 0.3 M sodium chloride at pH 7.5 with Ni^{2+} and Co^{2+} charged resins. (Buffer = 50 mM sodium phosphate buffer containing 50 mM imidazole and 0.3 M sodium chloride at pH 7.5 with, Sample volume = 6.8 ml, column volume = 0.6 ml, flow rate = 1 ml/min, resin material = Talon Superflow and Profinity IMAC, application by pump P960). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline. The volume values were corrected to start from the beginning of the elution buffer application with 0ml.

To achieve a better comparability of the chromatograms, the UV values are corrected by the baseline value. For this purpose, the baseline value is subtracted from all absorption values. This is used only for the graphical illustration of the chromatography curves.

Additionally, the volume values were corrected to achieve a standardized starting point for all chromatograms. The volume starts with 0ml from the beginning of the elution buffer application.

Figure 18 shows the chromatograms of the elution peaks of GlcNAc 2-epimerase purification with Profinity Ni^{2+} and TALON Co^{2+} resins in sodium phosphate buffer. The squares indicate the beginning and end of the collected fractions. The chromatograms show that the curve of the Profinity Ni^{2+} column with approx. 1150 mAU has a higher maximum absorption than the curve achieved with the TALON Co^{2+} column with 1100 mAU. The total area of the curve from the TALON Co^{2+} sample is 1687.7 mAU*ml. It is thus 10.2% larger than the area of the curve achieved with Profinity Ni^{2+} purified samples with 1514.8 mAU*ml. The fractions protein concentrations show similar results as seen in

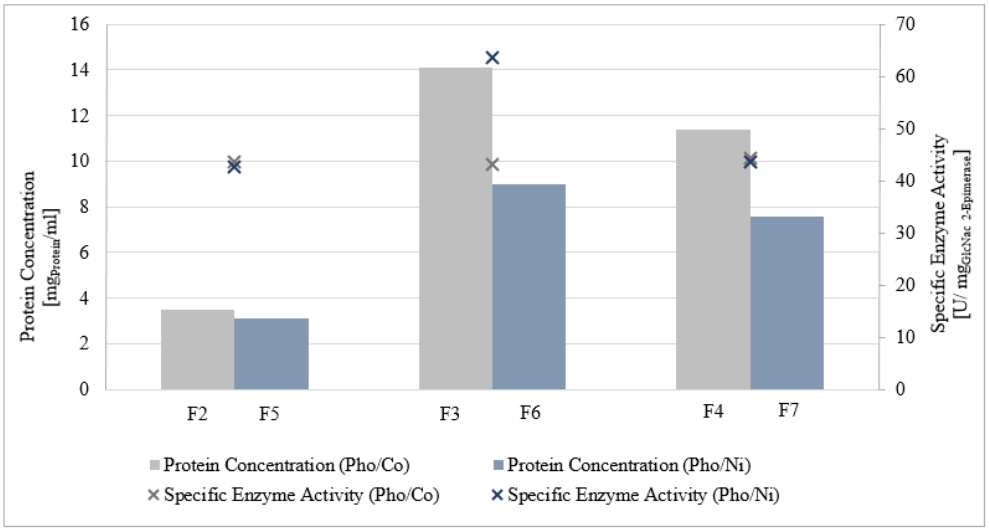

Figure 19: Protein concentration and specific enzyme activity of all GlcNAc 2-epimerase (F190043) elution fractions after IMAC with 50 mM sodium phosphate buffer with Profinity Ni^{2+} and TALON Co^{2+} resin. (F = Fractions of elution peak)

Figure 19. The protein concentrations of the 1ml fractions F2 to F4, purified with TALON Co^{2+}, have higher protein concentrations and thus higher protein masses. The protein concentrations in F2 was 3.5 mg/ml, in F3 14.1 mg/ml and in F4 11.4 mg/ml. Giving a total mass of 28.9 mg protein after elution. The protein concentrations eluted from the Profinity Ni^{2+} resin were lower. The following concentrations were measured in the fractions of Profinity Ni^{2+} purification: F5 with 3.1 mg/ml, F6 with 8.9 mg/ml and F7 with 7.6 mg/ml. This results in a total protein a mass of 19.6 mg protein, which is 32.2% lower compared to F2, F3 and F4. The first and last fractions show enzyme activity

values of about 43 U/mg. Here, no significant difference was found between the two purifications. The fraction F3 with 43.2 U/mg shows a 32% reduced activity compared to F6 with 63.6 U/mg.

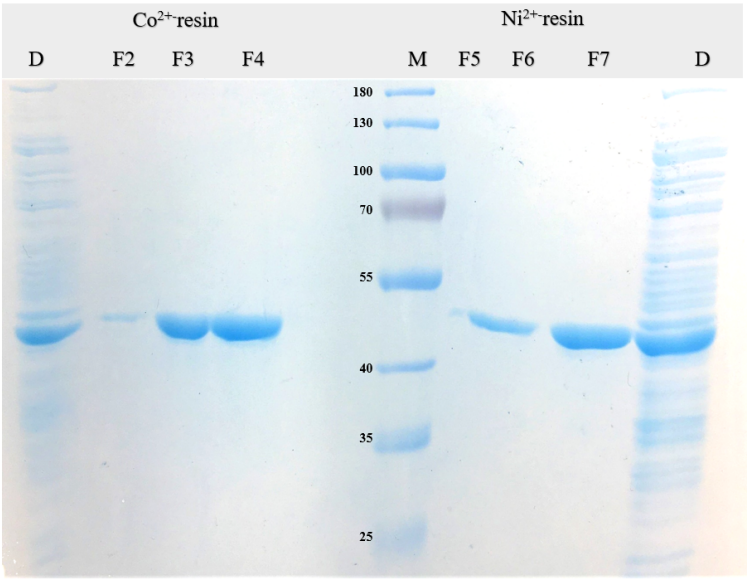

Figure 20: SDS-PAGE gel of GlcNAc 2-epimerase (fermentation F190043) fractions after IMAC with sodium phosphate buffer and Ni^{2+} or Co^{2+} resin. 10% SDS gel, all samples are diluted 1:10, 20 µl per sample and 5 µl of marker are applied. Stained with Coomassie Brilliant Blue R250. (M = marker with molecular masses in kDa, F = IMAC fraction, D = flowthrough out after sample application)

The gel of the SDS-PAGE shows the fractions F2 to F4 and F5 to F7 (Figure 20. The target enzyme GlcNAc 2-epimerase, with a mass of 46.8 kDa, is the only visible band in the fractions. GlcNAc 2-epimerase is also present in the flowthrough next to a large number of HCP bands. Figure 21 shows the elution peaks of the Tris samples with the Profinity Ni^{2+} and TALON Co^{2+} IMAC material. As in the previous example, the chromatogram has been corrected to enhance the comparability of the peaks. The squares show the beginning and end of the fractions used for analysis. The peaks of the Tris experiments show similar results as in Figure 18. The curve of the sample purified with the TALON Co^{2+} resin has with 950mAU a slightly smaller absorption maximum than the curve achieved from the Profinity Ni^{2+} purified sample with 1000 mAU, but shows a larger peak area. The sample peak purified with Profinity Ni^{2+} column has an area of 1124.7 mAU*ml and is thus 14.3% smaller than the TALON Co^{2+} purified sample peak with a stronger tailing, earlier elution and a total peak area of 1313.3 mAU*ml.

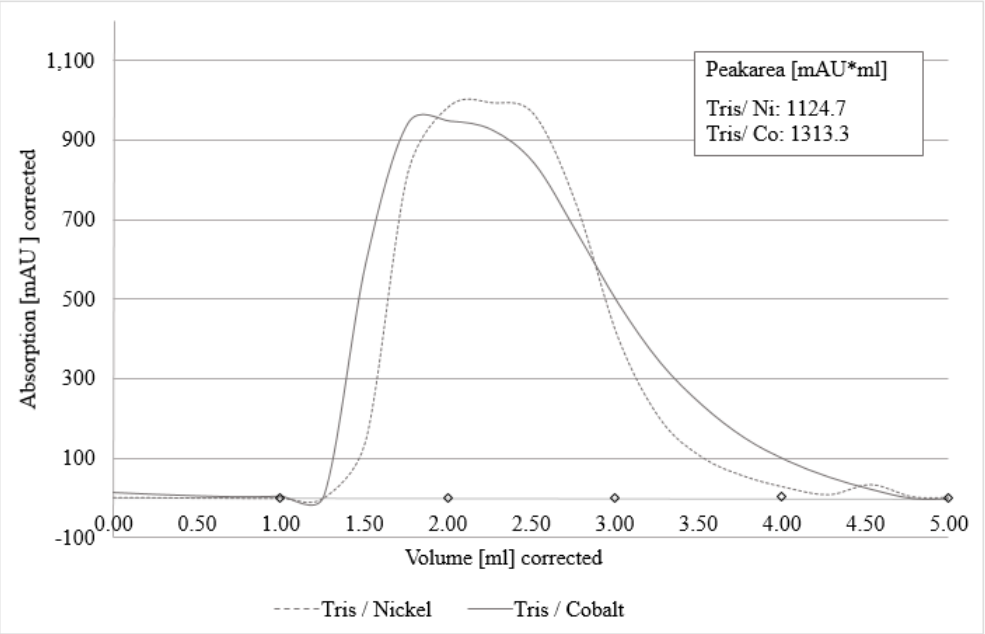

Figure 21: Comparison of IMAC elution curves for the purification of GlcNAc 2-epimerase (F190043) using 50 mM Tris buffer containing 50 mM imidazole and 0.3 M sodium chloride at pH 7.5 with Ni^{2+} and Co^{2+} charged resins. (Sample volume = 5.3 ml, column volume = 0.6 ml, flow rate = 1 ml/min, resin material = Talon Superflow and Profinity IMAC, application by pump P960). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline. The volume values were corrected to start from the beginning of the elution buffer application with 0ml.

The measured protein concentrations and enzyme activities of the individual fractions of both purifications with Tris buffer are shown in Figure 22. Due to the tailing of the curve from the sample purified with the TALON Co^{2+} resin, four fractions (F19 to F22) were collected and three (F12 to F14) for the curve from the purified samples with Profinity Ni^{2+} resin with 1ml each. This Figure shows that the fractions of the curve have a higher protein concentration but reduced enzyme activity. The fractions of the sample from TALON Co^{2+} purifications curve show in F19 with 2.0 mg/ml, F20 with 5.2 mg/ml, F21 with 4.6 mg/ml and F22 with 3.8 mg/ml a total protein mass of 15.6 mg. The fractions received after IMAC with a Profinity Ni^{2+} resin contain the following measured protein concentrations: F12 with 4.8 mg/ml, F13 with 7.2 mg/ml and F14 with 2.7 mg/ml with a total mass of 14.7 mg protein and thus a by 5.8% reduced protein mass compared to the samples received with the TALON Co^{2+} material. The specific enzyme activity of GlcNAc 2-epimerase in the fractions behaves contrary to the measured protein concentrations. The

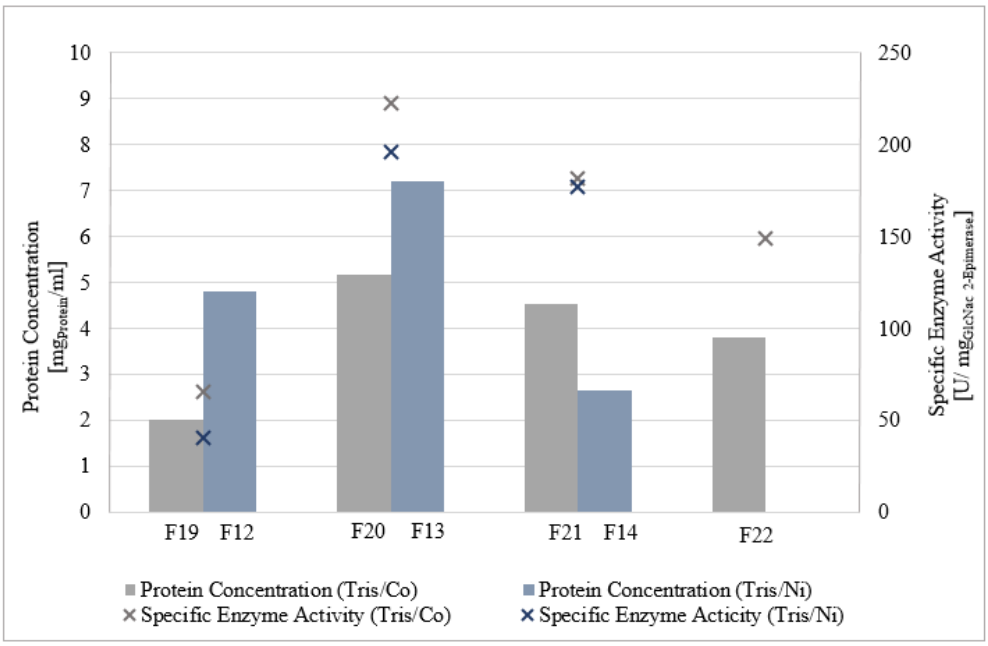

Figure 22: Protein concentration and specific enzyme activity of all GlcNAc 2-epimerase (F190043) elution fractions after IMAC with 50 mM Tris buffer with Profinity Ni^{2+} and TALON Co^{2+} resin. (F = Fractions of elution peak)

fractions F12, F13 and F14 with the higher protein concentrations show lower activity values. The samples of purified with TALON Co^{2+} resins have activities of F19 with 65.4 U/mg, F20 with 222.6 U/mg, F21 with 181.9 U/mg and F22 with 149.2 U/mg. The activities of the fractions of the curves from samples purified with Profinity Ni^{2+} with F12 40.1 U/mg, F13 with 196.3 U/mg and F14 with 177.2 U/mg are lower compared to the samples values purified with TALON Co^{2+} resins. The qualitative analysis of the target enzyme GlcNAc 2-epimerase in the elution fractions was performed by SDS-PAGE as seen in Figure 23. In Figure 23, the SDS-PAGE gel of the fractions F19 to F21 and F12 to F14 shows the GlcNAc 2-epimerase samples after purification via IMAC. With a molecular mass of 46.8 kDa, GlcNAc 2-epimerase is the only visible band in the shown fractions. In addition, a sample before IMAC application as well as the sample from flowthroughs are shown. The target protein is present before the IMAC and in smaller bands in the flowthrough. In order to compare all purifications performed with sodium phosphate buffer, Tris buffer and the Profinity Ni^{2+} and TALON Co^{2+} column material, the protein masses per pellet mass were calculated. The calculations from Table 29 show that purification with sodium phosphate buffer leads to the highest yields of the target enzyme. With a value of 18.4 mg/g, the purification of target enzyme with phosphate

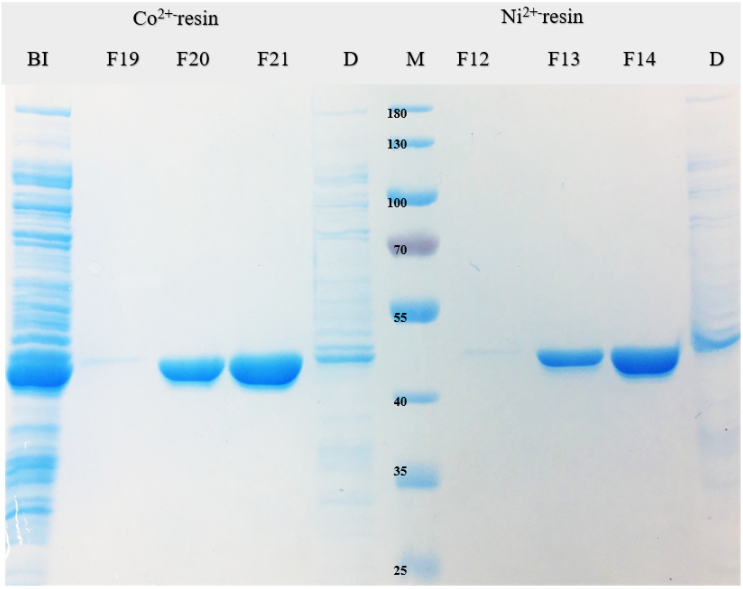

Figure 23: SDS-PAGE gel of GlcNAc 2-epimerase (fermentation F190043) fractions after IMAC with Tris buffer and Ni^{2+} or Co^{2+} resin. 10% SDS gel, all samples are diluted 1:10, 20 µl per sample and 5 µl of marker are applied. Stained with Coomassie Brilliant Blue R250. (BI = before IMAC, M = marker with molecular masses in kDa, F = IMAC fraction, D = flowthrough out after sample application)

and TALON Co^{2+} resin is most efficient. The Profinity Ni^{2+} material with 12.4 mg/g achieves a yield reduced by 32.6%. Purifications with the Tris buffer show a reduction of the purified enzyme mass in relation to the pellet mass. With values of 12.4 mg/g for samples purified with TALON Co^{2+}resin and 10.9 mg/g for samples from Profinity Ni^{2+} material, these purifications achieve a 32.6 and 40.8% lower yield of GlcNAc 2-epimerase compared to the phosphate and TALON Co^{2+} sample yield.

Table 29: Ratio of protein mass per wet cell weight. Calculated with the used pellet mass for the purification of GlcNAc 2-epimerase with sodium phosphate and Tris buffer as well as the measured protein mass of all elution fractions.

	Sodium Phosphate		Tris	
	Co^{2+}	Ni^{2+}	Co^{2+}	Ni^{2+}
WCW [g]	6.8	6.8	5.3	5.3
Protein Mass in Fraction Pool [mg]	28.9	19.6	15.6	14.6
Protein/WCW [mg/g]	**18.4**	**12.4**	**11.7**	**10.9**

In conclusion, these results show that a higher product yield is reached in the purification of GlcNAc 2-epimerase using the sodium phosphate buffer. The comparison of both

column materials shows that TALON columns loaded with Co^{2+} achieve a higher protein mass per wet cell mass.

4.2 Enzyme Stability

In order to produce the target proteins GlcNAc 2-epimerase and Neu5Ac lyase for the biocatalysis of Neu5Ac, storage of the enzymes for a short or even longer period of time is essential. The challenge here is to find optimal storage conditions for each enzyme. These conditions are very different depending on the enzyme characteristics and the storage conditions such as temperature. For the longer storage of both enzymes, lyophilization with different solvent (buffer and distilled water) and the influence of different excipients are investigated. However, lyophilization is too time-consuming to store proteins for a short time, so that storage of enzyme solutions in the refrigerator is preferred for this case.

4.2.1 GlcNAc 2-Epimerase Stability in Solution

GlcNAc2-epimerase from previous purifications showed significant losses in protein mass and enzyme activity when storing in solution in the refrigerator at 7 to 9 °C (data not shown). Therefore, the stability of epimerase in sodium phosphate and Tris buffer after purification with TALON Co^{2+} or Profinity Ni^{2+} columns were investigated. The used GlcNAc 2-epimerase (F190043) was purified with sodium phosphate or Tris buffer (see section 4.1.3). After purification, the enzyme was buffered via dialysis into sodium phosphate EQ or Tris EQ to remove imidazole. The solutions were filtered under a sterile workbench with sterile 0.2 µm syringe filters into previously autoclaved glass bottles. To determine the enzyme stability, the protein concentrations were determined at irregular intervals using Bradford and the enzyme activity was measured. The solutions were stored in the refrigerator at 7 °C during the whole observation period.
Figure 24 shows the measured protein concentrations of the enzyme solutions directly after dialysis and filtration (day 0) and after several measurements until day 49. The protein concentrations of the samples vary around the following mean values: Phosphate with Ni^{2+}: 3.47 mg/ml, Phosphate with TALON Co^{2+}: 6.07 mg/ml, Tris with Profinity Ni^{2+}: 3.74 mg/ml and Tris with TALON Co^{2+}: 3.56 mg/ml. All samples showed no loss of

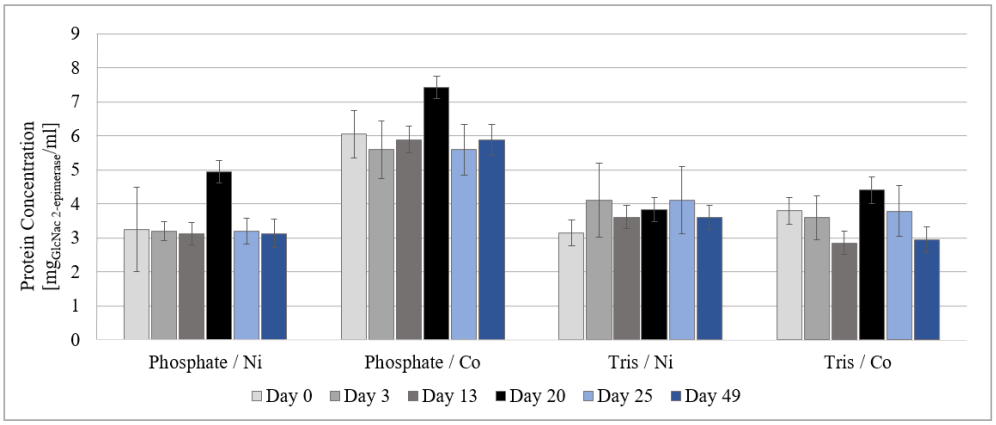

Figure 24: Protein Concentration of GlcNAc 2-epimerase solutions after several days stored in 50 mM sodium phosphate or 50 mM Tris buffer with 300 mM NaCl at pH 7.5 and purified with Co^{2+} or Ni^{2+} resins.

enzyme concentration during the entire storage period.
The measured enzyme activity of the samples over the entire storage period is shown in Figure 25. Also here, the activity was measured directly after dialysis and filtration and after several days of measurement up to day 49. Also here the measured values vary

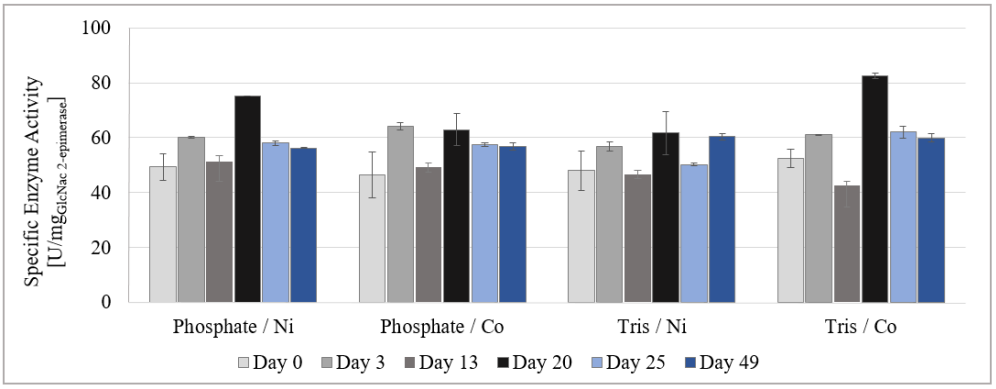

Figure 25: Specific enzyme activity of GlcNAc 2-epimerase solutions after several days stored in 50 mM sodium phosphate or 50 mM Tris buffer and purified with TALON Co^{2+} or Profinity Ni^{2+} resins.

with mean values around 58.4 U/mg for phosphate with Profinity Ni^{2+}, 56.1 U/mg for phosphate with TALON Co^{2+}, 5.9 U/mg for Tris with Profinity Ni^{2+} and 60.1 U/mg for Tris with TALON Co^{2+}. The activity values of all samples range between 45 U/mg and 60 U/mg. No loss of enzyme activity within the storage period is evident for all samples.

4.2.2 Enzyme Stability During Lyophilization

To determine the optimal conditions for the lyophilization of Neu5Ac lyase and GlcNAc 2-epimerase, both enzymes are freeze-dried with different concentrations of excipients. The stability of the enzymes after freezing or lyophilization is measured by the remaining enzyme activity with respect to the enzyme activity before, which was calculated according to equation 3.1. To understand whether the excipients have effects on enzyme stability during freezing or drying, these two phases of the process are considered separately. In all experiments, enzyme activity is determined after freezing as well as after freeze-drying. As can

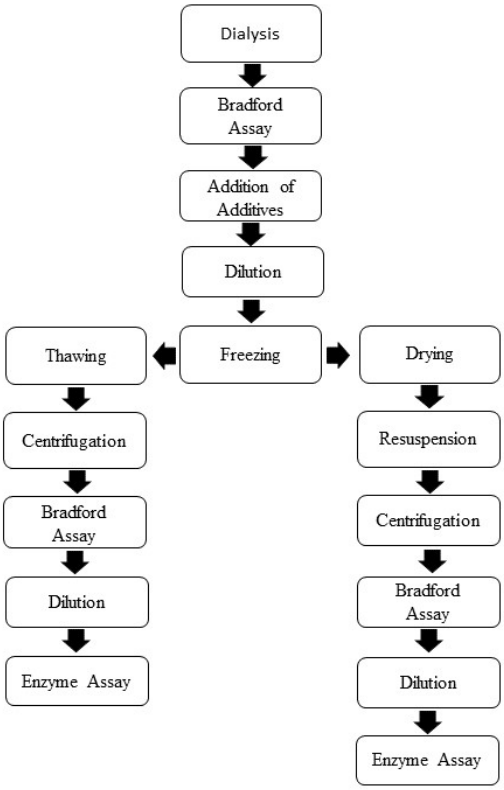

Figure 26: Steps for the preparation of GlcNAc 2-epimerase and Neu5Ac lyase samples for the determination of the enzyme stability after freezing and freeze-drying.

be seen in the following results, a high scattering of the measured values is visible, which is due to the measurement inaccuracies of several methods used. From the preparation of the sample to the measurement of the enzyme activity many steps are carried out (see Figure 26), when added up lead to a relatively high inaccuracy). In addition to the small sample volume during freeze-drying, handling errors during several dilution steps as well as measurement inaccuracies in the determination of protein concentration and enzyme activities play an important role. To put this into perspective, the number of measurements was increased to achieve the most accurate results possible.

4.2.2.1 GlcNAc 2-Epimerase

Purified GlcNAc 2-epimerase from different fermentation batches (F190046, F190040 and F190026) were rebuffered by dialysis in 20 mM Tris buffer with pH 7. After the addition of PEG and/or mannose, each sample had a GlcNAc 2-epimerase concentration of 2.5 mg/ml. In each run of experiments, nine different conditions were tested as shown in Table 17. Duplicates of 500 µl were prepared.
The results of the remaining enzyme activity of GlcNAc 2-epimerase after freezing and freeze-drying with different concentrations of excipients are shown in the Figures 27 and 28.
These Figures show the percentage of enzyme activity remaining in respect of the measured activity after dialysis and before freezing or freeze-drying (100% would be equal to no loss in activity). The effects of freezing of the samples and freezing with subsequent drying

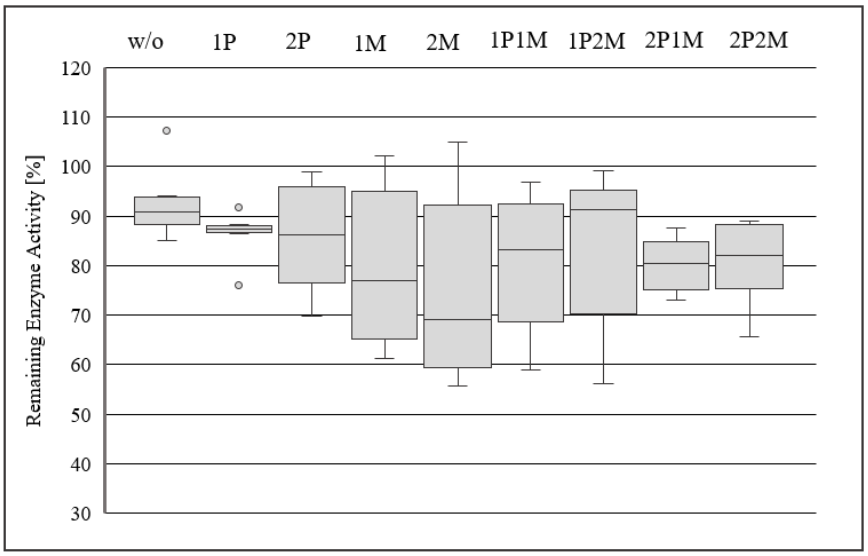

Figure 27: Percent remaining enzymatic activity of GlcNAc 2-epimerase in 20 mM Tris buffer with pH 7 after freezing with different PEG and mannose concentrations. Per tested condition n = 14. (w/o = without excipients, 1P = 1% PEG, 2P = 2% PEG, 1M = 10 mM mannose, 2M = 20 mM mannose)

have different effects on enzyme activity. For all samples except 1M and 2M the remaining enzyme activity after freezing is higher than after freeze-drying, with an average median of about 85%. Five out of nine tested conditions from Figure 27 shows large scattering of the measurements with an interquartile range of up to 30%. Based on the median, the samples 1M and 2M show the lowest enzyme activity after freezing with 77% and 69%.

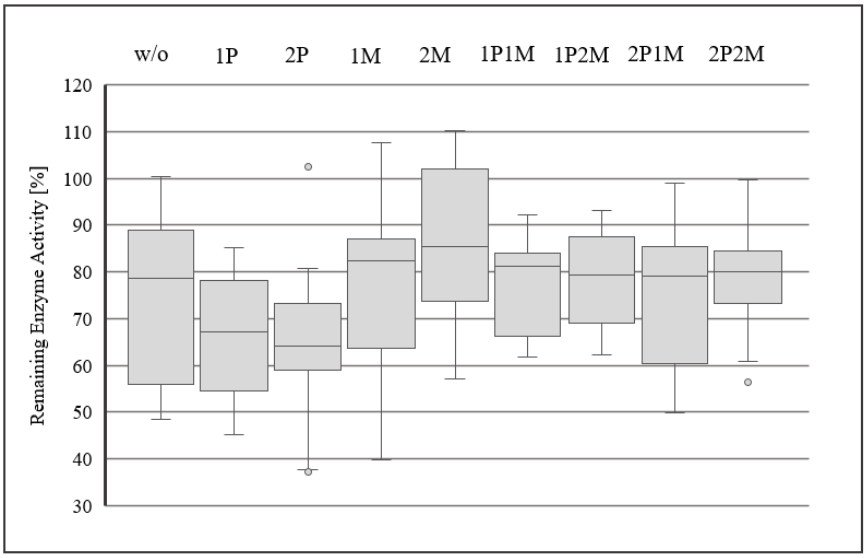

Figure 28: Percent remaining enzyme activity of GlcNAc 2-epimerase in 20 mM Tris buffer with pH 7 after lyophilization with different PEG and mannose concentrations. Per tested condition n = 14. (w/o = without excipients, 1P = 1% PEG, 2P = 2% PEG, 1M = 10 mM mannose, 2M = 20 mM mannose)

The highest measured activities can be found in the samples w/o (92%) and 1P1M (93%). The graphs in Figure 28 show an even distribution of the dispersion of the measured data after lyophilization with interquartile ranges from 10 to 30%. With a remaining average value of 78%, all samples show a reduction in the measures enzyme activity. In contrast to the measurements of frozen samples, the lyophilized sample 2M has the highest remaining enzyme activity with a median of 84%. The lowest enzyme activities are measured for the samples 1P (68%) and 2P (65%).
The results of stability determination after lyophilization of GlcNAC 2-epimerase show that the use of high mannose concentrations leads to the highest enzyme activity obtained.

4.2.2.2 Neu5Ac Lyase

Purified Neu5Ac lyase from fermentations of different batches (F190040 and F190031) were rebuffered via dialysis in 20 mM Tris buffer with pH 7 and distilled water. After the addition of PEG and/or mannose, each sample had a Neu5Ac lyase concentration of 3.0 mg/ml. In each run, nine different conditions were tested as shown in Table 17. Duplicates of 500 µl of each sample were prepared.
The Figures 29 and 30 show the remaining enzyme activity of Neu5Ac lyase in 20 mM Tris

buffer after freezing and freeze-drying. After several measurements with Tris buffered samples, low enzyme activities were obtained for all conditions. These Figures show the percentage of enzyme activity remaining after freezing or freeze-drying in respect of the measured activity after dialysis (in Tris or distilled water), where 100% would be equal to no loss in activity. In Figure 29, all samples show low remaining enzyme

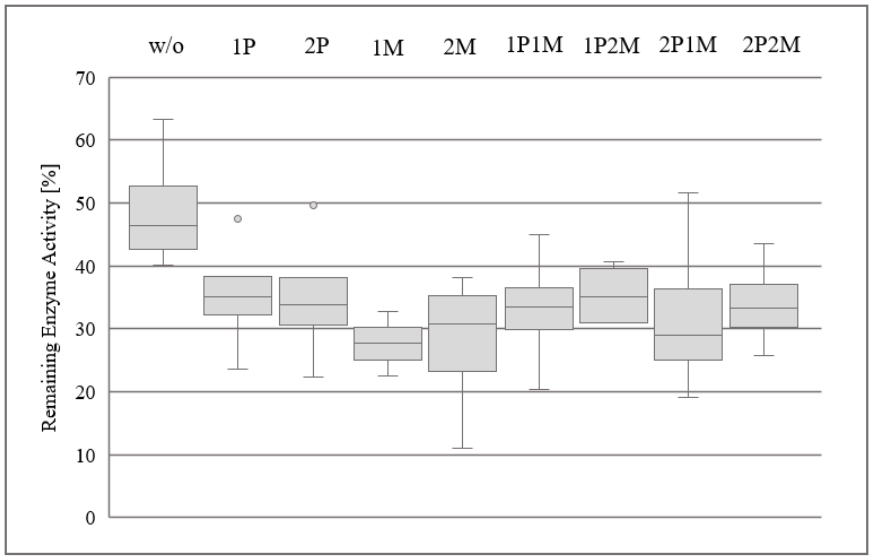

Figure 29: Percent remaining enzyme activity of Neu5Ac lyase in 20 mM Tris buffer with pH 7 after freezing with different PEG and mannose concentrations. Per tested condition n = 6. (w/o = without excipients, 1P = 1% PEG, 2P = 2% PEG, 1M = 10 mM mannose, 2M = 20 mM mannose)

activities after freezing. The mean median of the samples of all conditions is about 35%. An exception is the sample w/o, which shows a 11% higher remaining enzyme activity for Neu5Ac lyase. Overall, the measurements of the activities of Neu5Ac lyase show a lower scattering than for GlcNAc 2-epimerase. The measurements interquartile ranges between 5 to 10%. The samples 1M and 2P1M show the lowest activity values with 28 and 29%. Even after lyophilization of the samples, the sample condition w/o shows the highest remaining enzyme activity with 29% (see Figure 30). Similar to the activity measurements for GlcNAc 2-epimerase, a reduction of the remaining enzyme activity after freeze-drying can be determined for Neu5Ac lyase. Compared to the frozen samples, a 10% reduction (median of all conditions approximately at 25%) in remaining activities was measured. The minimum value in these measurements was found for sample 1P1M with 19% remaining enzyme activity. In addition, the activity of Neu5Ac lyase was determined in distilled water (see Figure 31). In contrast to the Tris buffered samples, Neu5Ac lyase in

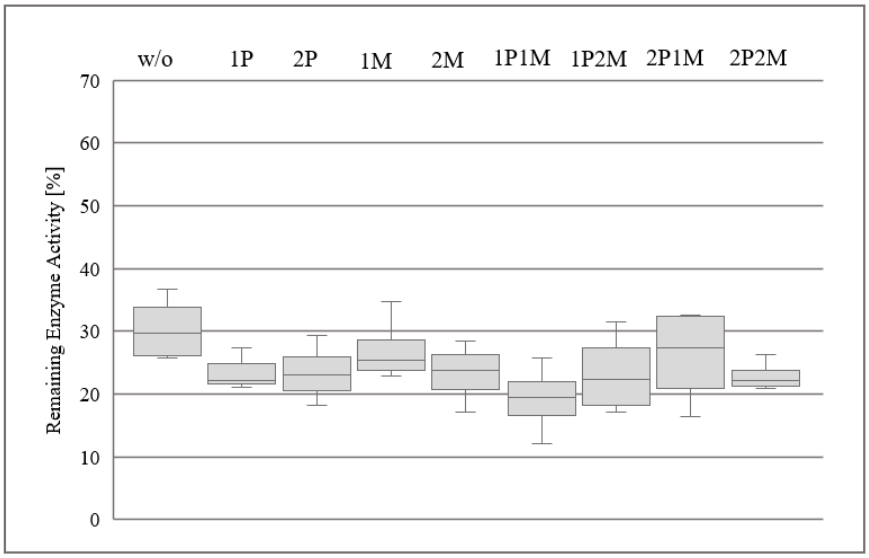

Figure 30: Percent remaining enzyme activity of Neu5Ac lyase in 20 mM Tris buffer with pH 7 after lyophilization with different PEG and mannose concentrations. Per tested condition n = 6. (w/o = without excipients, 1P = 1% PEG, 2P = 2% PEG, 1M = 10 mM mannose, 2M = 20 mM mannose)

distilled water shows significantly higher remaining enzyme activities. After freezing the sample, the measurements median was about 86%, and thus approximately 51% higher than the median of all conditions (except w/o) in Figure 29. Similarly, the sample in distilled water shows with a median of 81% a reduction of the remaining enzyme activity after lyophilization. Despite the high activity values obtained, the sample lyophilized in distilled water shows difficulties in resuspension in water as well as in Tris buffer for the enzyme activity assay. This problem has been observed in about 50% of the sample which were lyophilized in distilled water.

Lyophilization of Neu5Ac lyase in Tris buffer with different excipients, resulted in reduced values of remaining enzyme activity. The experiments showed that freezing and drying in distilled water leads to significantly higher activity values.

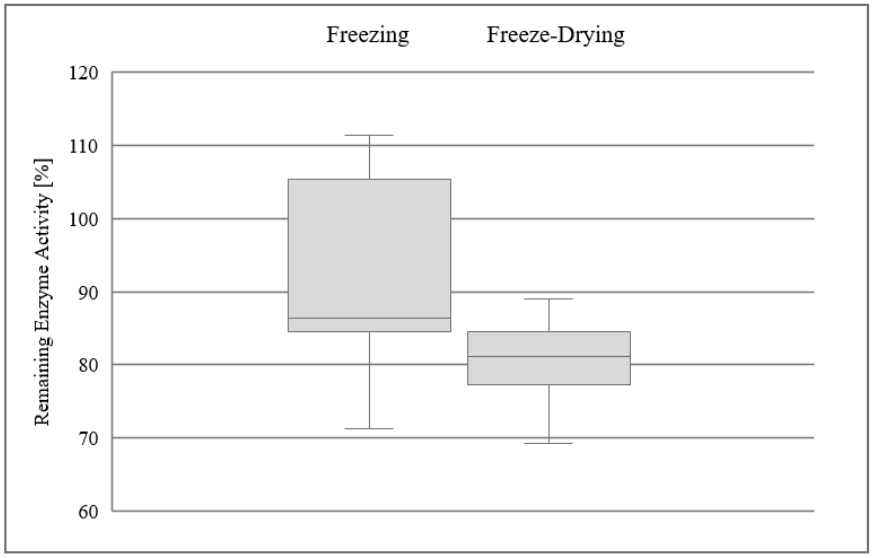

Figure 31: Percent remaining enzyme activity of Neu5Ac lyase after freezing and freeze-drying in distilled water. Per tested condition n = 8.

5 Discussion

This section focuses on the interpretation of the results for the optimization of purification and development of lyophilization for GlcNAc 2-epimerase and Neu5Ac lyase.

5.1 Purification of Neu5Ac Lyase and GlcNAc 2-Epimerase

The optimization of the purification strategy includes several issues. These involve implementing new filters, determining the binding capacities and lifetime of chromatography columns and improving GlcNac 2-epimerase purity.

5.1.1 Implementation of Filter Capsules

The implementation of the Whatman TC filter capsules has the goal to replace the previously used cellulose acetate filters and vacuum pump. This exchange decreases the duration of the time-consuming filtration step. The tested Whatman Polycap TC filter capsules are described as in-line filters for a safe and efficient filtration for critical biological and tissue culture solutions [Whatman Inc., 2019]. Each filter contains two layers of polyethersulfone (PES) membranes. One functions as a prefilter to ensure a longer filter life and high efficiency. Each filter has an effective filtration area (EFA) of 550 cm^2. Two different filter types were tested: one with a 0.6 µm prefilter and 0.45 µm filter and the second one with two 0.22 µm filters. Examination of the new filter system was necessary before integration it into the standard workflow. One value for the verification is the determination of protein binding or structural changes by filter adsorption effects. An important factor for these effects is the surface area of the filter. The surface area of filters combines the frontal area and the internal surface area which are described as EFA. With the surface area, the potential of protein binding increases. In addition, membrane materials show different adsorption potentials. The PES membranes are hydrophilic and have very low adsorption properties [McKinnon and Avis, 1993]. In experiments to determine protein binding in relation to the total surface area, PES membranes show very low values of bound protein compared to other conventional membrane materials (see Table 30).

Table 30: Bound BSA in relation to the membrane surface area for different membrane types.(BSA= bovine serum albumin, BET= membrane surface area determined trough *Brunauer, Emmett* and *Teller* principle. Data from Merck Online Learning Center [Merck, 2020])

Membrane	ng BSA bound/cm^2 BET area
PES	11.0
Nylon	353.0
Mixed esters of cellulose	2540.0

The mentioned BET is described as membrane surface area measured according to the *Brunauer, Emmett* and *Teller* method. With this principle the surface size is determined by gas adsorption onto a solid surface [Naderi, 2015]. Since gas molecules are significantly smaller than proteins, the area accessible to proteins is reduced. The BET area is equal to the EFA. Experiments were performed to determine protein binding or structural changes of GlcNAc 2-epimerase and Neu5Ac lyase. Protein binding was analysed by changes in enzyme mass. Structural changes are reflected in reduced enzymatic activity. Both filters were tested for the recovery of enzyme concentration and activity.
The experimental results in the tables 25 and 26 show that protein binding is present in small amounts, compared to the used protein mass. The mass of GlcNAc 2-epimerase is reduced by 3.9% after second filtration. For Neu5Ac lyase, a reduction of 4.6% was calculated. Both experiments show that the loss of protein mass is correlated with the loss of activity. A 1.9% reduced activity was detected for GlcNAc 2-epimerase and 4.8% for Neu5Ac lyase. It should be acknowledged that inaccuracies in protein concentration measurements by Bradford, as well as inaccuracies in sample volume determination, lead to variations in the measurement results. This has to be considered especially with such small deviations.

Table 31: Bound enzyme in relation to the membrane surface area calculated for Whatman TC filter capsules. Determined for GlcNAc 2-epimerase and Neu5Ac lyase after filtration with 0.6/ 0.4 µm and 0.2 µm filters.

Enzyme	ng enzyme bound/cm^2 EFA
GlcNAc 2-epimerase	98.18
Neu5Ac lyase	140.00

The calculated values for bound protein per EFA are higher (see Table 31) than the values for PES membranes with BSA from Table 30. Besides the protein binding of the membrane, adsorption effects of the capsule material and the pump tubes could play a part. It should be considered that adsorption effects vary with different proteins.
The filtration rate is another important value for the verification of a new filter system. In this case it is difficult to determine the exact filtration rate. The peristaltic pump has no precise speed regulation and the variability of the vacuum pump's flow rate also made a comparison of the two methods problematic. The flow rate at which the cell lysate is transported through the membrane is significantly dependent on the viscosity. This increases with increasing wet cell mass in the lysate. In this case, only a relative estimation of the saved time was done. Based on the extensive experience with the filtration of large volume cell lysates with cellulose acetate membrane filters, it can be said that a filtration time of two hours can be expected. The new filter capsules reduces the filtration time by at least 50%.
Due to the time saving and the acceptable loss of enzyme mass and activity, the filter capsules can be used for the filtration of large cell lysate volumes in the purification workflow of GlcNAc 2-epimerase and Neu5Ac lyase.

5.1.2 Column Performance of Ni and Co Charged IMAC Resins

In these experiments the binding capacities and lifetime of Co^{2+} and Ni^{2+} columns for GlcNAc 2-epimerase and Neu5Ac lyase were determined. The results showed significant differences between the enzymes and column materials. The Co^{2+} material showed a binding capacity of 26.7 mg/ml. Compared to the Ni^{2+} material (11.6 mg/ml), twice as much GlcNAc 2-epimerase was bound. The same was observed with the calculated binding capacity for Neu5Ac lyase. The Co^{2+} column binds up to 79.6 mg/ml and the Ni^{2+} column 56.5 mg/ml of enzyme.

Table 32: Data overview of TALON Superflow and Profinity IMAC resins. ([1] Data for the estimation of the binding capacites are given in the section 2.2.1 [Takara Bio, 2020; Bio-Rad Laboratories Inc., 2019])

	TALON Superflow	Profinity IMAC
Bound Metal Ion	Co^{2+}	Ni^{2+}
Binding Capacity[1] [mg/ml resin]	5 – 20	10 – 15
Metal Ion Capacity [µmol bivalent ion/ml]	≥ 12	12 – 30

The different binding capacities between the materials are based on the different properties of the column material as shown in Table 32. As already described by *Wong et al.* in 1991, the matrix of the column has a considerable influence on the stability of the complexes formed between the metal ion and the protein [Wong et al., 1991]. These include properties such as the pore size, the charge capacity of the surface area. The type of ion binding to the matrix and the number of exposed coordination sites of the metal ion also have effects [Bio-Rad Laboratories Inc., 2019]. The results show that the TALON material with Co^{2+} ions is capable of binding more target enzyme despite a reduced number of metal coordination sites compared to Profinity columns. According to *Clontech Laboratories*, the Co^{2+} loaded TALON reactive core has a higher binding affinity to (His_6)-tagged proteins than other Ni^{2+} loaded column materials [Clontech Laboratories, 2019a]. The higher binding capacities received in these experiments indicate a stronger binding of the recombinant enzymes to the TALON material.
The fact that approximately twice the amount of Neu5Ac lyase can be bound as GlcNAc 2-epimerase is unusual given the large molecular mass of the lyase. As a homotetramer with 34.3 kDa per subunit, Neu5Ac lyase has a molecular mass of 137.2 kDa. The homodimer GlcNAc 2-epimerase has 96.3 kDa. It can be assumed that Neu5Ac lyase binds to the column as a monomer and can occupy more binding sites as a smaller molecule. However, if the bound molecular mass per column volume is considered, the following results from Table 33 and 34 are obtained.

Table 33: Capacities and calculated molmasses bound per ml resin for multimeric Neu5Ac lyase structures. Calculations are based on the determined binding capacites for Co^{2+} charged and Ni^{2+} charged resin materials.

	Molecular Mass [kDa]	Binding Capacity [mg/ml resin]		Molmass Capacity [mmol/ml resin]	
Resin		Co^{2+}	Ni^{2+}	Co^{2+}	Ni^{2+}
Monomer	34.3	56.5	79.6	2.32	1.65
Dimer	68.6	56.5	79.6	1.16	0.82
Trimer	102.9	56.5	79.6	0.77	0.55
Tetramer	137.2	56.5	79.6	0.58	0.41

Table 34: Capacities and calculated molmasses bound per ml resin for monomeric and dimeric GlcNAc 2-epimerase structures. Calculations are based on the determined binding capacites for Co^{2+} charged and Ni^{2+} charged resin materials.

	Molecular Mass [kDa]	Binding Capacity [mg/ml resin]		Molmass Capacity [mmol/ml resin]	
Resin		Co^{2+}	Ni^{2+}	Co^{2+}	Ni^{2+}
Monomer	46.8	26.7	11.6	0.57	0.24
Dimer	93.6	26.7	11.6	0.29	0.12

These calculations show the bound molar mass per column volume. Assuming that GlcNAc 2-epimerase binds as a dimer, this gives 0.24 or 0.12 mmol/ml resin. With the molecular mass of the tetramer, Neu5Ac lyase yields 0.58 or 0.41 mmol/ml resin. If monomers of the lyase would actually bind to the column, this would result in bound molecular masses of 2.32 and 1.65 mmol/ml. Thus, the binding of Neu5Ac lyase as monomer would lead to a fivefold higher molar mass capacity compared to the capacity for dimeric GlcNAc 2-epimerase. However, the protein binding capacity of columns with Neu5Ac lyase is only twice as high. Based on these factors, it seems more likely that Neu5Ac lyase binds as a tetramer. The molar mass capacity of the columns for tetrameric Neu5Ac Lyase is twice as high as the capacity for GlcNAc 2-epimerase.

The point that Neu5Ac lyase binds better to the IMAC material despite a higher molecular mass, can be explained by the number and position of the (His_6)-tag (see Figure 3). The three-dimensional structure of the homotetramer shows four free accessible histidine residues at the N-terminal end. In contrast to the homodimer GlcNAc 2-epimerase, the Neu5Ac lyase has two additional binding sites for binding to metal ions. It can be assumed that the binding capacity of a metal chelate increases the more binding sites are present on the protein. The histidine groups are not permanently bound to the coordinating side, there is a balance of binding and dissociation [Porath, 1992]. A higher number of freely accessible His-tags thus increases the probability of binding to the metal chelate.

It is notable that for both enzymes significantly higher binding capacities were achieved than stated by the manufacturer (shown in Table 32). The highest values are 20 mg/ml for the Co^{2+} TALON column and 15 mg/ml for Ni^{2+} Profinity IMAC [Takara Bio, 2020; Bio-Rad Laboratories Inc., 2019]. The binding capacity depends on the size of the target protein, its three-dimensional structure, accessibility and number of polyhistidines and

also chromatographic parameters such as the flow rate [GE Healthcare, 2016]. Non-specific protein-protein or protein-matrix interactions are unlikely. Hydrophobic binding are reduced by the addition of NaCl in the buffers [Bornhorst and Falke, 2000]. Thus, the preferential binding of the target enzymes to the IMAC materials and also a slow flow rate lead to increased binding capacites.
Similar to the determination of the binding capacities, the determination of the IMAC material lifetime showed enzyme-specific results. The TALON Co^{2+} and the Profinity IMAC Ni^{2+} column showed no reduction in binding capacity after 15 and 16 chromatography runs with GlcNAc 2-epimerase. In contrast, both columns showed a significant reduction in binding capacity after nine to ten runs for Neu5Ac lyase. A precise reason for the enzyme-specific loss of binding capacity is difficult to identify. Many factors influence the protein-metal ion binding. A known factor for the loss of binding capacity is the process known as metal ion transfer (MIT). MIT depends on the binding affinity of the protein binding sites and the metal chelate. Due to the presence of a 'high affinity' binding site of the protein and possibly other coordination sites in the vicinity, the metal ion is preferentially bound to the protein [Sulkowski, 1989]. In contrast to metal ion leaching, which is caused by a weak binding of the ligand to the metal ion, this effect is enzyme-specific. This explains why the binding capacity is reduced after several chromatography runs with Neu5Ac lyase only. By binding metal chelates to the protein, they are removed from the column and the column binding sites are reduced.
The use of the TALON Co^{2+} material is favoured. The experiments showed higher measured binding capacities for TALON Co^{2+} columns for both enzymes and a longer lifetime of the material for Neu5Ac lyase. It should be noted that these results were obtained with pure enzyme solutions and the presence of HCPs affects both parameters.

5.1.3 Comparison of IMAC Resin and Buffer Variations for GlcNAc 2-Epimerase Purification

The comparison of the TALON Co^{2+} column with the Profinity IMAC Ni^{2+} column for the purification of GlcNAc 2-epimerase, shows significant differences in the enzyme yield using different buffers. With Tris buffer, the obtained protein yield after GlcNAc 2-epimerase purification is reduced. Usually sodium phosphate buffer is the first choice for the purification of proteins via IMAC. However, the most suitable buffer is enzyme specific and

should be investigated. Studies on the production of GlcNAc 2-epimerase by *Wong et al.* in 1991 used Tris buffers for the purification with IMAC and Profinity Ni^{2+} resin [Wong et al., 1991]. The results of *He et al.* from 2014 showed in a comparison, that Tris leads to a higher purity for the purification of miraculin. Miraculin is a tetrameric glycoprotein with a mass of 26.4 kDa per subunit [He et al., 2014]. However, the reduced yield of GlcNAc 2-epimerase from purifications experiments with Tris Buffer can be explained by the competitive binding of the present amines with the metal ions. These primary amines reduce bivalent metal ions and thus leads to a reduction of the freely available metal ions on the column. This effect depends on the pH value of the buffer, the Tris concentration and the binding strength of the proteins to the column material. Studies show that at pH 7.5, complex formation of Tris with TALON Co^{2+} or Profinity Ni^{2+} is weak (compared to Hg) but still present [Hanlon et al., 1966]. The decrease of freely available coordination sites of the TALON Co^{2+} and Profinity Ni^{2+} ions results in a loss of target protein and a reduced yield.
Experiments to investigate the binding capacity of TALON Co^{2+} reveals higher loadings of the target enzyme and as a result a higher yield. Similar to the results from the column performance experiments (as discussed in the previous section), this can be explained by a possible higher binding affinity. The experiments of column performance and the direct comparison of the values obtained from the purification experiments, indicate an increased preferential binding of the TALON Co^{2+} column for (His_6)-tagged proteins.
In addition to protein concentration, the specific enzyme activity of the fractions was determined. The enzyme activities of all fractions with sodium phosphate buffer are similar and indicate a comparable result of enzyme stability. Compared to these values, the measured activities of the fractions in Tris buffer are increased fourfold. If one considers these values in relation to the measured activities for the determination of enzyme stability in solution (section 4.2.1), the impression is strengthened that these values result from an error in the assay performance. The specific enzyme activities from the GlcNAc 2-epimerase samples for stability experiments with Tris for TALON Co^{2+} and Profinity Ni^{2+}, show values of about 50 U/mg after rebuffering. These values are comparable to the phosphate samples from the same experiments. Since the phosphate samples do not show any loss of activity caused by rebuffering and filtration, it can be assumed that the values of the Tris samples after rebuffering are representative. Therefore, a measurement error is responsible for the high activity values obtained in the fractions with Tris from the purification experiments.

With focus on prior purifications, a special interest was the optimization of the GlcNAc

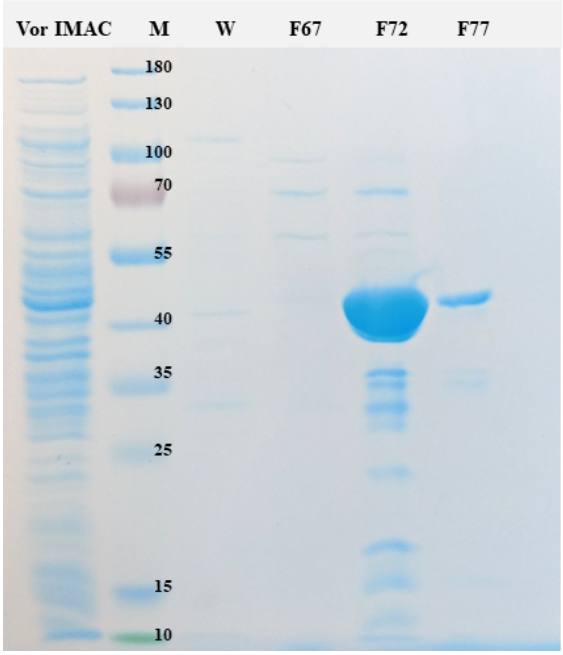

Figure 32: SDS-PAGE gel of IMAC fractions of GlcNAc 2-epimerase (F190025) containing impurities. IMAC was performed with a Co^{2+}-charged resin. Molecular mass of GlcNAc 2-epimerase = 46.8 kDa. 86.5 g WCW pellet were resupended in 400 ml equilibration buffer. 10% SDS gel, all samples are diluted 1:10, 2 µl per sample and 5 µl of marker are applied. Stained with Coomassie Brilliant Blue R250. (M = marker with molecular masses in kDa, W = wash out after sample application, F = IMAC fractions)

2-epimerase purity. Figure 32 shows an SDS-PAGE from previous GlcNAc 2-epimerase (fermentation F190025) purification via IMAC. It was assumed that the TALON Co^{2+} columns bind HCPs with easily accessible His residues and thus lead to impurities (Figure 32, lane 72). In the Figures 20 and 23, the gels from this purification experiments did not show detectable contaminations. The absence of contaminations can be caused by a variety of factors that influence the binding behaviour. An important factor here is the amount of protein applied in relation to the column volume (see Table 35). Higher protein masses of 554.2 mg/mL (phoshate buffer samples) and 249,6 mg/ml (Tris buffer samples) were applied in these experiments compared to 136,8 mg/ml with F190025. As can be seen in the gels of the SDS-PAGEs from the GlcNAc 2-epimerase purification experiments (see Figures 23 and 20), the binding capacity of the column was exceeded. Resulting in bands of the target protein appearing in the flowthrough. With such large masses of total proteins, a large amount of target protein is also applied to the column and is able to bind. Due to the preferential binding of (His_6)-tagged proteins on the columns, all freely

Table 35: Protein mass applied in relation to column volume. Comparison of the experimental results obtained from GlcNAc 2-epimerase purification (F190043) with previous purification (F190025). The protein mass is based on the total protein concentration and the sample volume applied on the column.

Sample	Protein Mass in Sample [mg]	CV [ml]	Protein Mass per CV [mg/ml]
Phosphate (F190043)	332.5	0.6	554.2
Tris (F190043)	149.8	0.6	249.6
Purification (F190025)	13,680.0	100	136.8

accessible binding sites of the metal ions are loaded with target protein, resulting in a small amount of bound HCPs. Another factor is the application of a lower flow rate due to the smaller column volume. This can influence the binding of target enzymes and HCPs. In addition, a different *E.coli* batch (fermentation F190043) was used for these experiments. By continuously changing the fermentation conditions to optimize the expression of GlcNAc 2-epimerase, some characteristics of the bacterial HCPs can be changed.

Due to the higher yield of GlcNAc 2-epimerase and the absence of other proteins in the fractions, the purification of GlcNAc 2-epimerase with 50 mM sodium phosphate buffer and the TALON Co^{2+} column material is recommended. When necessary, other approaches may be used to increase the purity of the target enzyme without changing the metal ions or buffer salts. Besides using the complete binding capacity to inhibit the binding of HCPs, imidazole can already be added to the equilibration buffer and during washing of the column. As described in the article by *Bornhorst* in 2000, the addition of < 20 mM imidazole in the sample buffer reduces the binding of non-(His_6)-tagged proteins to the metal ions [Bornhorst and Falke, 2000]. However, the exact concentration of imidazole depends on the strength of the protein-metal-bond and should be determined experimentally beforehand.

5.2 Enzyme Stability

Enzyme stability experiments include storage of GlcNAc 2-epimerase in solution and lyophilization of GlcNAc 2-epimerase and Neu5Ac lyase.

5.2.1 GlcNAc 2-Epimerase Stability in Solution

In order to investigate the enzyme storage stability of GlcNAc 2-epimerase in solution, the purified enzyme was re-buffered in sodium phosphate or Tris. The protein concentration and specific enzyme activity were determined at irregular intervals. In previous purifications of GlcNAc 2-epimerase (data not shown), the formation of precipitates was observed after several days of storage. This turbidity of the enzyme solution was accompanied by odour formation and loss of protein mass and activity. Measurements showed an average protein loss of 20% and a reduction in activity of 65%, after three weeks storage of GlcNAc 2-epimerase from F190026. The formation of turbidity and odour indicate microbial growth. The loss of protein concentration and activity indicates proteolytic activity caused by microbial contamination. Even small amounts of contaminated proteases can lead to large losses in activity [Ó'Fágáin and Colliton, 2017].
Samples from the GlcNAc 2-epimerase purification with Ni^{2+} and Co^{2+} columns as well as phossphate and Tris buffers were used for this study. To avoid contamination of proteases arising from microbial growth, the samples were sterile filtered into autoclaved flasks under a safety cabinet. Additionally to the sterile conditions, the samples are stored separately to determine a difference in stability depending in the purification conditions. The results of the protein concentration and enzyme activity assays show no reduction over a period of 49 days. The reduced protein concentration of the samples, compared to the fractions of the purification, is caused by protein loss and dilution during rebuffering and sterile filtration. The concentration values remain constant over the entire storage period. The results for the specific enzyme activity are undergoing severe deviations. These are due to the conditions under which the GlcNAc 2-epimerase assay is performed (see section 4.2.2). The constant values indicate that the previously observed turbidity and loss of activity are entirely a result of proteolytic activities caused by microbial contamination. An influence of the buffer salts on the enzyme stability is not proven.
Due to the stable results of all enzyme solutions over a period of 49 days, the use of sterile vessels and filtration under sterile conditions for storage is recommended. Tris

and sodium phosphate are suitable buffers for storage. However, due to the fact that Tris is not suitable for the purification of GlcNAc 2-epimerase, as determined in previous experiments, rebuffering after purification is preferred.

5.2.2 Enzyme Stability During Lyophilization

The aim of these experiments was to determine suitable excipients and concentrations to maintain a high activity of GlcNAc2-epimerase and Neu5Ac lyase after lyophilization. Both enzymes were observed with different concentrations of mannose and PEG for their stability after freeze-thawing and freeze-drying. The results of the remaining enzyme activities show significant differences between the two proteins. GlcNAc 2-epimerase shows high recovery after freeze-thawing and lyophilization. Neu5AC lyase loses most of its activity in the presence of Tris, mannose and PEG.
To avoid pH shifts during the freezing process by formation of eutectic mixtures, a low concentrated Tris buffer was chosen. Other buffer types, like the frequently used phosphate buffer, show pH shifts of up to 3 units. This shift arises from the precipitation of the low soluble Na_2HPO_4 salt [Jiang and Nail, 1998]. Results of *Gloger* from 2002 show, that the use of Tris buffer for lyophilization leads to significantly lower changes in the pH value [Gloger, 2002].
The results for the stability of GlcNAc 2-epimerase show that the addition of mannose alone leads to low recovery. The highest remaining activity is achieved by the buffer alone or addition of 1% PEG and 20 mM mannose. The results support the hypothesis of *Carpenter et al*. from 1993. Sugars are not suitable as cryoprotectant in low concentrations and only stabilize sufficiently during drying in combination with at least 1% PEG [Carpenter et al., 1992]. However, this increase in stability is dependent on concentration and varies for every protein. In the stabilization experiments it is achieved with a concentration of 20 mM mannose and 1% PEG. Other excipients tested show slightly reduced activities. The high remaining activity of the enzyme in presence of the Tris buffer only, suggests that the regulation of pH is sufficient to maintain the enzyme in the active form during freezing and thawing. As expected, the samples show a loss in activity after dehydration. The results show a high loss of activity in the samples without the addition of mannose. Samples with 20 mM mannose showed the highest loss after freezing and thawing but achieved the highest recovery after drying. Samples with 10 mM mannose and mannose in combination with PEG, show comparable values. This supports the thesis that PEG does not stabilize

the protein sufficiently during drying. Only by formation of hydrogen bonds between the sugar molecules and the protein, stabilization can take place [Gloger, 2002; Carpenter et al., 1992]. The samples with 10 mM and 20 mM mannose show higher activities after lyophilization in comparison to freezing and thawing. This suggests that the thawing process in particular is a critical point for the stabilization of GlcNAc 2-epimerase with mannose. When thawing is avoided, it is shown that mannose serves as a lyoprotectant even without PEG. This can be attributed to the structure of mannose in aqueous solution. In solutions, mannose forms pyranoses, which have a ring structure. The most common form is α-D-mannopyranose with > 67% [Angyal, 1968]. In this ring form, mannose has a strong similarity with the substrate, since GlcNAc 2-epimerase catalyses the reaction with GlcNAc in both directions (see Figure 33). The use of substrates, ligands or co-factors to

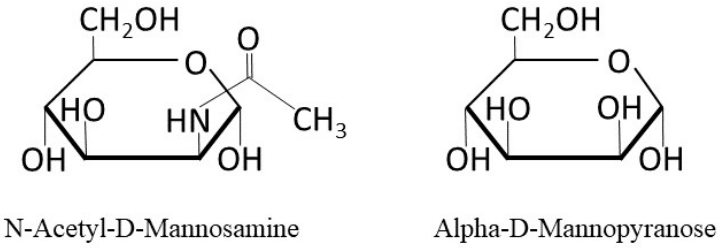

Figure 33: Structure of the GlcNAc 2-epimerase product N-acetyl-D-mannosamine and α-D-mannopyranose. In aqueous solution, mannose may form a pyranose sugar.

stabilize proteins in aqueous solution and during lyophilization is described by *Ó'Fágáin and Colliton*. By occupying the active site, a conformational change of the enzyme is induced [Ó'Fágáin and Colliton, 2017]. This change is small, but leads to a tighter, more stable packing of the protein and thus shifting the equilibrium to the native side.
The results for Neu5Ac lyase after freezing and thawing show similar trends in the effects but show a much greater loss of activity. The sample without the addition of excipients shows the lowest decrease in activity. Similar to the GlcNAc 2-epimerase results, samples without addition of PEG achieved the lowest remaining activity values. After freeze-drying, the samples without additives and with 2% PEG and 10 mM mannose, have the highest values. However, the enzyme recovery is rather small compared to GlcNAc 2-epimerase and is insufficient for the use of Neu5Ac lyase as biocatalyst. Tests of freeze-thawed and freeze-dried Neu5Ac lyase samples in distilled water show a high recovery. After freezing and thawing 85% and after drying 82% enzyme activity could be found. It seems that the presence of Tris during freezing has an destabilizing effect. This could be explained by (small but present) pH shifts during freezing in the presence of Tris.

Due to the high enzyme activity recovery of GlcNAc 2-epimerase samples, lyphilization in 20 mM Tris buffer with pH 7 and 20 mM mannose is recommended. The here examined highest mannose concentration lead to the highest recovery. It can be assumed that the recovery would increase with the mannose concentration. But this has to be determined experimentally. For Neu5Ac lyase, freeze-drying in distilled water reveals the highest recovery and is thereby the favoured enzyme preparation.

6 Outlook

In this thesis, issues concerning the optimization of purification and the development of a lyophilization method were investigated and discussed. The study of these objectives leaves some open issues and opens new research questions. The binding behaviour of proteins during chromatography is influenced by many factors. On order to gain a better understanding of the behaviour of GlcNAc 2-epimerase and Neu5Ac lyase, further aspects can be investigated. One aspect is the structure of bound Neu5Ac lyase on the column. The results of this thesis show high binding capacites for both column materials. To understand this preferential binding, it would be interesting to investigate the enzyme structure in EQ buffer. Analysis by size exclusion chromatography or native PAGE could provide information regarding the distribution of monomeric or multimeric Neu5Ac lyase under chromatography conditions. The second aspect is the apparently reduced binding capacity caused by the use of Tris buffer. The purification of GlcNAc 2-epimerase showed a reduction in yield independent of the column material when using Tris. The promotion of enzyme retention by competitive binding of amines can be confirmed by studies with other enzymes or Tris buffers with different ionic strengths. The studies on enzyme stability during lyophilization achieved promising recoveries. For the experimental determination of optimal lyophilization conditions, small sample quantities were advantageous. By lyophilization of large bulk quantities under favoured conditions, the stability can be verified under more realistic laboratory conditions. Studies of GlcNAc 2-epimerase stability showed that high mannose concentrations stabilize the enzyme during freezing and drying. One idea for further investigations is to achieve higher recovery by increasing the mannose concentration.

7 Appendix

A Data for the Column Performance of Ni and Co IMAC Resins

Table A1: Data for chromatography to determine the binding capacity and lifetime of Co^{2+} and Ni^{2+} column resins with GlcNAc 2-epimerase and Neu5Ac lyase.

	GlcNAc 2-epimerase		Neu5Ac Lyase	
	Co^{2+}	Ni^{2+}	Co^{2+}	Ni^{2+}
Sample Volume [ml]	2.0	2.5	1.5	2
Protein Concentration [mg/ml]	23.5	3.95	37.2	21.9
Applied Protein Mass [mg]	47.0	9.9	55.8	43.8
Number of Runs [-]	15	16	14	15

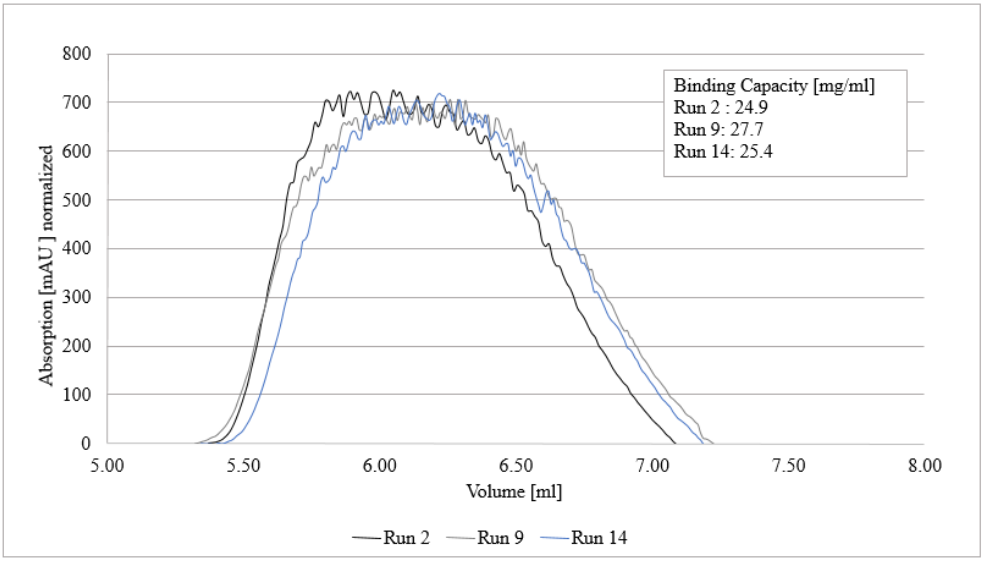

Figure A1: Curves of the elution and binding capacities of the chromatography runs 2, 9 and 14 with Co^{2+} IMAC resin for GlcNAc 2-epimerase. (Resin= Talon Superflow, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

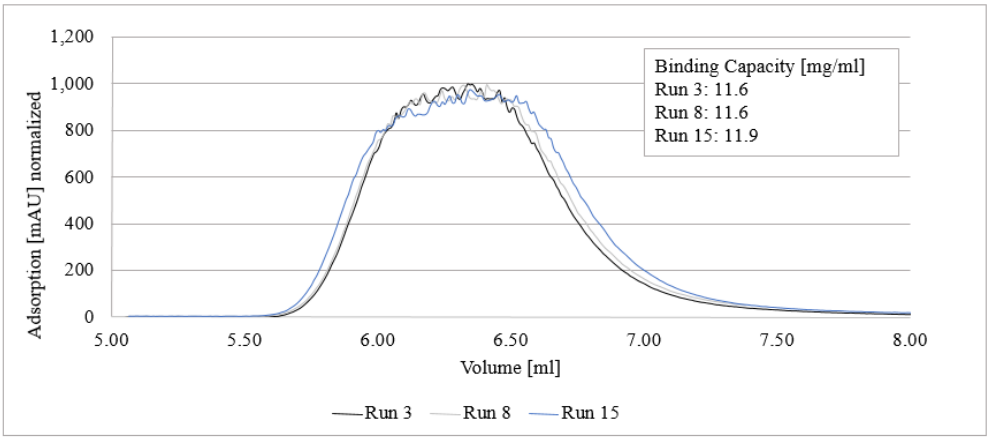

Figure A2: Curves of the elution and binding capacities of the chromatography runs 3, 8 and 15 with Ni^{2+} IMAC resin for GlcNAc 2-epimerase.(Resin= Profinity IMAC, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

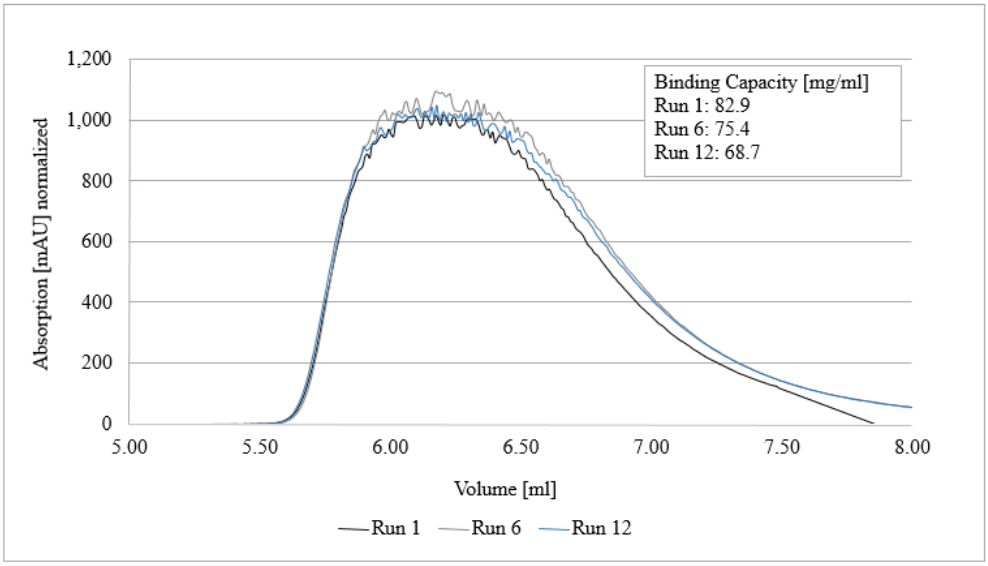

Figure A3: Curves of the elution and binding capacities of the chromatography runs 1, 6 and 12 with Co^{2+} IMAC resin for Neu5Ac lyase.(Resin= TALON Superflow, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

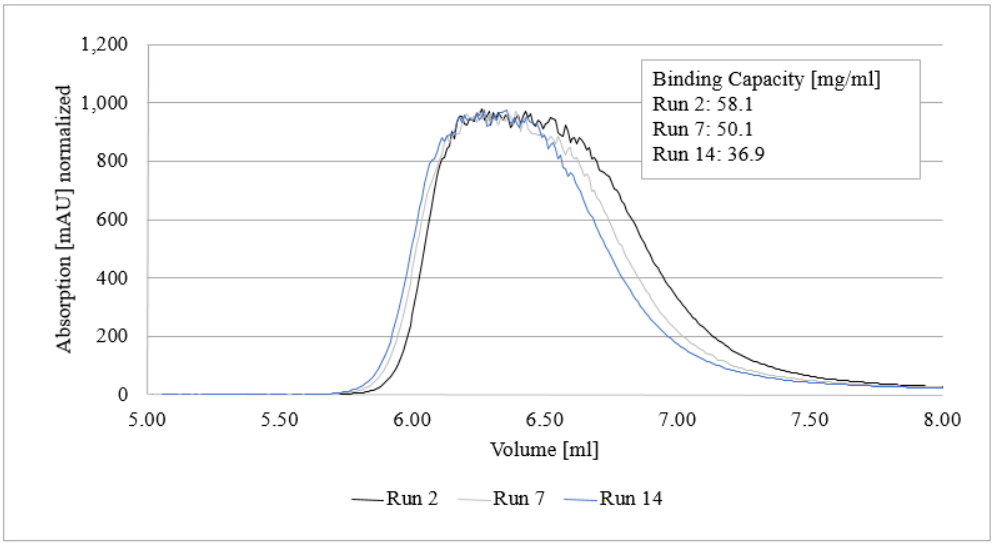

Figure A4: Curves of the elution and binding capacities of the chromatography runs 2, 7 and 14 with Ni^{2+} IMAC resin for Neu5Ac lyase.(Resin= Profinity IMAC, column volume= 0.6 ml, flow rate= 0.5 ml/min, application via 2 ml sample loop at 0.2 ml/min). For a standardized baseline, the absorption values of the curves were corrected by the value of the baseline.

B Others

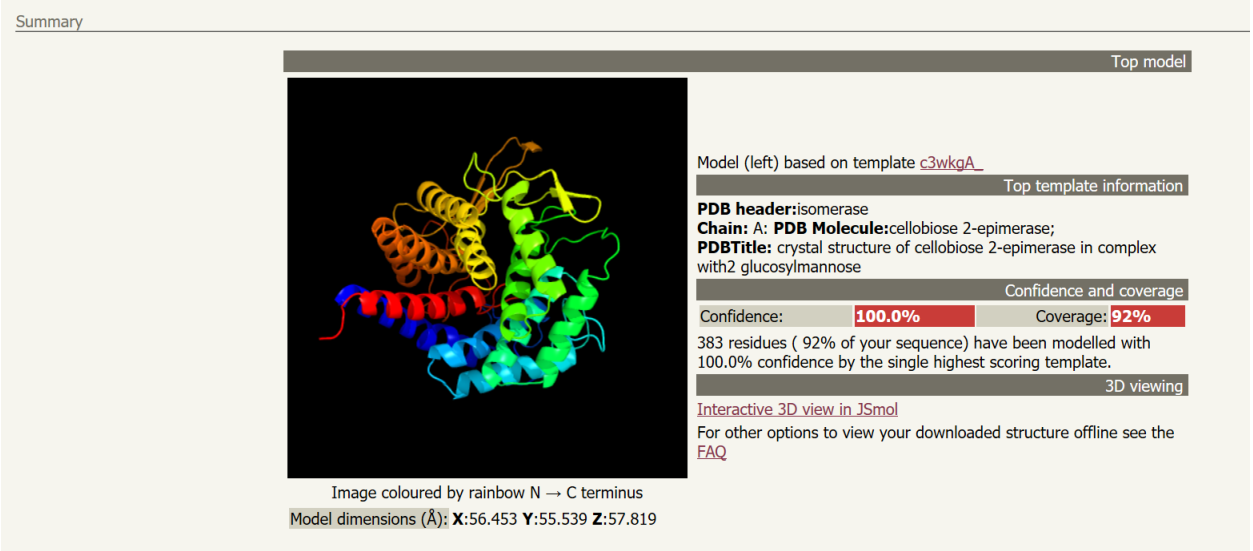

Figure B5: Summary of the structural prediction of GlcNAc 2-epimerase from *Pedobacter heparinus* based on the amino acid sequence. Created at 04.02.2020 with the Protein Homology/ analogy Recognition Engine V 2.0 (Phyre^2) from the Structural Bioinformatics Group. URL: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

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