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Further Purification of an Isolated Nitrile Hydratase Fraction With Asparaginase and Glutaminase Activity from Rhodococcus Rhodochrous and Evaluation of its Kinetics and Properties as a Potential Treatment for Human Leukemia

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FURTHER PURIFICATION OF AN ISOLATED NITRILE HYDRATASE FRACTION WITH ASPARAGINASE AND GLUTAMINASE ACTIVITY FROM *Rhodococcus rhodochrous* AND EVALUATION OF ITS KINETICS AND PROPERTIES AS A POTENTIAL TREATMENT FOR HUMAN LEUKEMIA

By

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Under the Direction of George Pierce, PhD

ABSTRACT

L- Asparaginase is capable of hydrolyzing L-asparagine into L-aspartic acid and ammonia. This catalytic function is used for the treatment of children with acute lymphoblastic leukemia resulting in the depletion of L- asparagine in the leukemia cells. Bacterial asparaginase used in current leukemia therapies has side effects and short serum half-life. This study presents the purification and characterization of nitrile hydratase (NHase) with asparaginase (ASNase) and glutaminase (GLNase) from *Rhodococcus rhodochrous DAP 96253* and *96622.* The influence of pH, lysis buffer composition, dialysis process, anion exchange chromatography's flow rates and pH were incorporated into the approach to purify NHase exhibiting ASNase activity. A NHase purified at pH 7.6, using 50 mM phosphate buffer, 5mM 2-mercaptoethanol as a lysis buffer, then dialyzed, followed by anion exchange chromatography and subsequent size exclusion chromatography showed a higher ASNase and GLNase activity and lower NHase activity. This enzyme shows potential as leukemia treatment.

INDEX WORDS: Asparaginase, Glutaminase, Nitrile hydratase, Leukemia, *Rhodococcus*, Anion Exchange Chromatography

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DEDICATION

To my lovely family and friends.

Thanks to my parents who accompanied me through their daily and powerful prayers which resulting in the guidance of God. Thanks to my brothers for taking care of my parents during the time that I was abroad doing this research and for their constant and emotional support. To my lovely German who carefully listened to me despite his unfamiliarity about protein purification and *Rhodococcus* helping me to advance in my research with his thoughtful advises. Finally, I dedicate this work to Andrea Rodriguez who inspired to me and demonstrate that never is late to follow the dreams and achieve proposed goals.

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LIST OF ABBREVIATIONS

AEX: ..... Anion exchange chromatography

ALL ..... Acute Lymphoblastic Leukemia

ASN: ..... L- asparagine

ASNase: ..... L-asparaginase

CFL ..... Cell free Lysate

GF: ..... Gel filtration

GLN: ..... L- glutamine

GLNase: ..... L- Glutaminase

IEX: ..... Ion Exchange Chromatography

NHase: ..... Nitrile hydratase

PB: ..... Phosphate buffer

SEC: ..... Size exclusion chromatography

1 INTRODUCTION

1.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a malignant disease of the bone marrow, blood and extramedullary sites which is the result of a genetic alteration of lymphoid precursor cells and its subsequent abnormal proliferation. The uncontrolled growth and immortality of immature lymphocytes cause failures in the bone marrow as anemia, leukopenia and thrombocytopenia, increasing the risk of infections. Other symptoms include fever, night sweats, weight loss, fatigue, dyspnea and easy bleeding or bruising (Terwilliger & Abdul-Hay, 2017). In general, genetic alterations observed in leukemia cells include dysregulation of gene expression, activation of protooncogenes, deletion or functional inactivation of tumor-suppressor genes along with gene mutations and numerical changes(Tedesco & Vecchione, 2012)

According to the National Cancer Institute, in 2018, there were 5960 estimated new cases of ALL in the USA. The estimated number of deaths was 1470 (Howlader et al., 2018).Though ALL incidence has a bimodal distribution with a first peak in childhood and a second peak around the age of 50, It has a significant relevancy in children because Juvenile ALL is one of the most significant pediatric cancers, constituting approximately 75-80% of childhood leukemias compared to 20% of adult leukemias and causing mortality among pediatric patients with cancer.(Tedesco & Vecchione, 2012).

1.2 L-Asparaginase

L- asparaginase (ASNase E 3.5.1.1) is an enzyme that hydrolyzes the amino acid L - asparagine (ASN) to ammonia and aspartate as is shown in figure 1.1 (Avendaño & Menéndez, 2008). Due to this physiological role, ASNase is used in pediatric acute lymphoblastic leukemia (ALL) trials because this enzyme depletes serum ASN, which is not produced by ALL cells, but

it is required by ALL cells for protein production. As a result, leukemia cells undergo apoptosis. Unlike normal cells, leukemia cells' survival depends on ASN from serum due to the fact that ALL cells are unable to produce ASNase (Hong, Holland, & American Association for Cancer Research, 2010).

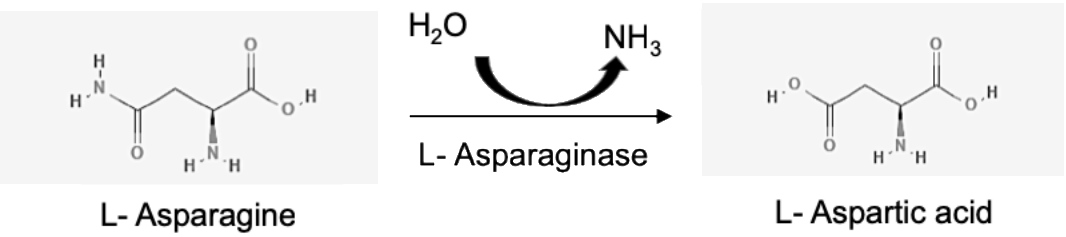


Figure 1.1Hydrolysis of L-asparagine by L-ASNase

Although L- ASNase is produced by several organisms such as animals, plants, bacteria, fungi, algae and yeast, bacterial ASNase is current approved and used for Juvenile leukemia treatment(Batool, Makky, Jalal, & Yusoff, 2016).

The enzyme structure and kinetics characteristics depend on the ASNase source. While the molecular size of *E coli* ASNase is reported between 133 and144 kDa, the molecular weight of other asparaginases ranges between 140 to 150 kDa. The structure of L - ASNase from *Erwinia carotovora* is defined as a dimer of dimers. This enzyme has two homotetramer composed of 8 monomers (A to H) with four active sites. The homotetramer consists of two subunits of the protein forming an intimate dimer, AC or BD pair (Figure 1.2). In each pair of dimers there are two active sites for glutamine which, according to computational studies, are enough for providing catalytic activity to the enzyme; while other studies consider ASNase activity is only when the enzyme is in the tetrameric state (Mezentsev et al., 2007) (Ramya, Doble, Rekha, & Pulicherla, 2012).

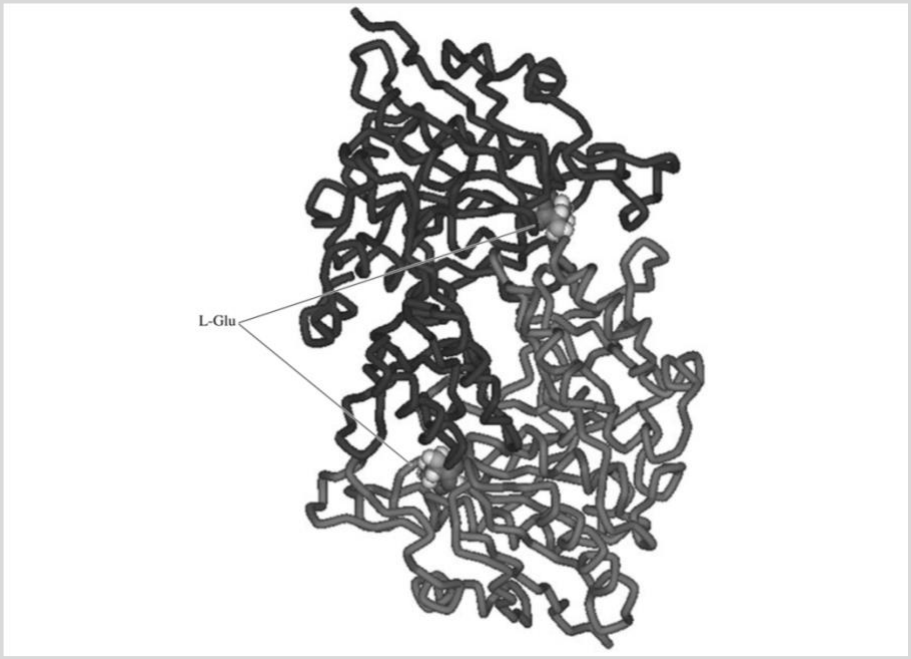


Figure 1.2 AC dimer of L-ASNase with two Active Sites with Substrate L-glutamine. This structure was identified for L- ASNase from *Erwinia carotovora* (Mezentsev et al., 2007)

Although bacterial ASNase treatment has been used for more than 50 years as one of the major therapies in children with ALL, the inhibition of ASNase during chemotherapy is one of the most important concerns because it makes the treatment ineffective for ASN depletion. ASNase inhibition has been attributed to: ASNase antibody production, degradation of ASNase by peptidases, and expression of asparagine synthetase (ASNS) in certain cancer cell lines by synthesizing ASN from other amino acids such as glutamine/glutamate(Marini, Perissinotti, Bixby, Brown, & Burke, 2017). Finally, the repetitive administration of ASNase is the result of inhibition of ASNase and its short serum half-life (Husain, Sharma, Kumar, & Malik, 2016b).

Currently, Juvenile ALL is treated with three different commercial asparaginases. Two of them, Elspar® and Oncaspar®, are derived from *Escherichia coli*, but Oncaspar® is a polyethylene-conjugated formulation, polyethylene glycol (PEG)- ASNase that has a longer half-life than the native *E. coli* ASNase (Elspar®). The third ASNase (Erwinaze®) comes from *Erwinia chrysanthemi*. Those asparaginasases used in clinical trials have shown ASNase

inhibition and some side effects such as pancreatitis, hypercoagulability, liver dysfunction, and allergic reactions(Koprivnikar, McCloskey, & Faderl, 2017)(Lanvers-Kaminsky, 2017).

In addition, clinically available ASNase preparations possess significant GLNase activity and some studies have looked for GLNase -free ASNase. It is argued that the intrinsic GLNase activity of both *E. coli* and *E. crysanthemi*-derived ASNase causes major side effects such as neurological seizures, hyperglycemia, immunosuppression, pancreatitis, leucopenia, and thromboembolysis (Husain et al., 2016b). However, some studies concluded that GLNase activity is essential to obtain an effective ASNase therapy for treatment of leukemia due to adequate glutamine (GLN)depletion caused by GLNase may prevent that ASN be synthesized from glutamine/glutamate by ASNS-positive ALL cells (Marini et al., 2017)(Richards & Kilberg, 2006)

The microbial source and growth conditions cause the ASNase variability of properties such as: molecular weight, affinity to its substrate (K_m), optimum pH, temperature, and structural diversity. These variations in enzyme properties also can affect the immunogenicity of ASNase and its side effects in clinical trials. (Krishnapura, Belur, & Subramanya, 2016).

A diversity of studies has been done in order to reduce the immunogenicity of ASNase and increase its half-life. The majority of these studies have included the alteration of the structure by chemical modification or genetic modification in order to change the amino acid sequence(Krishnapura et al., 2016)(Nguyen, Su, & Lavie, 2016). The use of novel enzymes with ASNase -like activity can avoid the costs of structure modification and obtain a new treatment based on ASN depletion to reduce the immunogenicity of ASNase.

Leukemia statistics presented above, side effects and resistance to current commercial

enzymes are significant reasons to look for new microbial sources. It is necessary to maintain efforts to looking for new microbial sources of ASNase, other than *E. coli* and *E. chrysanthemi*, that may improve the treatment of emergent cases and decrease the mortality rate caused by ALL.

1.3 Characteristics of *Rhodococcus rhodochrous*

Rhodococcus rhodochrous is a nonmotile, aerobic Gram-positive rod which may form substrate, vegetative mycelium (not aerial) which is fragmented into regular roads and cocci. Colonies of this bacteria are visible after 1- 3 days of incubation at 30°C and pH of 7.4 ± 0.2. *R. rhodochrous* growth on 0.1% (w/v) acetamide and ASN as sole carbon and nitrogen sources (Bergey & Holt, 2000).

The genome of *R. rhodochrous* has 6,869,887 bp, of which 53 are RNA genes and the others are protein-coding genes. While 5186 of these genes have a putative function, the remaining genes are associated with proteins of unidentified functions. In addition to the genetic diversity, this ubiquitous microorganism has a metabolic versatility. *R. rhodochrous* possesses a large group of enzymes such as, hydratases and oxygenases giving this microorganism an enormous metabolic ability which is used in biodegradation and biotransformation process(Chen, Otten, Resch, Muyzer, & Hanefeld, 2013).

R. rhodochrous might be one of the new microbial ASNase sources due to its diverse physiology and metabolism. These properties and its large suite of enzymes make of *R. rhodochrous* a significant tool in clinical environmental and industrial areas(Chen et al., 2013).

1.4 Nitrile Hydratase

Nitrile hydratase (NHase EC 4.2.1.84) hydrates nitriles to the corresponding amide as is shown in figure 1.3 (Yamada & Kobayashi, 1996). This enzyme has a minimal hetero-tetramer

structure composed of α- and a β-subunits (24-mer structure shown by Du 2013) and an active site bound to either a reduced cobalt or an iron ion. Subunit α allows the metal ions binding through two modified cystine residues present in this subunit (Lan et al., 2017) Although NHase is principally a bacterial enzyme produced by a diversity of genera from Proteobacteria, purifications of NHase from *Rhodococcus* spp. have been the most reported (Prasad & Bhalla, 2010).

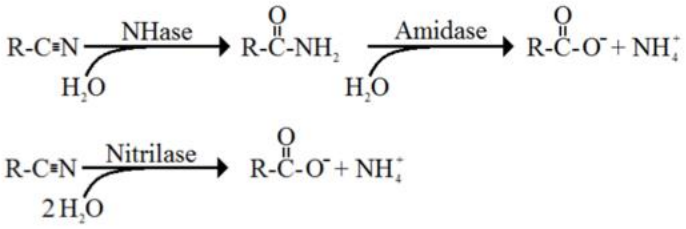


Figure 1.3 Enzymatic Conversion of Nitriles
(Du, 2013)

Rhodococcus rhodochrous J1 cultured with specific metal ions concentrations synthetizes a low-mass(L-NHase) and a high-mass (H-NHase) molecular weight NHase with 94 and 504 kDa respectively with α-subunits of 26 kDa and β-subunits of 29 kDa. The number of subunits for L- NHase is between 4 and five, while H-NHase has around 18 -20 subunits(Yamada & Kobayashi, 1996) and according to Due, 2013 it has 24 subunits.

R. rhodochrous DAP 96622 and DAP 96253 when cultivated in induction media, with cobalt (II) chloride and urea, overexpress nitrile hydratase production where urea is an inducer of NHase production and Co^{+2} activates and overexpress the enzyme. NHase purifications made from these induced cells resulted in the isolation of known NHase α- and β-subunit monomers (26 and 29kDa) and novel NHase subunits around 50 KDa and 20 KDa (Figure 1.4). For *R.*

rhodochrous DAP 96622 two subunits of 50 kDa were identified as NHase αβ dimers (Figure 1.4-A), while for *R. rhodochrous* DAP 96253 (Figure 1.4 -B) the 50 kDa was recognized as a NHase complex with glutamine synthetase and the 20 kDa protein band was identified as a NHase subunit fragment(Du, 2013).

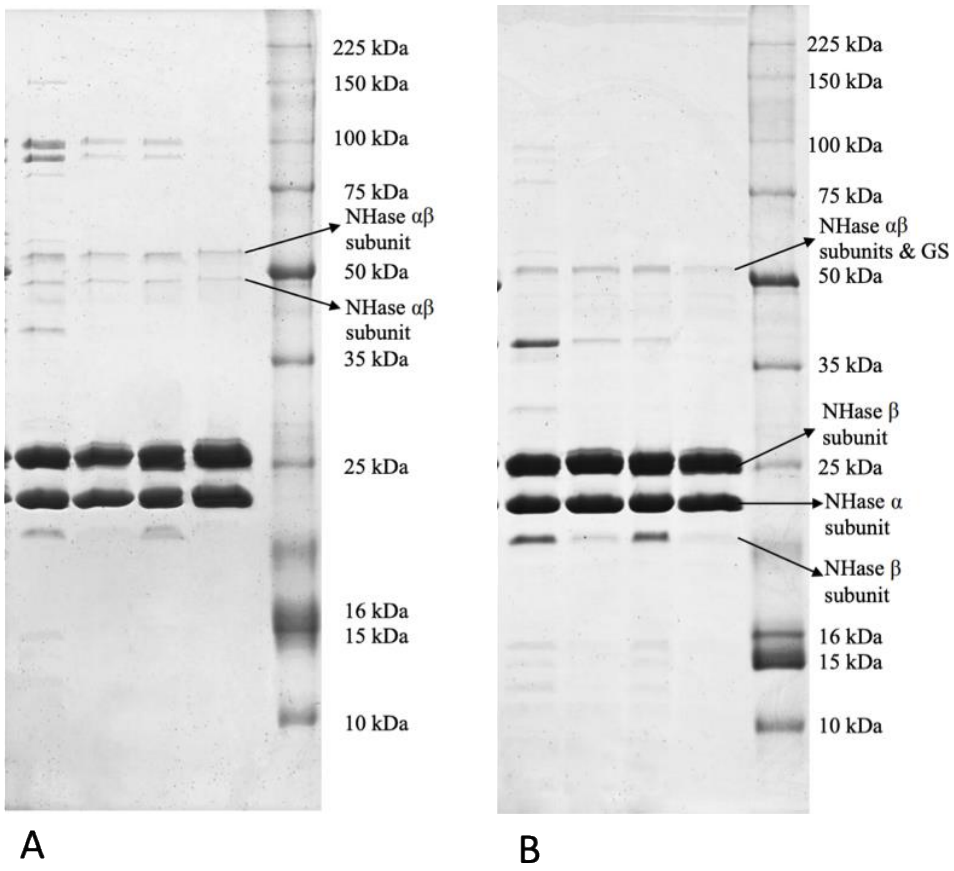


Figure 1.4 NHase purified from R. rhodochrous DAP 96622 and R. rhodochrous DAP 96622 (Du, 2013)
Figure A shows the protein bands obtained in the last four purification steps of NHase from *R. rhodochrous* DAP 96622 and figure B presents purification steps of NHase from *R. rhodochrous* DAP 96253.

Although the major interest in the purification of NHase is promoted by the traditional and wide application of this enzyme in industrial production of commercial amides such us acrylamide, nicodamide and 5- cyanovaleramide, alternative studies have been shown that

molecular structure of Nitrile -hydrolases, such as NHase, provides the enzyme function and new properties that allow the utilization of those enzymes not only in production of acrylamide from the hydration of acrylonitrile, but also in potential future applications (Kobayashi & Shimizu, 2000).

Some microbial enzymes are able to catalyze alternative substrates to well-characterized substrates in metabolic pathways. As a result, some enzymes present a diversity of substrate affinities. Hydrolases with α-β structures are an example of enzymes with features that catalyze an array of different reaction (O'Brien & Herschlag, 1999). Unpublished experiments about purification of nitrile hydratase with ASNase activity, conducted in the laboratory of Applied and Environmental Microbiology at Georgia State University have shown that high-mass NHase purified from induced cells of *R. rhodochrous* DAP 96253 has the ability to use GLN and/or ASN as substrate(s).

1.5 L- Glutaminase

L- glutaminase (GLNase, E 3.5.1.2) hydrolyzes the amino acid L -glutamine into L-glutamic acid and ammonia (Figure 1.5). The enzyme is broadly produced by animals, plants and microorganisms. The bacterial source is the most used in industrial production of GLNase for food and therapeutic applications(Binod et al., 2017). The native molecular mass of bacterial GLNase varies between a range of 40 to 148 kDa and the molecular size of the GLNase subunits are between 26.9 and 55 KDa. Both the small (40 kDa) and large native protein were purified from *Pseudomonas spp.* while the smallest subunit (26.9 kDa) was purified from *Rhizobium etli* and the largest (55 kDa) was reported for *Bacillus pasteurii* (Nandakumar, Yoshimune, Wakayama, & Moriguchi, 2003).

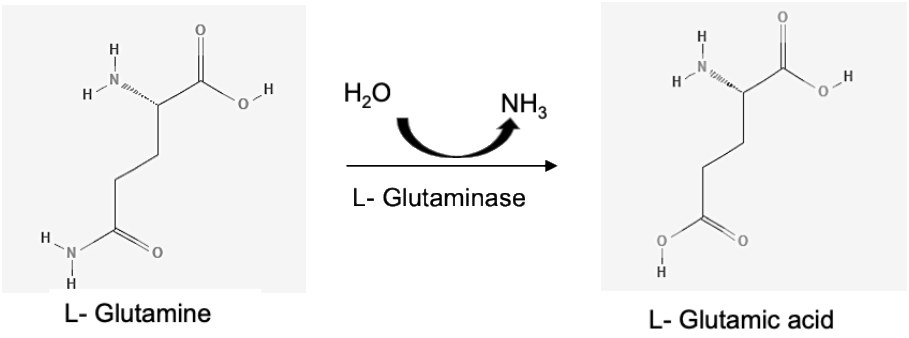


Figure 1.5 Hydrolysis of L-Glutamine Catalyzed by L- GLNase.

The therapeutic application of GLNase have been focused in cancer treatments due to the fact that recent developments have demonstrated that GLNase has antitumor properties specially for ALL(Binod et al., 2017). ALL cells are GLN dependent and GLN depletion in blood by GLNase cause selective death of tumor cells(Reda, 2015). Depletion of GLN in blood also alters the novo synthesis of ASN mediated by asparagine synthetase because this enzyme use GLN as an amino group source to synthesize ASN, a vital amino acid for leukemia cell growth (Parmentier et al., 2015).

1.5.1 Ion Exchange Chromatography

Ion exchange chromatography (IEX) is a protein purification technique based on the surface charge protein properties. The surface charge of proteins varies according to its specific structure and the pH which surrounds the protein. This variation influences the adsorptive properties of charged proteins to an inversely charged resin. When the net charge of a protein is zero, the interaction of the protein with charged resin will be absent. The pH where proteins are uncharged is called the isoelectric point. Above a certain protein's isoelectric point it has a negative charge, while below this pH the net surface charge is positive. Thus, the selection of pH is one the most critical variables in IEX because pH can cause a higher charge difference between the target protein and contaminant proteins present achieving a separation by binding or

elution of the specific polypeptide. Others critical factors in IEX are the amount of protein loaded into the column, the fluid velocity in which the IEX is run and the salt concentration used to elute the protein previously bound to the column. IEX is comprised of anion exchange chromatography (AEX) and cation exchange chromatography (CIEX). AEX separates negatively charged proteins and CIEX is used to purify positively charged proteins (Roe, 2001).

AEX uses adsorbent resin positively-charged or anion exchangers, which adsorbs proteins with net negative surface charges. Some anion exchangers used in AEX are quaternary groups (Q), diethylaminoethyl (DEAE) and diethylaminopropyl (DEAP). These are packed into a column forming a packed bed which is equilibrated with buffer that fills the free spaces in the column such as matrix pores. In an IEX an equilibration step is followed by the sample application and a subsequent wash step. After the target protein is bound, elution is done by increasing the ionic strength of the buffer or changing the pH. The regeneration process is the last step of IEX to eliminate remaining molecules into the column (GE Healthcare Life Sciences, 2016).

1.5.1.1 Size Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel filtration (GF) is a protein purification technique based on the size of the molecules which are separated from a solution as they pass through a column packed with a gel that acts as a chromatographic media. The gel media has a continuous liquid phase within a solid phase composed of a controlled pores size. The size of the pores helps to separate the proteins by diffusion. Small proteins can slowly diffuse into the pores of the gel, while large proteins have less diffusion into the pores moving faster through the column. Proteins with size considerably bigger than pores size are unable to diffuse into the gel moving down through the column with the elution buffer is flowing. Two

significant variables in SEC are the sample volume and the flow rate which provides better resolution when it is low. The applications of SEC in purification process of protein include: fractionation by size, separation of monomers from dimers, native molecular weight estimation and desalting protein samples from upstream steps with higher salt concentration (GEhealthcare, 2010).

1.6 Enzyme Activity

The Enzyme activity is a property of the enzyme molecule which is expressed as μmol/min. A unit of active enzyme is defined as the amount of enzyme that transforms 1 μmol of substrate in one minute or as the amount of enzyme that produces 1 μmol of product per minute. The specific activity of a purified fraction or a preparation is based on the total mg of protein. The specific activity is an indicator of the purity of the enzyme. Higher specific activity is associated to purity. The majority of methods used to measure the enzyme activity (enzyme assays) are based on detect changes caused by the enzyme in the substrate or products in the UV/visible region of the spectrum (Roe, 2001).

1.7 Hypothesis

Type of fermentation paste, and variables used in purification process including: buffer composition, basic pH, flow rates higher than 0.3 ml/min, and short storage time of whole cells improve the ability of NHase purified from induced *R. rhodochrous* to use ASN as substrate and its enzyme kinetics.

1.8 Specific Aims

- To further purify nitrile hydratase with ASNase and GLNase activity from *R. rhodochrous* using different column flow rates, pH and buffer composition.
- To evaluate the kinetics and properties as a treatment for human leukemia of NHase with ASNase activity purified from induced *Rhodococcus rhodochrous* cells.

2 MATERIALS AND METHODS

2.1 Enzyme Purification

2.1.1 Cell Suspensions

Whole cells of *R. rhodochrous* DAP 96253-C6 and *R. rhodochrous* DAP 9662 were suspended in lysis buffers using a relation of 1 g of whole cell (packed wet weight) per 1 ml of lysis buffer. Cell suspensions were vortexed in 50 ml conical centrifuge tubes (Falcon™) until cells were completely suspended. Three different types of fermentation pastes of *R. rhodochrous* were used as starter material for purifications. Induced cells, *R. rhodochrous* cells DAP 96253-C6 fermented in media with glucose, urea and Co^{+2} (Harvested on 01-08-18, 08-10-18 and 02-07-19), non-induced cells of *R. rhodochrous* DAP 96253-C6 fermented in media only with glucose and urea (Harvested on 09-07-18) and induced cells of *R. rhodochrous* DAP 96622 (Harvested on 02-18-19)

2.1.2 Lysis

Three different lysis buffers were used to evaluate the effect of these on ASNase activity: 20 mM phosphate buffer (PB) pH 5.8, 50 mM TRIS HCl pH 8. 50mM PB pH 7.2 and 50mM PB+ 5 mM 2-mercaptoethanol pHs 7.2, 7.4 7.6 and 8.

Cell suspensions made in the buffers mentioned above were poured into a 20 ml beaker, which were set on ice and sonication was done for 20 minutes at 1 second on 1 second off using

a Fisher® 550 Sonic Dismembrator [with Minonix incorporated convertor and a horn with a ½" diameter tip]. Sonicated cells were centrifuged for 30 minutes at 13000 rpm and 4°C using a Beckman Avanti™ J-25 centrifuge and a Beckman JA-25-50 rotor. The centrifugation was repeated three times. After each centrifugation, the supernatant was recovered as cell free lysate (CFL).

The enzyme activity of NHase, GLNase and ASNase for the lysate was measured following the procedure described in section 2.3.

2.1.3 Dialysis

Lysates made using 2-mercaptoethanol as a component of the lysis buffer were dialyzed. Approximately 7- to 10 ml of lysate was loaded in a Spectra/Por® 3 dialysis membrane, Standard RC Tubing of 3550 MWCO and was dialyzed for 40 hours with stirring at 4°C against 300 ml of dialysis buffer (Appendix A3). 0.05 MHCL- TRIS and 50 mM PB buffer were tested as dialysis buffers to evaluate the effect on ASNase activity.

2.1.4 Anion Exchange Chromatography (AEX)

Anion exchange chromatography was performed using a Harvard Apparatus syringe pump (Cat #55-5920 115/230, serial Nº B-13140) and ToyoScreen® Super Q-650 M 5 ml column a polypropylene column packed with 65µm hydroxylated methacrylic polymer with an ammonium ion. Prior to the chromatography process, the column was pre-conditioned by washing with 10 ml of 1 M sodium hydroxide (NaOH), loaded with a 10 ml and 15 mm diameter syringe. Then 10 ml of 2 M sodium chloride (NaCl) and 20 ml of double deionized water (ddi H2O) were loaded, using 1ml/ min flow rate which was adjusted in the syringe pump setting the diameter at 15mm and the flow rate at 1ml/min. The column was equilibrated with 30 ml of equilibration buffer at 1ml/min. Lysates previously dialyzed were diluted (1:2) with equilibration buffer, prefiltered using a Captiva Premium Syringe Filter, polypropylene housing,

polyethersulfone (PES) membrane, 25 mm diameter, 0.45µm pore size and filtered using a 0.2 µm filter. 10 ml of diluted sample was loaded through the column using a specific flow rate (0.3,0.6 or 1 ml/min,).

After loading the sample, the column was washed with 1 column volumes (cv) of equilibration buffer. The protein was eluted by a step elution process, using 5 ml of 0.1, 0.2, 0.5 and 1M NaCl added to the equilibration buffer. The same flow rate used during sample loading was employed for protein elution. The column was washed using the process described above and re-equilibrated with 5 cv of equilibration buffer. Samples were collected from each elution in order to determine protein concentration, enzyme activity and molecular weight by SDS-PAGE.

2.1.4.1 Effect of Lysis Buffer pH on ASNase, NHase and GLNase Activity

R. rhodochrous cells DAP 96253-C6 fermented in a medium with glucose, urea and Co^{2+} (induced cells), harvested on 01-08-18 were lysed using two different lysis buffers: 20 mM phosphate buffer (PB) pH 5.8 and 50mM PB pH 7.2. After lysis an AEX was done using 25 mM monosodium phosphate (NaH_2PO_4)/10mM sodium butyrate (Aldrich), pH 7.2 as an equilibration buffer. Enzyme activity (ASNase, GLNase and NHase) was assayed using the method described in section 2.3.

2.1.4.2 Effect of AEX Flow Rate on ASNase, NHase and GLNase Activity

Using free lysate cells from *R. rhodochrous* DAP 96253-C6 as starting material for AEX, three different flow rates were tested for loading the sample and protein, 0.3ml/min, 0.6 ml/min and 1ml/min. Enzyme activity was assayed using the method described in section 2.3.

2.1.4.3 Effect of Storage Time of Fermentation paste on ASNase, NHase and GLNase Activity

Lysis at pH 7.2 followed by AEX at pH 7.2 were repeated at several time points after the harvest with cold storage. Whole cells of *R. rhodochrous* DAP 96253- C6 from fermentation 01 18 18 were lysed and purified by AEX after 1, 86 days, 150, and 154 days of storage at 4°C. Enzyme activity was assayed using the method described in section 2.3.

2.1.4.4 Effect of 2- Mercaptoethanol and Dialysis Process on ASNase Activity

Induced cells of *R. rhodochrous* DAP 96253-C6 induced with urea and Co^{+2}, harvested on 01-08-18, were lysed using 2-mercaptoethanol as a reducing agent and a lysis buffer free of 2-mercaptoethanol (50mM PB- 5 mM 2- mercaptoethanol pH 7.2 and 50mM PB pH 7.2)

In order to verify the efficiency of dialysis step in purification process, one AEX purification was performed using a lysate containing 2- mercaptoethanol which previously was dialyzed (see section 2.1.3) and another purification was done using the same lysate without dialysis process. Enzyme activity was assayed using the method described in section 2.3.

2.1.4.5 Effect of Buffer Exchange Between Purification Steps on Enzyme Activity

Using induced cells of *R. rhodochrous* DAP 96253-C6 fermented in media with glucose, urea and Co^{2+}, harvested on 01-08-18, three different NHase purifications were made in order to evaluate the effect of buffers used during the purification process on ASNase activity. The buffers and step purification in which these buffers were used are recorded in table 2.1

Table 2.1 Buffers used during Purification.
Buffers used during three NHase purifications to test the effect of changing buffers on Enzyme Activity

Buffer	**Purification using the same buffer for all purification steps**	**Purification using three different buffers**	**Purification using two different buffers**
Lysis Buffer	0.05 M TRIS - HCl 5mM	50 mM PB 5mM 2-mercaptoethanol	

	2mercaptoethanol pH 8	pH 8
Dialysis Buffer	0.05 M TRIS HCl 5mM 2-mercaptoethanol, 1.5 % glycerol, 1% $MgS0_4$ $7H_2O$ pH 8	50 mM PB 5mM,2-mercaptoethanol, 1.5 % glycerol, 1% $MgS0_4$ $7H_2O$ pH 8
Equilibration Buffer for AEX	0.05 M TRIS HCl pH 8	10mM Sodium butyrate, 25mM NaH_2PO4 pH 8
Elution Buffer for AEX	0.05 M TRIS HCl pH 8.0 incorporating 0.1, 0.2, 0.5 and 1 M NaCl	10mM Sodium butyrate, 25mM NaH_2PO4 incorporating 0.1, 0.2, 0.5 and 1 M NaCl

2.1.4.6 Effect of the Type of AEX column on ASNase Activity

Two AEXs were done using a ToyoScreen® Super Q-650 M 5 ml, a polypropylene column packed with 65µm hydroxylated methacrylic polymer with an ammonium ion and a HiTrap™ amine (Q) strong anion exchange group HP anion exchange chromatography column, a Q Sepharose High Performance strong quaternary ammonium anion exchange resin 5ml column. The AEXs were done simultaneously, fixing two syringes in the syringe pump and connecting each syringe to each column. Enzyme activity was assayed using the method described in section 2.3.

2.1.5 Size Exclusion Chromatography (SEC)

Fractions recovered from AEX with high ASNase activity and the highest protein concentration were polished using GE AKTAExplorer™ model 100 (Amersham Pharmacia, Piscataway, NJ) fast protein liquid chromatography (FPLC) controlled using the UNICORN app 5.11, employing a a120 ml size exclusion column Hiprep™ 16/60 Sephacryl™ S100 HR, a propylene column packed with dextran acrylamide particle platform allyl dextran and N, N methylenebisacrylamide. 50 mM PB at pHs of 7.4, 7.6 and 8 were used to equilibrate the column and elute the protein during SEC. The column was washed with 120 ml of 100 mM PB at 1ml/min. After washing the column, 5 ml of AEX fraction was loaded at 0.5 ml/ min onto size

exclusion column. After loading the sample, approximately 150 ml of 50 mM PB was loaded until protein was detected at wavelength between 300 and 1400 nm. Samples were collected and stored on ice into a fridge at 4ºC for characterization assays.

2.1.5.1 Effect of pH used during purification process on ASNase, GLNase and NHase activity.

In order to know the effect of pH buffers used during lysis, dialysis , AEX and SEC process, several purifications were made from *R. rhodochrous* cells DAP 96253-C6 fermented in media with urea and Co^{2+} (Harvested on 01-08-18, 08-10-18 and 02-07-19) and no induced cells of *R. rhodochrous* DAP 96253-C6 fermented in media with Urea (Harvested on 09-07-18) The purifications were made using 50mM PB- 5 mM 2- mercaptoethanol as a lysis buffer, 50 mM PB 5mM,2-mercaptoethanol, 1.5 % glycerol, 1% $MgS0_4\,7H_2O$ as a dialysis buffer and 10mM Sodium butyrate/25mM NaH_2PO4 as equilibration buffer. The pHs tested were 7.2, 7.4 7.6 and 8.

2.2 Protein Quantification

The protein concentration for each elution was determined through the bicinchoninic acid assay (BCA), using Pierce™ BCA protein assay reagent A, B, and bovine serum albumin standard from 0.32- 2 mg/ml protein (Thermo Scientific™). Lysates and dialyzed lysates were diluted 1/8, 1/16 and 1/32. For fractions from AEX the dilutions used were 1/2, 1/4 and 1/8. For the fractions from SEC, the dilution used were 0, and 1/2.

2.3 Enzyme Assays

2.3.1 ASNase and GLNase Activity Assays

For ASNase activity determination 10µL of each purification fraction was suspended in 990µL of 1000 ppm ASN (TLC grade, Sigma) and for GLNase activity100 ppm GLN (TLC

grade, Sigma) and mixed for 2 min at 25°C. The substrates were dissolved either in 50mM PB 7.2 or 50mM PB 7.6. The reaction was made in triplicate. After time reaction 10 μL of 4 N sulfuric acid(H_2SO_4) to degrade enzymes and stop the reaction. After vortex it for 30 seconds, the reaction was neutralized with 4 N Sodium hydroxide (NaOH). After neutralization, color reaction to measure ammonium was done by a modification of Fawcett and Scott method (Fawcett & Scott, 1960). 1 ml of the neutralized enzyme reaction, 2 ml 2.5% sodium phenate, 3 ml 0.01% sodium nitroprusside and 3 ml 0.15% sodium hypochlorite were added into a glass test tube in that order. The test tubes were vortexed for 30 seconds. After 30 minutes of dark incubation at 25ºC the absorbance was measured using a PW Victor 3 V (Wallac1420) plate reader at 630 nm and compared ammonium concentration of 0-5 ppm.

2.3.2 NHase Assay

The NHase activity was determined by adding 2 μL of the purification sample to 998 μL of 1000 ppm acrylonitrile (Aldrich) at pH 7.2 or 7.6. The enzyme reaction was conducted as was described in section 2.3.1 including and additional step after neutralizing of the reaction. 10μL of 1:50 amidase from *Pseudomonas aeruginosa* (Sigma Aldrich) was suspended into the neutralized enzyme reaction and incubated at 37ºC for 30 minutes to complete the conversion of amides in their respective acids and ammonium. The ammonium production was measured by the modification of method of Fawcett and Scott Face (see section 2.3.1). After 30 minutes of dark incubation at 25ºC the absorbance was measured using a PW Victor 3 V (Wallac1420) plate reader at 630 nm and compared ammonium concentration of 0-20 ppm.

2.4 Determination of Molecular weight of NHase with ASNase Activity.

2.4.1 Native Gel Electrophoresis

Fractions loaded through SEC column and fractions collected from SEC were diluted to obtain 5-10 μg/ml protein concentration using 2.5 μl of Native PAGE® LDS Sample Buffer (4X), and distilled water up to 15 μl. 3μl Invitrogen ™ Native Mark Unstained protein standard and 15 μl of each sample were loaded in an Invitrogen ™ Non- Denaturing Nu PAGE® gel, using Native PAGE ™ Anode Buffer (1X) and Native PAGE ™ Light Blue cathode Buffer (1X) at 150 V constant using a XCell *SureLock* ™ Mini-Cel for electrophoresis . The gels were stained with Coomassie staining.

2.4.2 SDS -PAGE

Samples from purification of NHase with ASNase activity were diluted to obtain 5-10 μg/ml protein concentration using 2.5 μl of NuPAGE™ LDS Sample Buffer (4X), 1μl of NuPAGE™ Reducing Agent (10X) and distilled water up to 15- 20μl. After preparation, samples were loaded in precast NuPAGE® Novex Bis-Tris gel, using NuPage 1x SDS Running MOPS buffer, at 200 V to identify their molecular weight. The Thermo scientific™ PageRuler™ Plus protein Ladder was used to identify the Molecular weight of loaded proteins. Proteins bands were visualized by silver and Coomassie Blue staining.

2.5 Determination of Isoelectric Point of NHase with ASNase activity.

The isoelectric point of nitrile hydratase with ASNase activity was determined by isoelectric focusing (IEF) using an Invitrogen ™ Novex ® IEF gel. After SEC, samples with highest ASNase activity were prepared with 5 μl of Novex®IEF Sample Buffer and water until reach a protein concentration of 5μg/ml. After assembling the gel into a XCell *SureLock* ™ Mini-Cel for electrophoresis, 200 ml 1X Novex® IEF Cathode Buffer 1X Novex® IEF Anode Buffer were used to fill the inner and outer chamber respectively, 25 μl of samples and 5 μl of

Invitrogen ™ IEF marker 3-10 were loaded. The gel was run at 100 V constant for 1 hour, followed by 200 V constant for 1 hour, and 500 V constant for the last 30 minutes.

2.6 Determination of ASN and GLN K_m

The Michaelis constant (*Km*), and maximal velocity (*Vmax*) of purified NHase with ASNase activity were determined using ASN and GLN as substrate in the range of 0.05– 20mM. The ASNase and GLNase velocity were determined using 10 µl of purified sample and the enzyme assay method described in section 2.3. The *Km* and *Vmax* were determined using the Lineweaver-Burk plot and the following equation (See appendix D).

$$\frac{1}{V} = \frac{KM}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax}$$

2.7 Determination of Thermal and pH Stability on ASNase Activity

2.7.1 Thermal Stability

Purified samples of NHase with ASNase activity were incubated at 25,30 and 37 °C for 15 minutes and the residual ASNase activity was assayed using the method described in section 2.3.

2.7.2 pH Stability

In order to determine the effect of pH on ASNase activity 20 µl of purified enzyme was mixed with 20 µl of buffers at 6.5, 7.0, 7.2, 7.4, 7.6, 8, 9 and 10 pH s. The buffers used for pHs 6–7.5 and 8–10 were 50 mM phosphate buffer (pH 6–7.5) and 0.05 M Tris–HCl respectively.

(Tabandeh & Aminlari, 2009). The ASNase activity was determined at pH 7.6 which was the pH used for purification process.

2.8 Determination of Half-life of ASNase Activity

The half- life of ASNase activity was determined through protease and serum treatment. 0.5 ml of purified NHase with ASNase -like activity was mixed with 2.5 ml of human serum (Sigma) at a final concentration of 50 U/ml and sample was incubated at 37°C. The activity of ASNase was assayed every 5 hours until the ASNase was undetectable.

The resistance of ASNase activity to trypsin was measured using a solution of 50 IU trypsin (Sigma) in 50 mM buffer phosphate (pH 7.4) containing 0.5 ml of purified NHase with ASNase activity (50 U/ml). After homogenization and incubation at 37°C, the ASNase activity was measured every 10 minutes until the enzyme was no longer detectable (Husain et al., 2016b).

2.9 Determination of GLNase and ASNase Stability after Storage

After purification, SEC fractions were stored on Ice at 4ºC and ASNase and GLNase activity was measured in different time periods after storage. The ASNase and GLNase activity measured immediately after SEC was termed as initial activity and was used as a reference to evaluate the stability of the enzyme. Enzyme assays were done as was described in section 2.3

3 RESULTS

3.1 Purification of NHase with ASNase activity

3.1.1 Effect of Lysis buffer pH on ASNase Activity

As is illustrated in figure 3.1, the ASNase activity for the protein purified by AEX from a cell-free lysate made with 20mM PB pH 5.8 was undetectable and NHase activity showed a specific activity between 29 ± 1.6 and 39 ± 1 $IUmg^{-1}$ where a unit of enzyme per mg of protein

present in the total volume of the purified fraction is equal to the micromoles of ammonium produced per minute. Contrarily, when *R. rhodochrous* cells were suspended in 50 mM PB pH 7.2 lysis buffer, the ASNase activity after an AEX was 256 ± 13 $IUmg^{-1}$ and NHase activity was 1.5 $IUmg^{-1}$ (Figure 3.2). This suggests that the structure of NHase's active site changes at pH 7.2 resulting in a better binding to ASN as substrate.

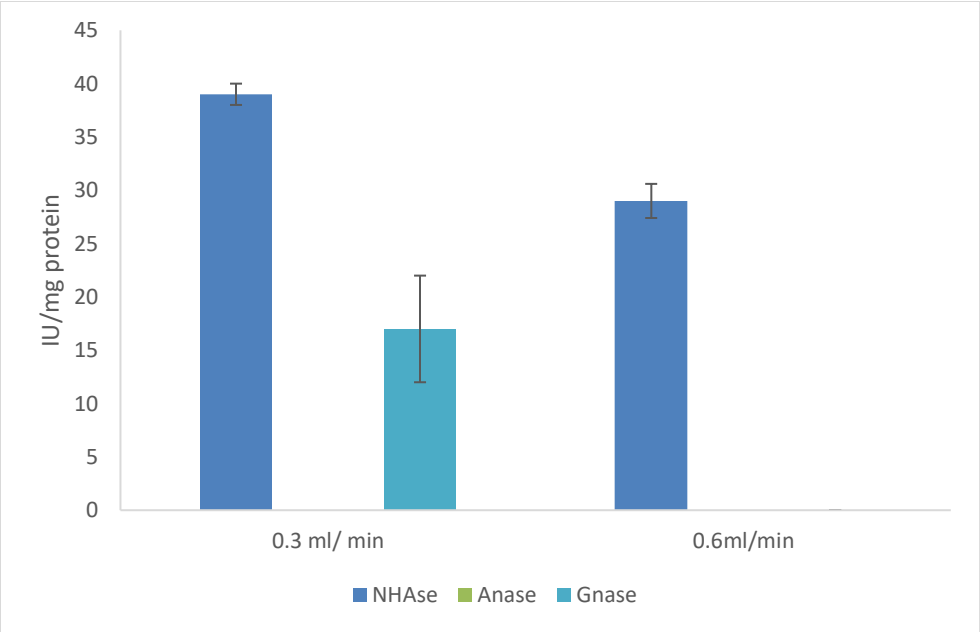


Figure 3.1 Effect of Lysis Buffer 20mM PB pH 5.8 on Enzyme activity.
Enzyme activity for nitrile hydratase purified from induced cells of R. rhodochrous DAP 96253 C6, harvested on 01-08-18 and stored at 4ºC for 1 day after harvest. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

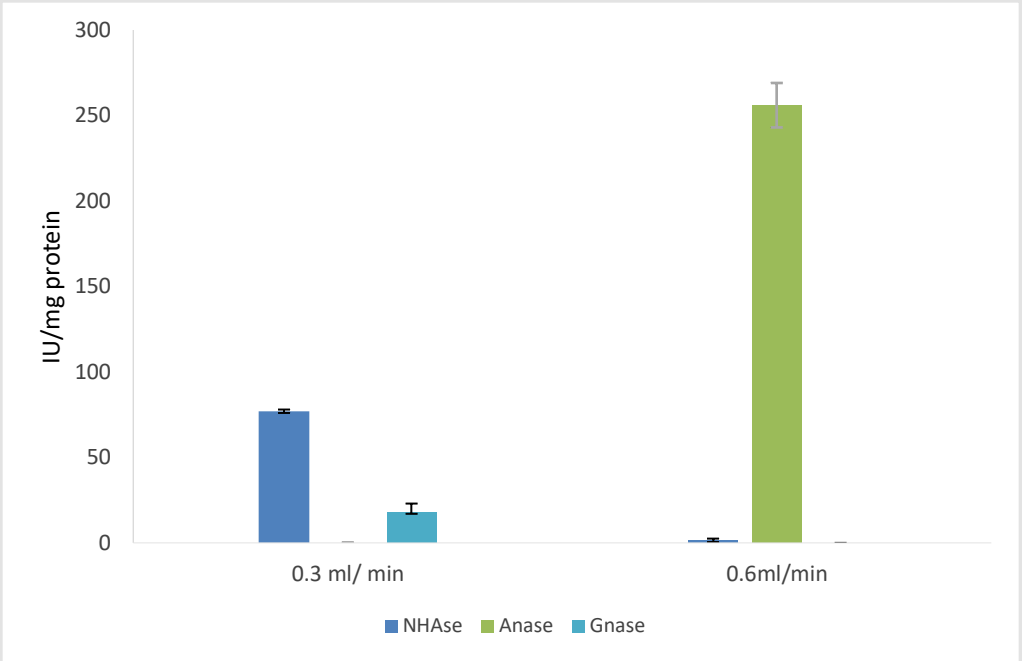


Figure 3.2. Effect of Lysis buffer 50 mM PB pH 7.2 on Enzyme activity of NHase
The enzyme was purified from fermentation paste of induced R. rhodochrous *DAP 96253 C6, stored at 4ºC for 1 day and lysed by sonication in 50 mM phosphate buffer pH 7.2*. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

3.1.2 Effect of Purification Flow rate on ASNase Activity

The ASNase activity for the purified protein from *R. rhodochrous* DAP 96253 C6 was higher (256± 13 $IUmg^{-1}$) when the anion exchange chromatography (AEX) was performed at 0.6 ml/min flow rate (figure 3.3)

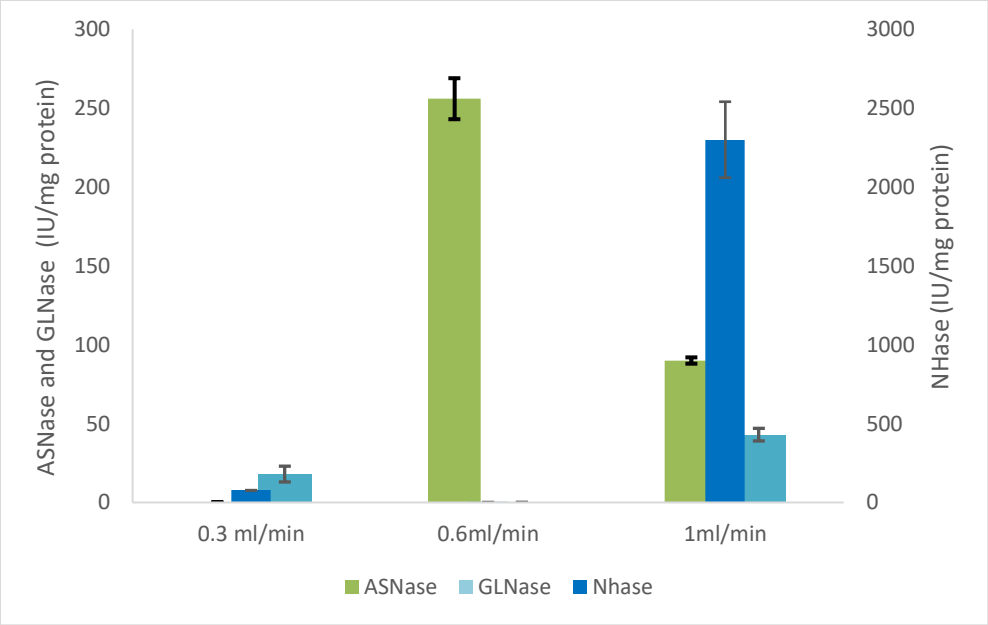


Figure 3.3. Effect of AEX Flow Rate on Enzyme Activity of the Purified Protein.
Lysis buffer and equilibration buffer used in these three sets of purifications was 50mM PB pH 7.2 and the starter material for these came from induced *R. rhodochrous* DAP 96253-C6 harvested on 01-18-18. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

3.1.3 Effect of 2- Mercaptoethanol and Dialysis on ASNase Activity

Using a 50 mM PB; 5mM 2-mercaptoethanol; pH 7.2 as a lysis buffer and dialysis the NHase preparation showed a post AEX ASNase activity of 557 ± 3 UI/mg and 249 ± 6UI/mg were purified in AEX fractions 0.1 and 0.2 M NaCl respectively. However, the ASNase activity was undetectable for the purification that used a 2-mercaptoethanol free lysis buffer and for the purification made using 50 mM PB 5mM 2-mercaptoethanol pH 7.2 as a lysis buffer without dialysis process (Fig. 3.4).

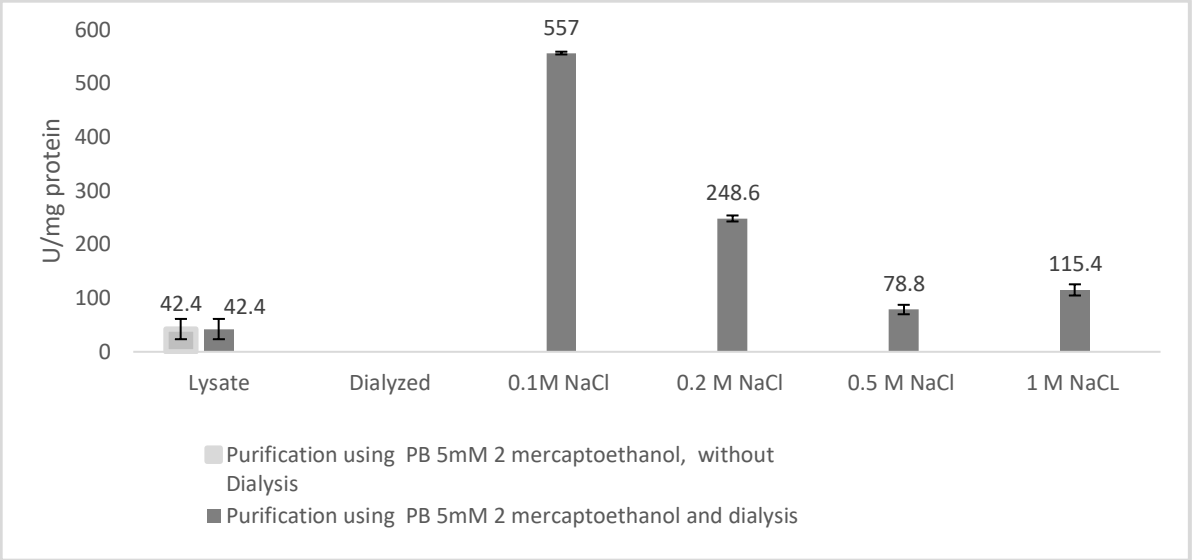


Figure 3.4. Effect of 2- mercaptoethanol and Dialysis Process on ASNase Activity. Purifications were made from induced cells of *R. rhodochrous* DAP 96253- C6 harvested on 01-18-18. One unit (IU) of ASNase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

3.1.3.1 Effect of storage conditions of fermentation paste on ASNase, NHase and GLNase Activity

Figure 3.5 shows that when fermentation paste is stored at 4ºC for 18 days, the ASNase activity in purified fractions decrease. However, the activity was detectable after 150 and 250 days using 2- mercaptoethanol as a reducing agent.

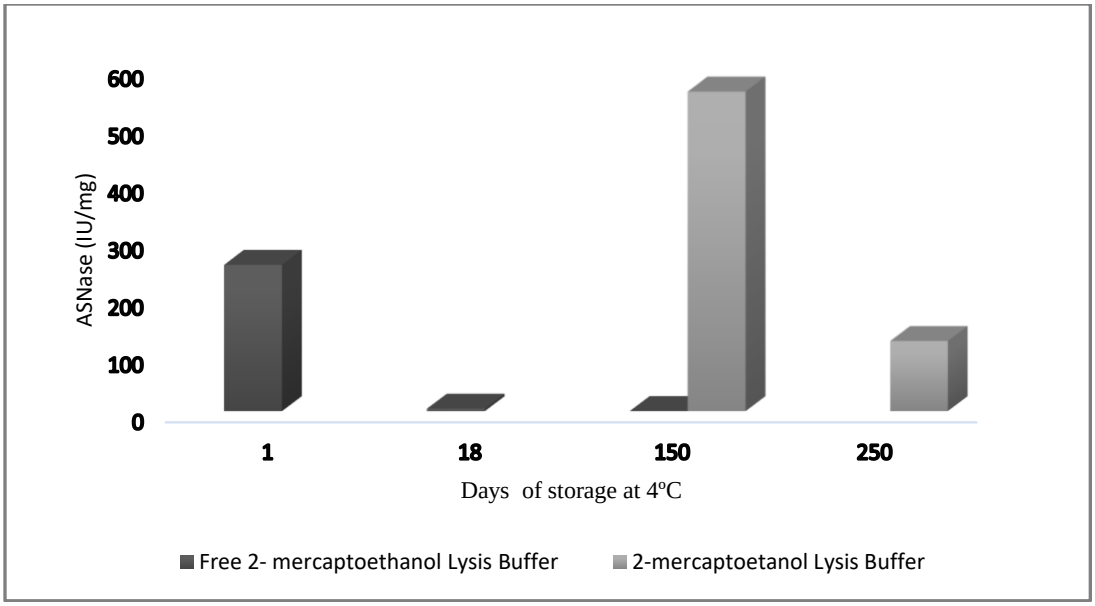


Figure 3.5 Effect of storage time of cells and 2- mercaptoethanol on ASNase activity.
The lysis buffer free of 2- mercaptoethanol was used for purifications done after 1, 18 and 150 days of storage. The purifications 150 and 250 days postharvest were done using 2-mercaptoethanol as a component of lysis buffer. The buffers used during purifications were adjusted at pH 7.2. One unit (IU) of ASNase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

3.1.4 Effect of buffer Exchange between Purification Steps on ASNase Activity

A set of purifications of NHase with ASNase activity from fermentation paste of *R. rhodochrous DAP 96253- C6* harvested on 08 -10-18 were made to compare the effect of the changing types of buffer during purification process to the effect of using a unique buffer for the entire process (Lysis, dialysis and AEX).

Figure 3.6 shows the enzyme activity (ASNase, GLNase and NHase) for starting material and purified fractions from a purification which used 0.05 M Tris-HCl pH 8.0 in all purification steps. For this purification ASNase activity was considerably lower than NHase activity.

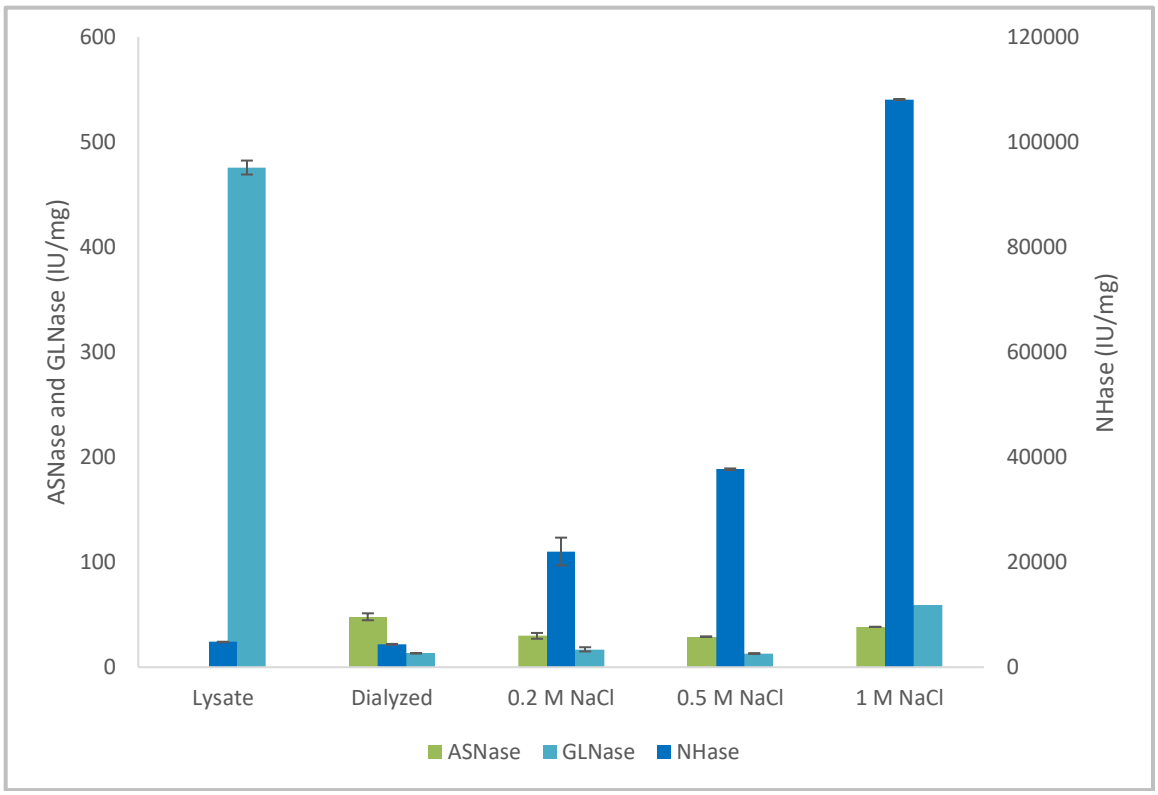


Figure 3.6 Effect of 0.05M TRIS-HCl pH 8 as the Unique Buffer for all Purification Steps on Enzyme Activity

Purification was performed from Induced cells of *R. rhodochrous DAP 96253*- C6. 0.05 M TRIS-HCl buffer pH 8 was used for lysis, dialysis, equilibration and elution steps. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

Figure 3.7 shows the results for purification made from fermentation paste harvested on 08 -10-18 and using different buffers for each purification step. The ASNase activity approximately was 2 folds higher when 0.05M TRIS-HCl pH 8 was used only for dialysis buffer and other buffers, such as PB and sodium butyrate were used in lysis and AEX.

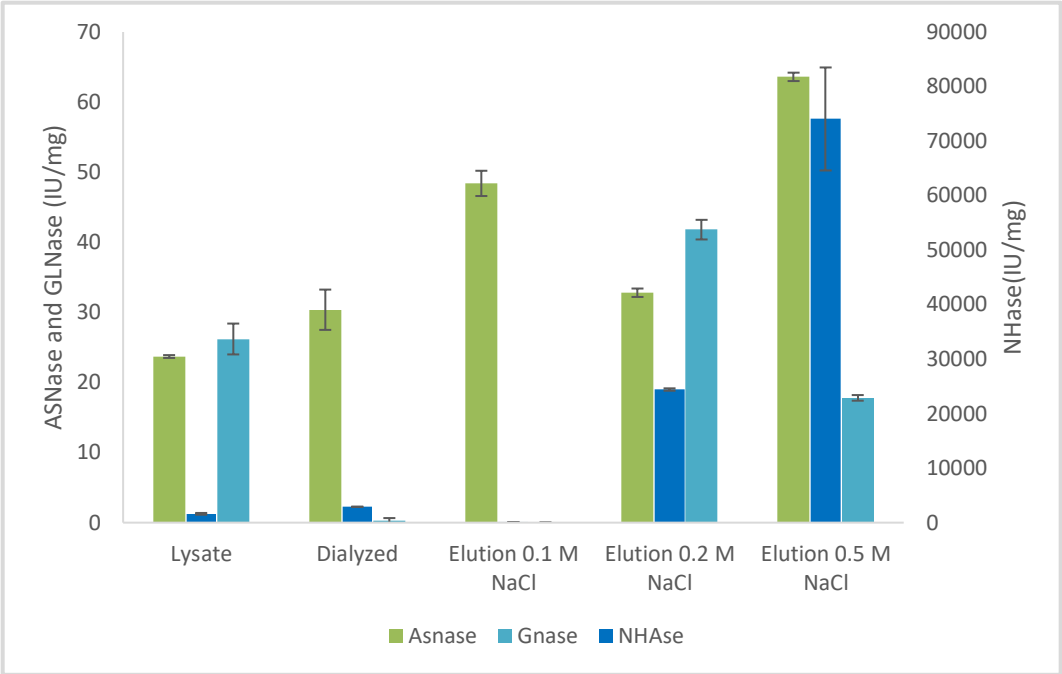


Figure 3.7 Enzyme Activity Using Different Buffers During Protein Purification.
50 mM PB-2mercaptoethanol was employed for lysis, 0.05M TRIS- HCl for dialysis and sodium butyrate /sodium monophosphate buffer for AEX. The Purification was made from induced cells of *R. rhodochrous* DAP 96253-C6. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

Figure 3.8 shows that the ASNase from purified fractions is more active when PB is part of the composition of lysis, dialysis, equilibration and elution buffer compared with the activity showed in purifications in which 0.05M Tris-HCl was used during purification process.

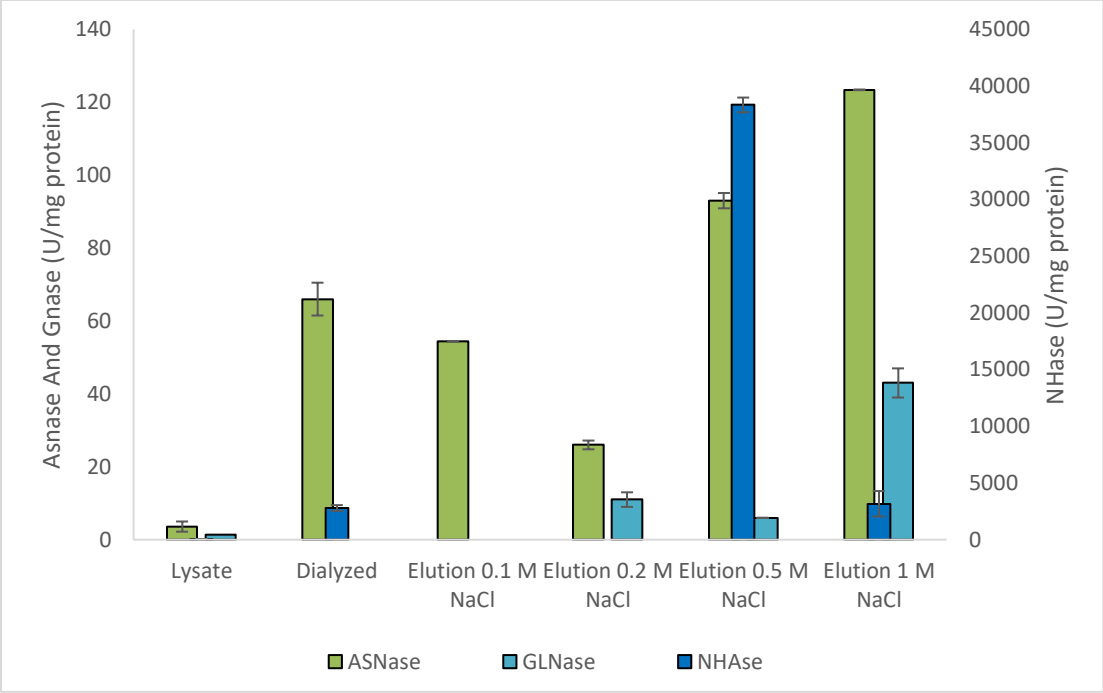


Figure 3.8Effect of PB pH 8 as a Component for Lysis and Dialysis Buffer on Enzyme activity NHase activity was not determined for flow-through, washing, 0.1 and 0.2 M NaCl. The Purification was made from induced cells of *R. rhodochrous* DAP 96253-C6 lysed in 50mM PB- 2 mercaptoethanol, dialyzed against a buffer 50m MPB dialysis buffer (5-mM 2mercaptoethanol, glycerol and $MgSO4\ 7H_2O$) and the protein was eluted with 10 mM sodium butyrate/ 25 mM sodium monophosphate during AEX. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

The effect of interchanging buffers used for lysis, dialysis and AEX on enzyme activity is shown in figure 3.9. this figure summarizes results presented in table 3.6 to 3.8. The fraction that exhibited the highest ASNase and lowest NHase activity was purified using 50 mM PB as a lysis and dialysis buffer and 10 mM sodium butyrate- 25mM NaH_2PO_4 as equilibration and elution buffers (Purification method 3).

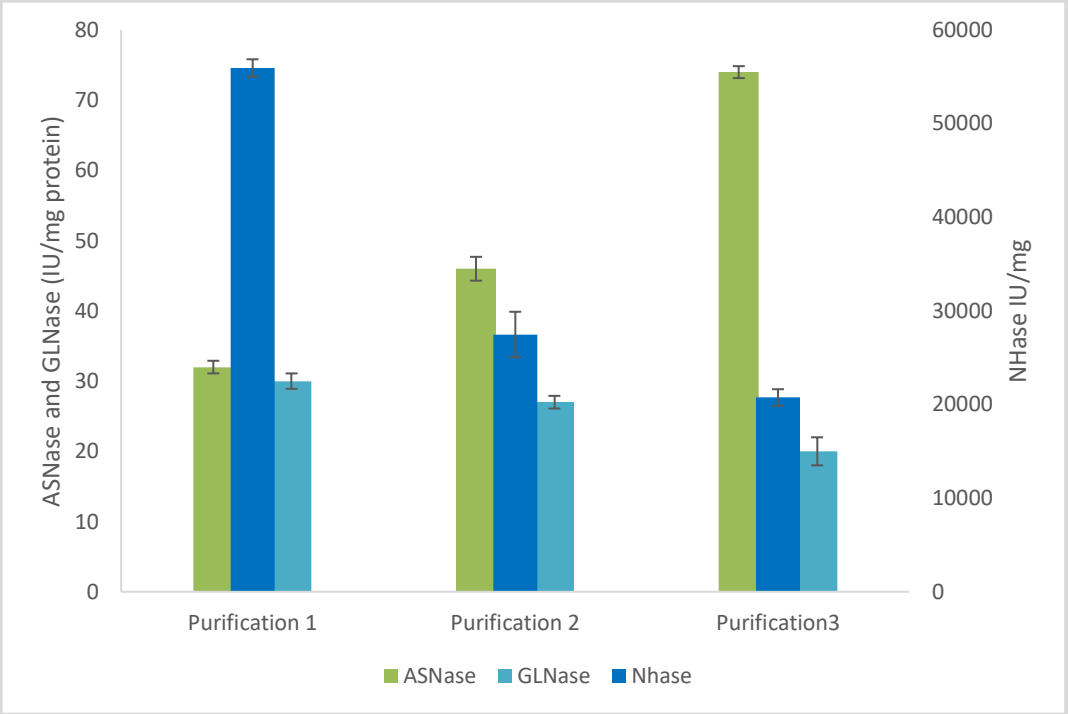


Figure 3.9 Effect of Interchange Buffers During Purification Process on Enzyme Activity. **Purification 1** was performed using 0.05 M TRIS- HCl for all purification steps. **Purification 2** used 50 mM PB- as a lysis buffer, 0.05 M TRIS- HCl for dialysis buffer and 10 mM sodium butyrate- 25mM NaH_2PO_4 as equilibration and elution buffer. **Purification 3** 50 mM PB as a lysis and dialysis buffer and 10 mM sodium butyrate- 25mM NaH_2PO_4 as equilibration and elution buffers.

3.1.5 Purification Process pH and ASNase, GLNase and NHase activity

Figures 3.10 and 3.11show the enzyme activity average for purifications made at pHs 7.2, 7.4, 7.6 and 8 using induced cells of R. *rhodochrous* DAP 96253 C6 suspended in PB -2 mercaptoethanol Lysis Buffer. Purifications made at pHs 7.6 confer higher ASNase activity while purifications at pH 8 produce purified fractions with high activity of NHase.

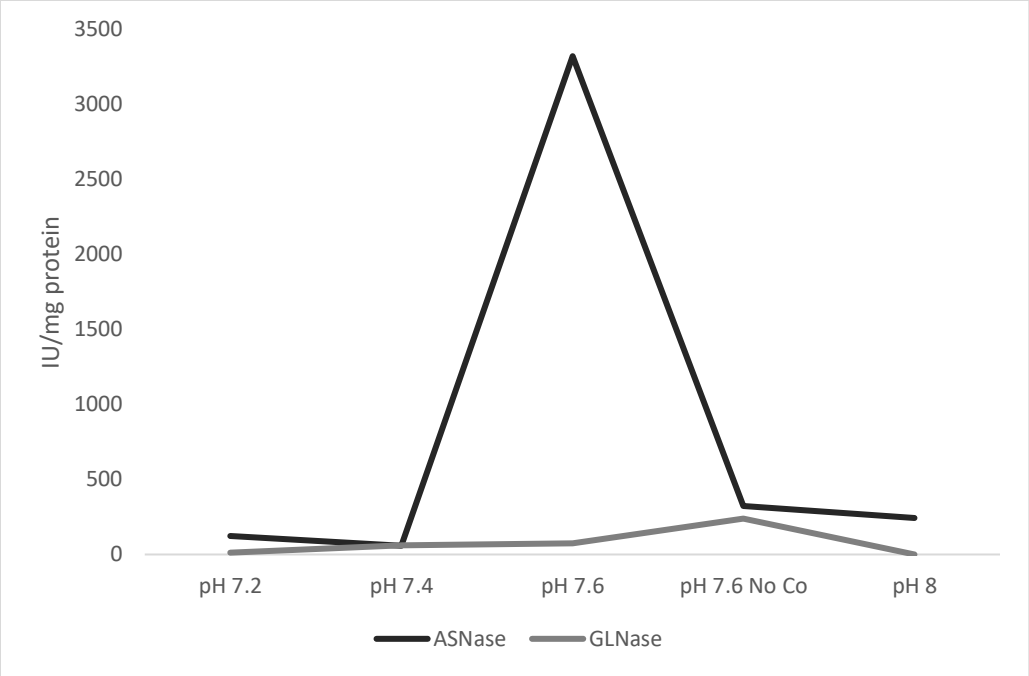


Figure 3.10 Effect of pH Purification on ASNase Activity and GLNase activity.
Purification at 7.2 was done by AEX and the other purifications were made by AEX followed by SEC. The results of pH 7.6 No Co^{2+} and pH 8 No Co^{2+} were obtained from purifications from non- induced cells of *R. rhodochrous* DAP 96253-C6 fermented in media with urea harvested on 09-07-18. One unit (IU) of ASNase or GLNase is defined as the amount of enzyme able to produce 1 μmol of ammonia per minute at pH 7.2 and 25°C.

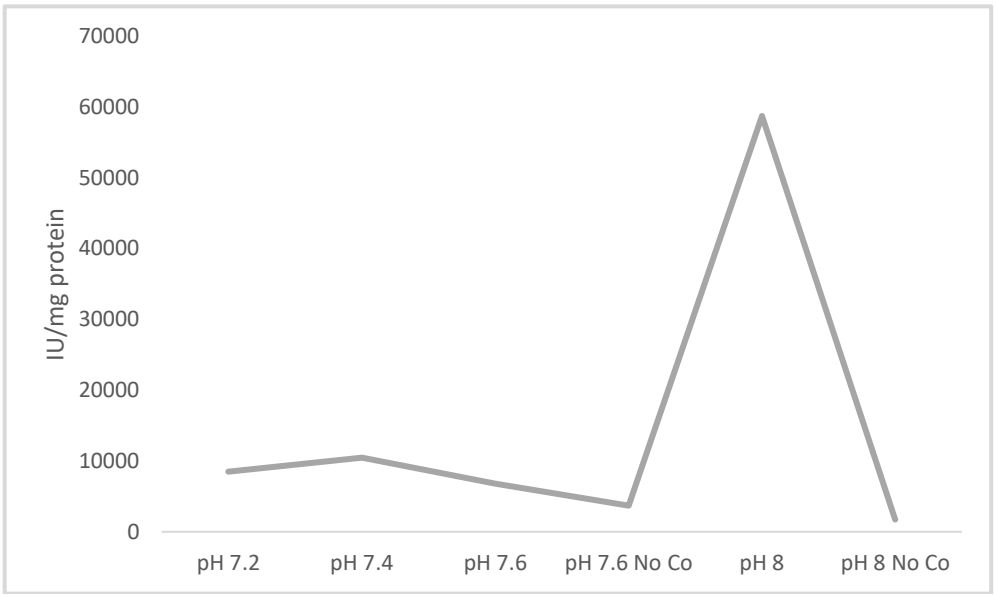


Figure 3.11 Effect of pH Purification on NHase activity.
Purification at 7.2 was done by AEX and the other purifications were made by AEX followed by SEC. The results of pH 7.6 No Co^{2+} and pH 8 No Co^{2+} were obtained from purifications from non-induced cells of *R. rhodochrous* DAP 96253-C6, fermented in media with urea harvested on 09-07-18. One unit (IU) of NHase is defined as the amount of enzyme able to produce 1 μmol of ammonia per minute at pH 7.2 and 25°C.

3.1.6 Effect of the Type of AEX column on ASNase Activity

Two purifications simultaneously were made from non $GUCo^{+2}$ induced cells from *R. rhodochrous* DAP 96253C6 to compare the results of ASNase activity using two different AEX columns, a HiTrap™ Q column and a Toyopearl® super Q -650M column. Tables 3.1 and 3.2 show that Toyopearl Super Q column allows the purification of a protein with higher specific activity and a fold purification of 16.8 than the enzyme purified using HiTrap™ Q column.

Table 3.1Summary for Purification of NHase with ASNase Activity Using HiTrap Q Column

Purification step	Total activity (IU)		Protein (mg)	Specific activity (IU/mg)		Fold purification		Yield (%)	
	NHase	ASNase		NHase	ASNase	NHase	ASNase	NHase	ASNase
Lysate	87844.5	5062.5	405	216.9	12.5	1.00	1.00	100	100
After dialysis	82320	8070	300	274.4	26.9	1.3	2	94	159
Elution 0.5 M NaCl	85326	622	17	5019.2	36.6	23	3	97	12

Table 3.2 Summary for Purification of NHase with ASNase Activity Using Toyopearl® Super Q Column.

Purification step	Total activity (IU)		Protein (mg)	Specific activity (IU/mg)		Fold purification		Yield (%)	
	NHase	ASNase		NHase	ASNase	NHase	ASNase	NHase	ASNase
Lysate	87844.5	5062.5	405	216.9	12.5	1.00	1.00	100	100
After dialysis	82320	8070	300	274.4	26.9	1.3	2.	94	159
Elution 0.5 M NaCl	1881	236	1.12	1679.6	210.6	8	17	2.1	5

3.1.7 Purification of NHase with ASNase and GLNase activity from No induced cells of R. rhodochrous DAP 96653 C6

A SEC was done with 5 ml of a fraction with ASNase activity of 3 ± 0.01 IU $mg^{-}1$, eluted with 0.5 M NaCl during an AEX which used non induced cells of *R. rhodochrous DAP* 96253 C6 as starter material (see table 3.3). After SEC, 9 fractions of 13 samples collected presented ASNase activity. As is depicted in figure 3.12, fraction 12 presented the highest ASNase activity 755 ± 26 IU mg^{-1}, but GLNase was more active with 990 ± 52 IU mg^{-1}. The purified protein with ASNase activity recovered in fraction 10 present better properties for ALL treatment because its

GLNase activity is 36 ± 5.2 IU mg^{-1} and the NHase activity is considerably lower compared to the other SEC fractions obtained in this purification.

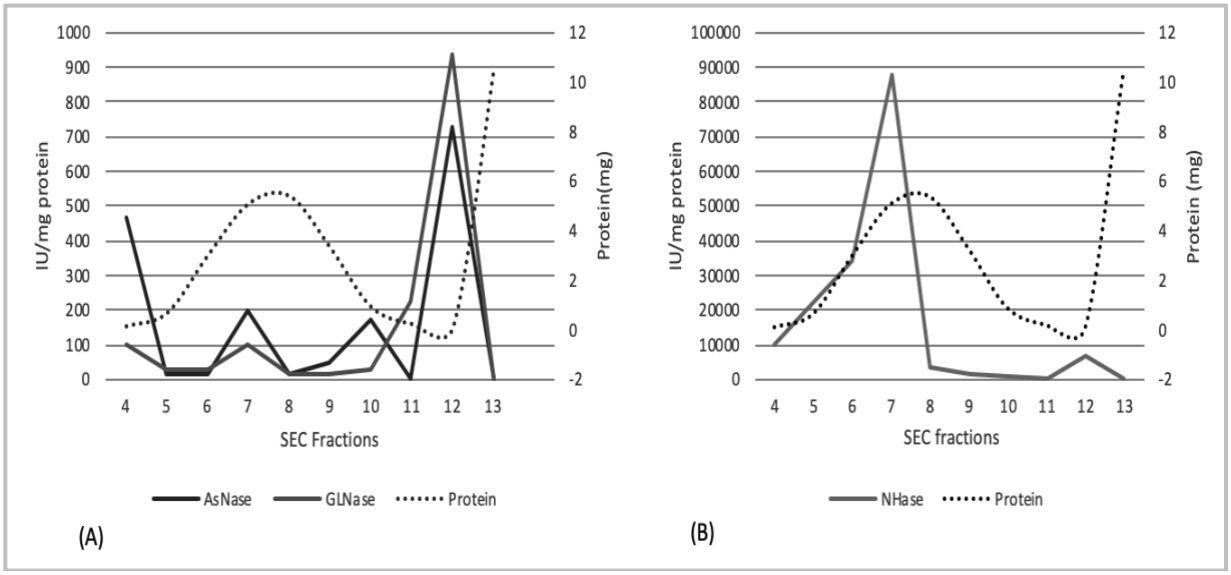


Figure 3.12 Enzyme Activity After SEC Using Uninduced Cells of R. rhodochrous DAP 96253 C6.
(A) Specific activity of ASNase and GLNase for SEC fractions. (B) Specific activity of NHase for SEC fractions. The purification was done at pH 7.6 and the enzyme activity was assayed at pH7.2. The NHase activity was no determined for fraction 11 and 13. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 μmol of ammonia per minute at pH 7.2 and 25°C.

The yield percentage for the ASNase purified from non $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96253 C6 was approximately 3 and 68f old higher than GLNase and NHase respectively (Table 3.3)

Table 3.3 Summary for Purification of NHase with ASNase and GLNase Activity from Non-induced Cells of R. rhodochrous DAP 96253

Purification step	Total activity (IU)			Protein (mg)	Specific activity (IU/mg)			Fold purification			Yield (%)		
	NHase	ASNase	GLNase		NHase	ASNase	GLNase	NHase	ASNase	GLNase	NHase	ASNase	GLNase
After dialysis	209415	230	115	115	1821	2	1	1.0	1.0	1.0	100	100	100
Elution 0.5 M NaCl	251526	99	99	33	7622	3	3	4.2	1.5	3.0	120.1	43	86
SEC fraction 10	722	157	27.9	0.9	802	174	31	0.4	87.0	31	0.3	68	24

3.1.1 Purification of NHase with ASNase and GLNase activity from GUCo^{2+} induced cells of R. rhodochrous DAP 96622

A NHase with ASNase, GLNase and NHase activity was purified after lysis, dialysis and AEX steps at pH 7.6 using a flow rate of 0.6ml/min. As is presented in figure 3.13 the highest specific activity for the three enzymes of interest was obtained after a second elution with 0.1M NaCl (0.1M2). After polishing fraction 0.1M2 by SEC, five of 10 fractions collected had ASNase activity. These fractions were tested for NHase and GLNase activity and NHase activity was founded undetectable while GLNase was active in fraction 1,3 and 4. Fraction 4 presented the highest ASNase and GLNase activity which was higher 10247 ±141IU mg^{-1} compared to ASNase 8555 IU mg^{-1}(Figure 3.14).

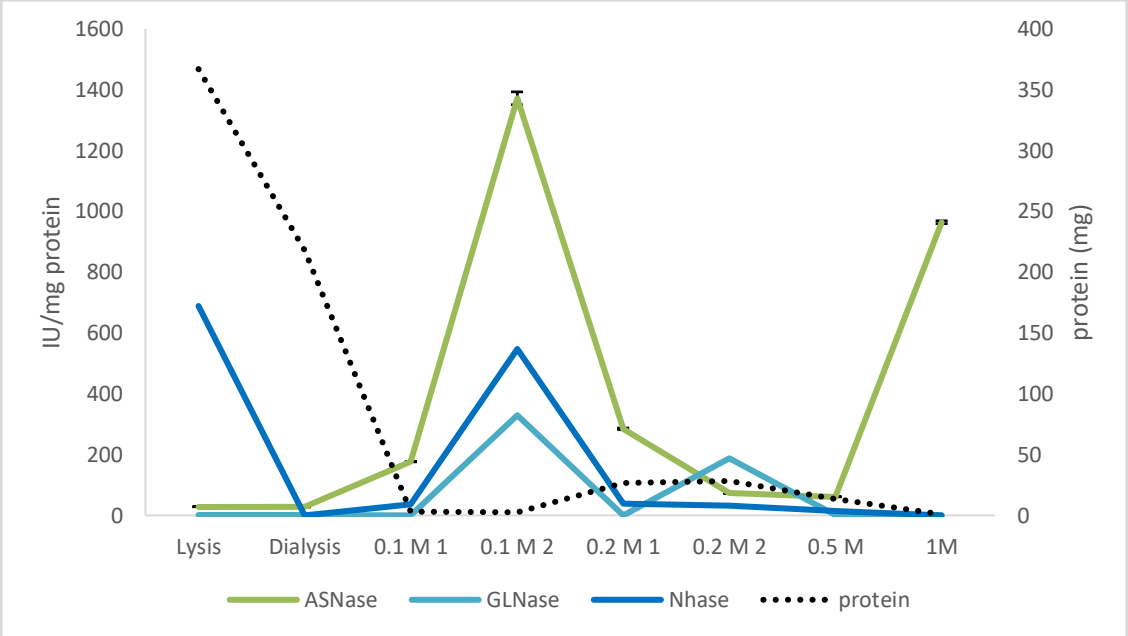


Figure 3.13 Enzyme Activity after AEX using Induced R. rhodochrous DAP 96622
ASNase, GLNase and NHase activity of starter material and purified fractions from AEX. The NHase activity for dialysis was not tested. The fraction 0.1 M2 was used to load the SEC. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

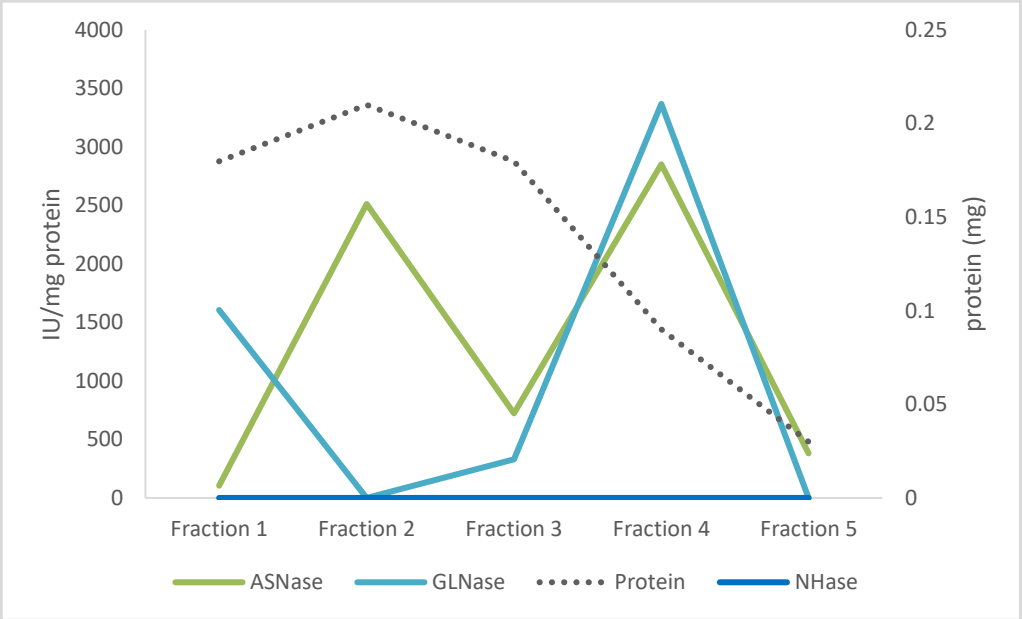


Figure 3.14 Enzyme Activity after SEC using Induced R. rhodochrous DAP 96622
The enzyme assay was done at pH 7.6 and NHase activity using 1000 ppm acrylonitrile was undetectable. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

The fold purification and percentage of yield for the purification of NHase with ASNase and GLNase activity from induced *R. rhodochrous* DAP 96622 were calculated using the enzyme activity and protein concentration obtained in each step of purification process as is presented in table 3.4 and the summary for the SEC fraction in which GLNase was undetectable is presented in table 3.5. The fraction without GLNase activity presented less fold purification and yield percentage for ASNase.

Table 3.4 Summary for Purification of NHase like ASNase and GLNase Activity from Induced Cells of R. rhodochrous DAP 96622.

Purification step	Total activity (IU)		Protein (mg)	Specific activity (IU/mg)		Fold purification		Yield (%)	
	ASNase	GLNase		ASNase	GLNase	ASNase	GLNase	ASNase	GLNase
Lysate	7707	513.8	367	21	1.4	1.0	1	100	100.0
After dialysis	5232	174.4	218	24	0.8	1.1	0.05	67.9	33.9
AEX Fraction 0.1M 2	3483	822.5	2.5	1393	329	66.3	21.93	45.2	160.1
SEC fraction 4	257	303	0.09	2852	3369	136	225	3.3	59.0

Table 3.5 Summary for Purification of NHase with ASNase without GLNase Activity from Induced R. rhodochrous DAP 96622.

Purification step	Total activity (IU)		Protein (mg)	Specific activity (IU/mg)		Fold purification		Yield (%)	
	ASNase	GLNase		ASNase	GLNase	ASNase	GLNase	ASNase	GLNase
Lysate	7707	513.8	367	21	1.4	1.00	1	100.0	100.0
After dialysis	5232	174.4	218	24	0.8	0.64	0.05	67.9	33.9
AEX Fraction 0.1M 2	3483	822.5	2.5	1393	329	22.45	21.93	45.2	160.1
SEC fraction 2	527	0	0.21	2511	0	23.9	0.00	6.8	0.0

3.1.2 Purification of NHase with ASNase and GLNase activity from $GUCo^{+2}$ induced cells of R. rhodochrous DAP 96253 C6

The enzyme activity after lysis, dialysis and AEX purification steps using cobalt-urea induced cells of *R. rhodochrous* DAP 96253-C6 is shown in the figure 3.15. After eluting the sample with 5 ml of 0.5M NaCl, the specific activity for ASNase and GLNase was 75 ± 4 and 44 ± 2 $IUmg^{-1}$ with a total protein concentration in the 5 ml of the sample of 10.75 mg.

Figure 3.16 illustrates the enzyme activity of SEC fractions obtained after loading into SEC columns the elution 0.5 M NaCl from AEX. This fraction was chosen for polishing by SEC because it was the fraction presented ASNase and GLNase activity, low NHase activity and higher protein concentration. 11 Fractions of 15 SEC fractions collected presented similarly ASNase and GLNase activity, but NHase activity only was detected in fractions 12, 13 and 14. The highest specific ASNase and GLNase activities were shown in fraction 4 with values of 9078 ± 349 IU mg^{1} and 8962 ±349 IU mg^{1} respectively. Fractions 7 -10 presented lower values for ASNase and GLNase specific activity and higher protein concentration. Due that the fact that volumes of SEC fractions were 3 ml and fraction 8 and 9 presented similar protein

concentrations (0.41 and 0.42 mg/ml) and ASNase activities (335±40and 274 ± 25U $mg^{-1)}$, those fractions were pooled to characterize the purified enzyme.

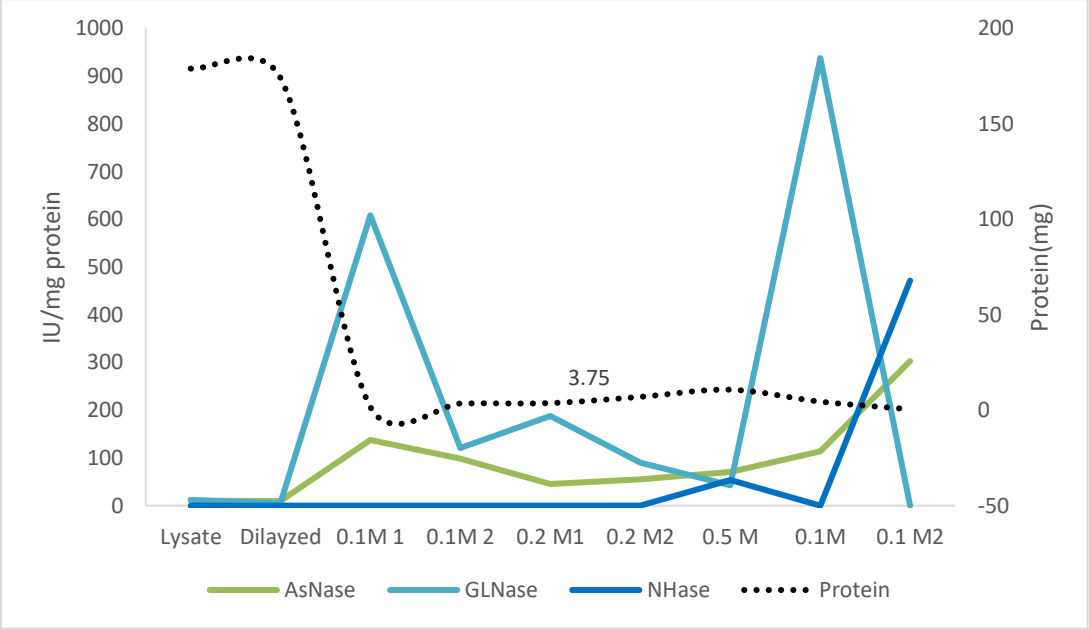


Figure 3.15Enzyme Activity after AEX Using Induced R. rhodochrous DAP 96253 C6
The enzyme assay was done at pH 7.6. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

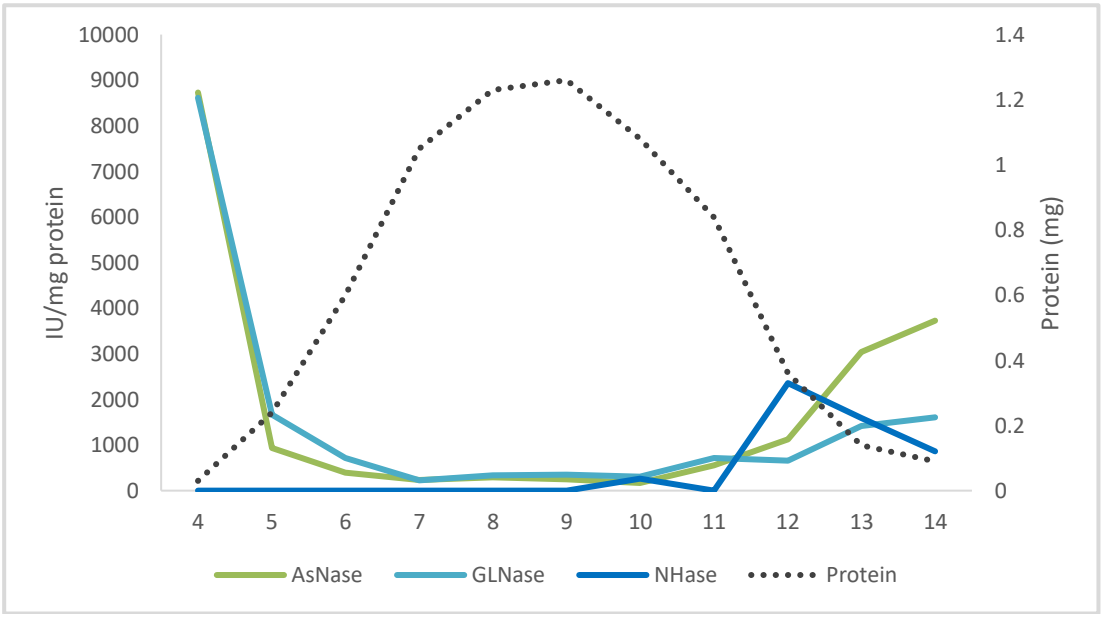


Figure 3.16 Enzyme Activity after SEC Using Induced R. rhodochrous DAP 96253 C6
The enzyme assay was done at pH 7.6. The NHase activity was undetectable using 1000 ppm Acrylonitrile at pH 7.6. One unit (IU) of ASNase, GLNase, or NHase is defined as the amount of enzyme able to produce 1 µmol of ammonia per minute at pH 7.2 and 25°C.

Table 3.6 summarizes the results of the four steps for the purification from induced cells of *Rhodococcus rhodochrous* DAP 96253 C6. The fold purification and percentage of yield for ASNase were in a range of 26.4 to 839 and 14.1 % to 25% for ASNase, while for GLNase was between 28 to 718 and 9.2 to 28 %. An 839-fold purified enzyme was obtained in fraction 4 with the highest ASNase activity (8729 IU mg^{-1}) with an overall recovering of only 14% of the total activity, which is the specific activity times the total mg protein in preparation. However, the enzyme purified in fraction 13 presented a better yield of 22.8% with ASNase activity of 3036 IU mg^{-1}.

Table 3.6 Summary for Purification of NHase with ASNase and GLNase Activity from Induced R. rhodochrous DAP 96253 C6.
For the SEC step purification three different fractions were included for the summary.

Purification step	Total activity (IU)		Protein (mg/ml)	Specific activity (IU/mg)		Fold purification		Yield (%)	
	ASNase	GLNase		ASNase	GLNase	ASNase	GLNase	ASNase	GLNase
Lysate	1861.6	2148	179	10.4	12	1.0	1.0	100.0	100.0
After dialysis	1670.4	1044	174	9.6	~~6~~	0.9	0.5	89.7	48.6
AEX Fraction 0.5 M	763.25	473	10.75	71	44	6.8	3.7	41.0	22.0
SEC Fraction 4	261.87	258.39	0.03	8729	8613	839	718	14.1	12.0
SEC Frac8 &9	330	403	1.2	275	336	26.4	28.00	17.7	18.8
SEC Fraction 11	464.52	600.6	0.84	553	715	53	60	25.0	28.0
SEC Fraction 13	425.04	198.38	0.14	3036	1417	292	118	22.8	9.2

3.2 Properties and Kinetics of Nitrile hydratase with ASNase and GLNase activity

3.2.1 Molecular Weight Determination of Nitrile hydratase with ASNase Activity

The identification of molecular weight and purity of the enzyme purified from cells of *R. rhodochrous* was done using native and SDS- electrophoresis gel.

Figure 3.17 shows the protein bands for the fractions s purified from no induced $GUCo^{+2}$ cells of *R. rhodochrous* DAP 96253 C6. All fractions presented a ~ 72 KDa protein subunit

which only was prominent for fractions five to ten. Fraction six to seven, which presented the higher NHase activity showed two prominent additional subunits of ~ 23 and ~28 kDa. Fraction 12 which presented the highest ASNase activity, but higher GLNase activity has 2 slightly protein subunits of ~ 72 and ~ 48 KDa while fraction 10 which ASNase activity approximately is five fold higher than GLNase and NHase activity is low compared to the other fractions (figure 3.11) showed 3 slightly bands of ~23, ~ 48 and ~ 72 (figure 3.17- Lane 5).

According to figure 3.18, fractions 4 to 7 and 12 present a native molecular weight ~ 242 kDa, while fractions 8 to 11 present a prominent band around 242 KDa and multiple bands under this molecular weight.

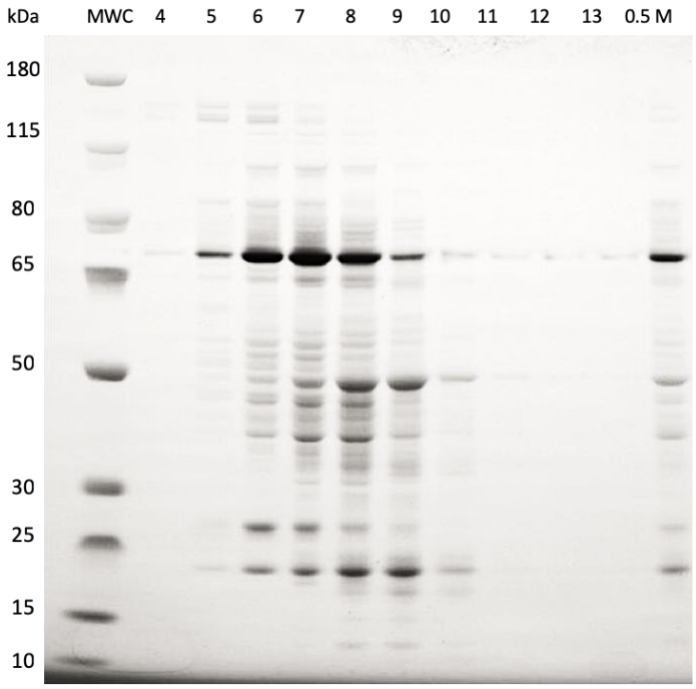


Figure 3.17 SDS-PAGE for SEC Fractions from Uninduced GUCo^{+2} Cells of R. rhodochrous DAP 96253 C6.
First lane shows the protein ladder, Lane 2 to 10 shows the protein bands for the SEC fractions (4 to13) that presented ASNase activity. In the last lane are the protein bands obtained for the AEX elution 0.5 M, used for the SEC.

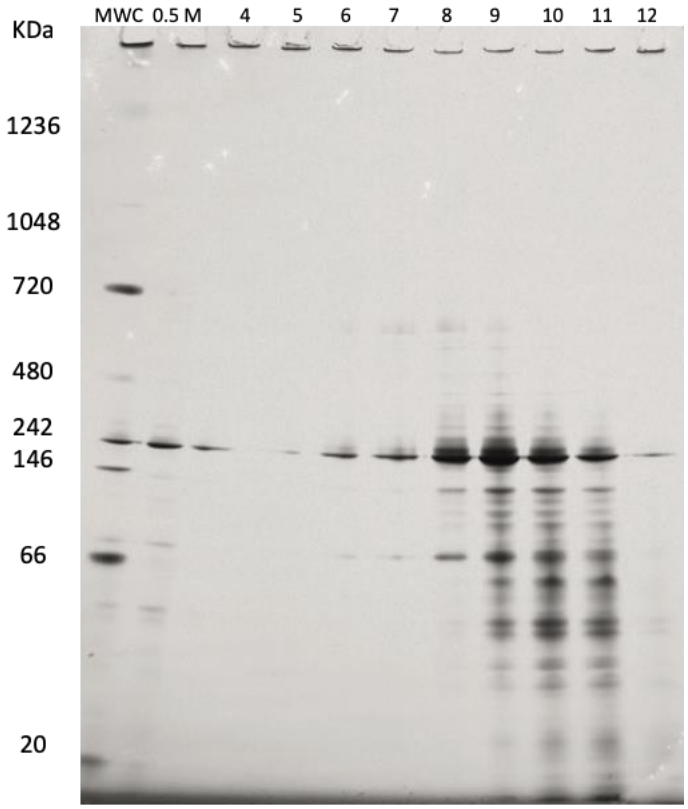
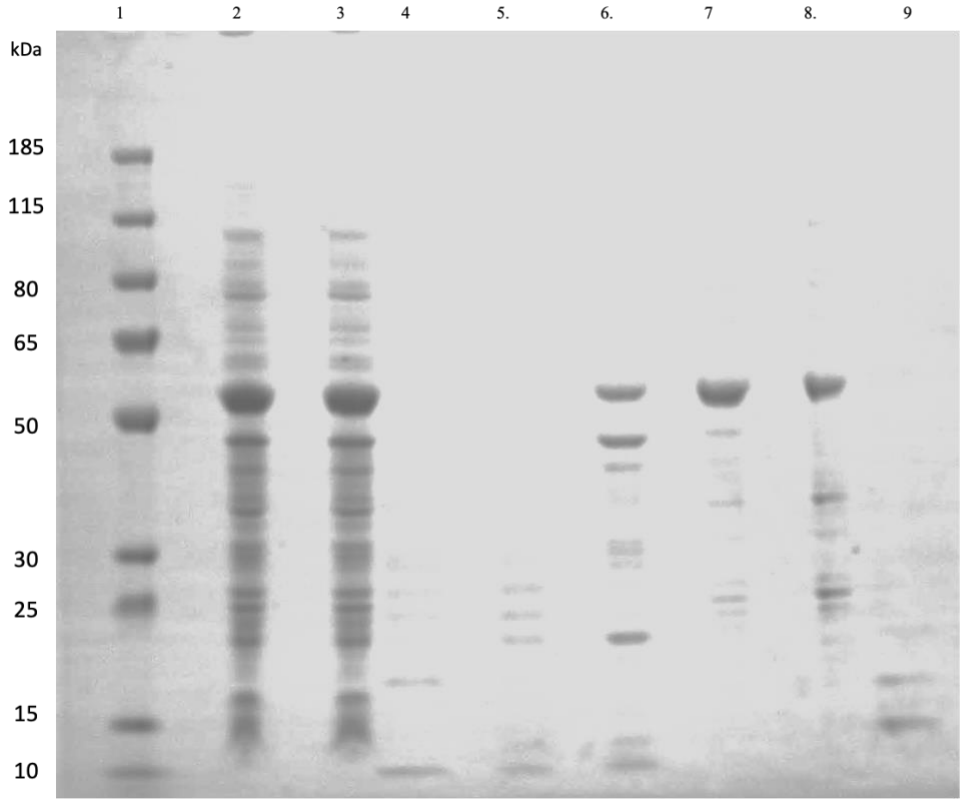


Figure 3.18 Native-PAGE for SEC Fractions from Non -induced GUCo^{+2} cells of R. rhodochrous DAP 96253 C6

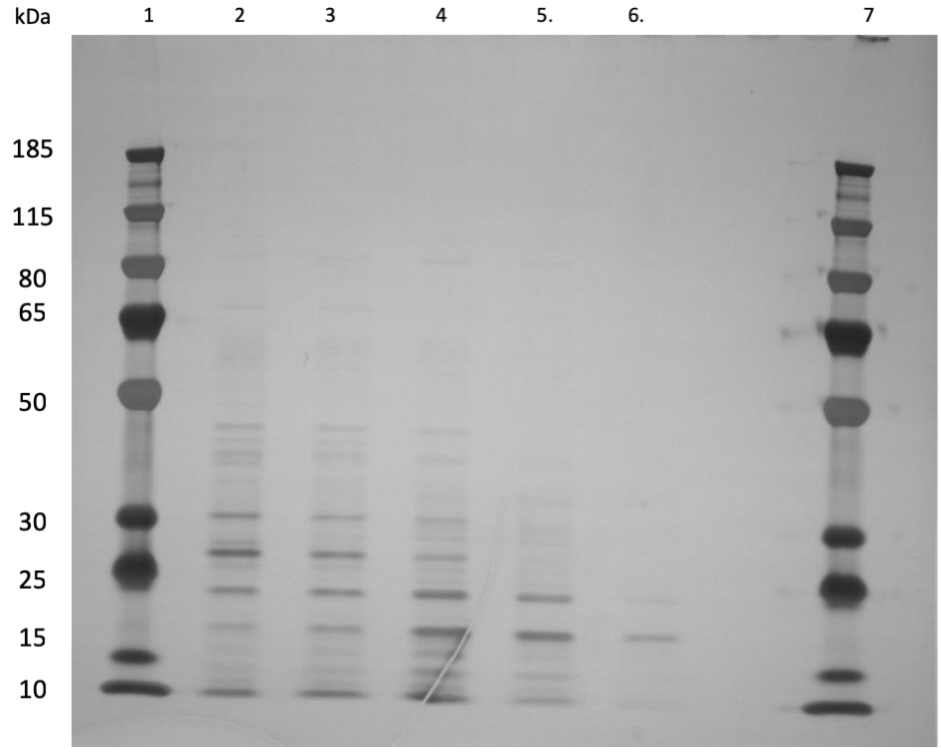
The NHase with ASNase and GLNase activities purified from *R. rhodochrous* DAP 96622 by AEX in the elution 0.1M 2 presented 4 protein subunits with molecular weight of ~28, ~ 25 ~ 23 and ~10 kDa (figure 3.19- Lane 5). The elution 0.1M 2 was used as tstarting material for SEC.



Lane	Sample
1	Ladder
2	Lysate
3	Dialyzed lysate
4	0.1 M 1
5	0.1 M 2
6	0.2 M1
7	0.2 M2
8	0.5 M
9	1M

Figure 3.19 SDS-PAGE for AEX Fractions from Induced R. rhodochrous DAP 96622. Fraction 0.1 M 2 was used as starter material in SEC.

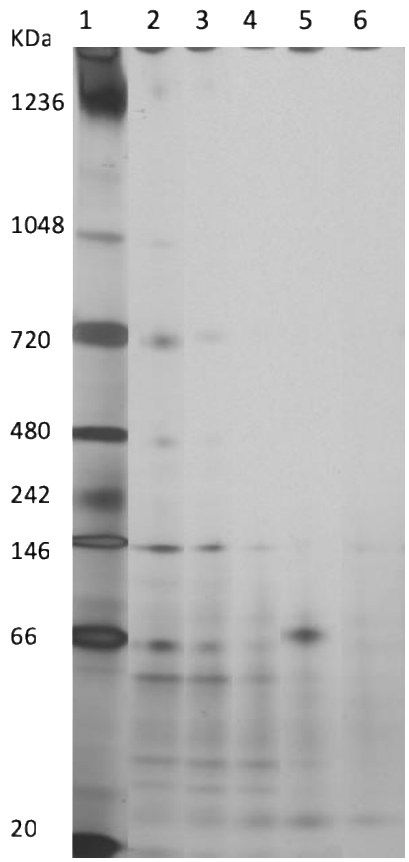
After SEC, fraction 4 presented three bands of ~ 25 ~ 20 and ~10 kDa, while fraction 5 showed only one prominent band around 20 kDa and the band at 25 kDa was slightly showed suggesting that 10 kDa band is the protein that provides the GLNase activity because in fraction 5 the GLNase was undetectable (Figure 3.20)



Lane	sample
1 and 7	Ladder
2	Fraction 1
3	Fraction 2
4	Fraction 3
5	Fraction 4
6	Fraction 5

Figure 3.20 SDS-PAGE for SEC Fractions from Induced R. rhodochrous DAP 96622

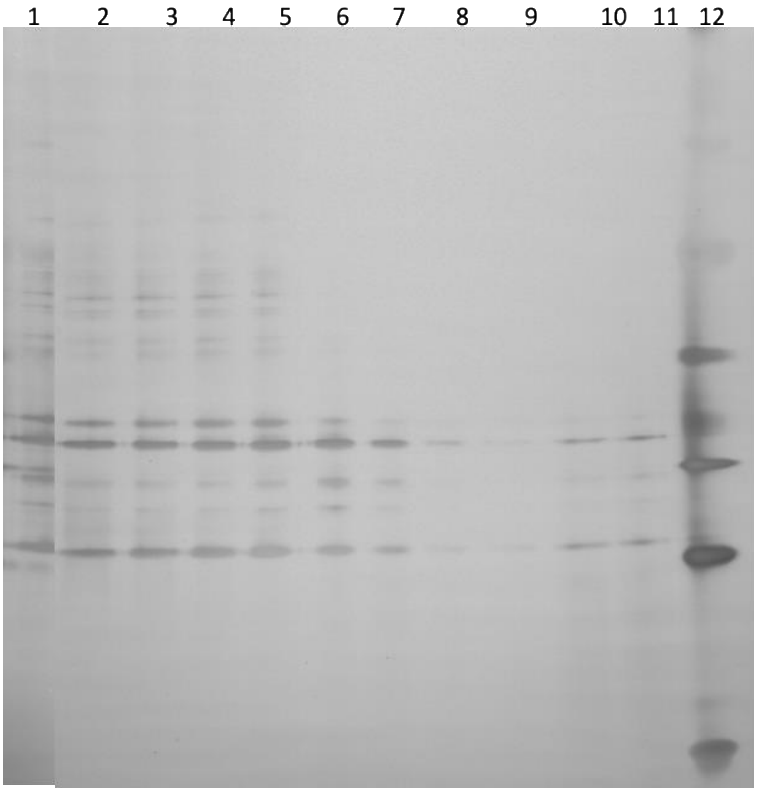
As illustrated in Figure 3.21, fraction 4 from *R. rhodochrous* DAP 96622 presents a prominent native protein band of 66 kDa and a slightly band of ~ 22 kDa. According to figure 3.20, the prominent band might be composed for 2 subunits of ~ 25 and ~ 20 kDa and the slightly band might be composed of protein subunits of ~ 11 kDa



Lane	sample
1	Ladder
2	Fraction 1
3	Fraction 2
4	Fraction 3
5	Fraction 4
6	Fraction 5

Figure 3.21 Native -PAGE for SEC Fractions from Induced R. rhodochrous DAP 96622
3 µl of Native Mark Unstained Protein Standard was used as a ladder

Figure 3.22 shows the molecular size of the native protein bands of purified fractions from *R rhodochrous* DAP 96653 C6. The AEX fraction used to run the SEC presented at least 10 native protein bands of which only three bands were prominent showing molecular weights of ~66, ~210 and ~242 kDa. After polishing by SEC, these 3 bands were present in fractions 4 to 8 and in the fractions 9 to 14 a protein with a molecular weight of ~210 and ~66 were shown.



Lane	Sample
1	0.5 M
2	Fraction 4
3	Fraction 5
4	Fraction 6
5	Fraction 7
6	Fraction 8 /9
7	Fraction 10
8	Fraction 11
9	Fraction 12
10	Fraction 13
11	Fraction 14
12	Ladder

Figure 3.22 Native- PAGE for SEC Fractions from Induced R. rhodochrous DAP 96253 C6
Fraction 8/9 were pooled using fraction 8 and 9 from SEC

The enzyme with ASNase and GLNase activity purified from induced cells of *R. rhodochrous* DAP 96253 C6 presented protein bands on SDS-PAGE with molecular weights of ~ 72, ~28 and ~23 kDa. Fractions 10 and 11 showed only the bands with ~ 72 and ~ 23 kDa (Figure 3.23). Fraction 12 and 13 is shown in figure 3.21 B in which a slightly ~ 72 protein band is observed.

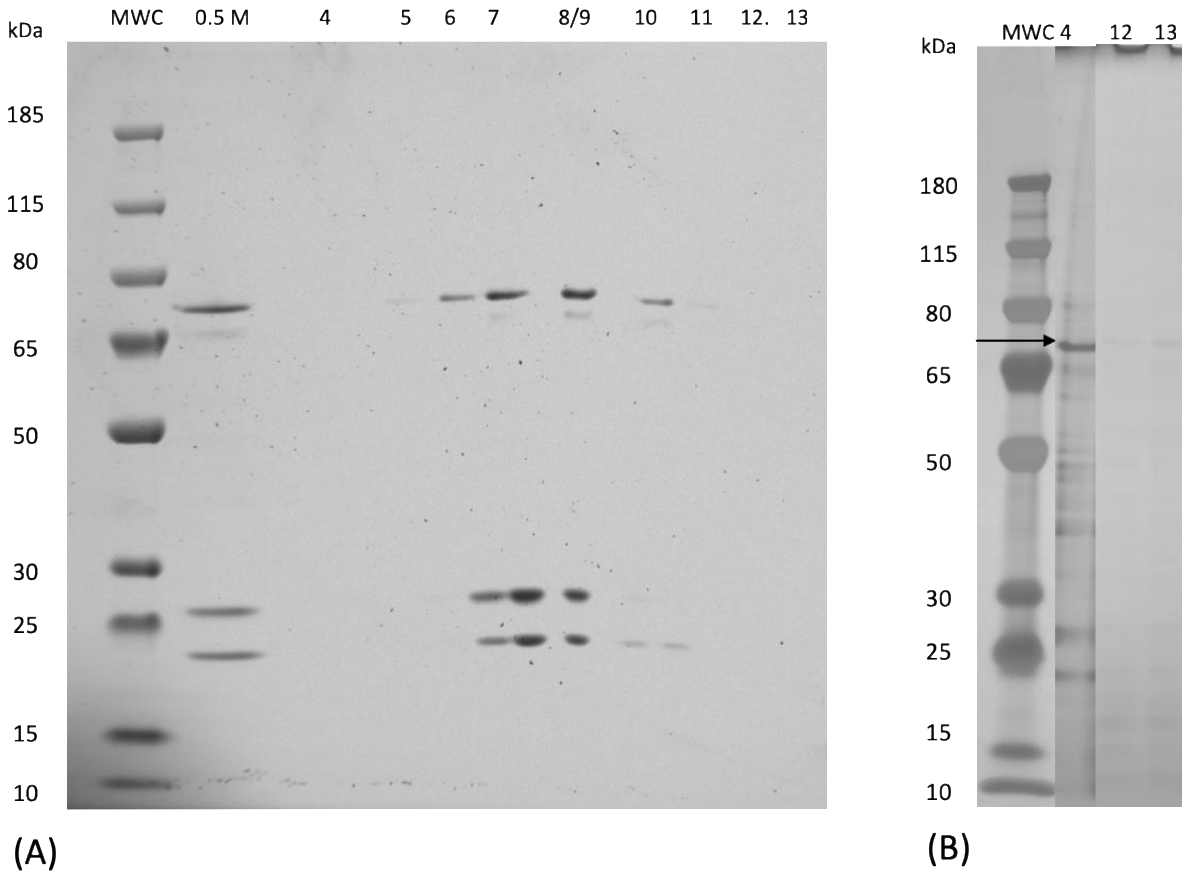
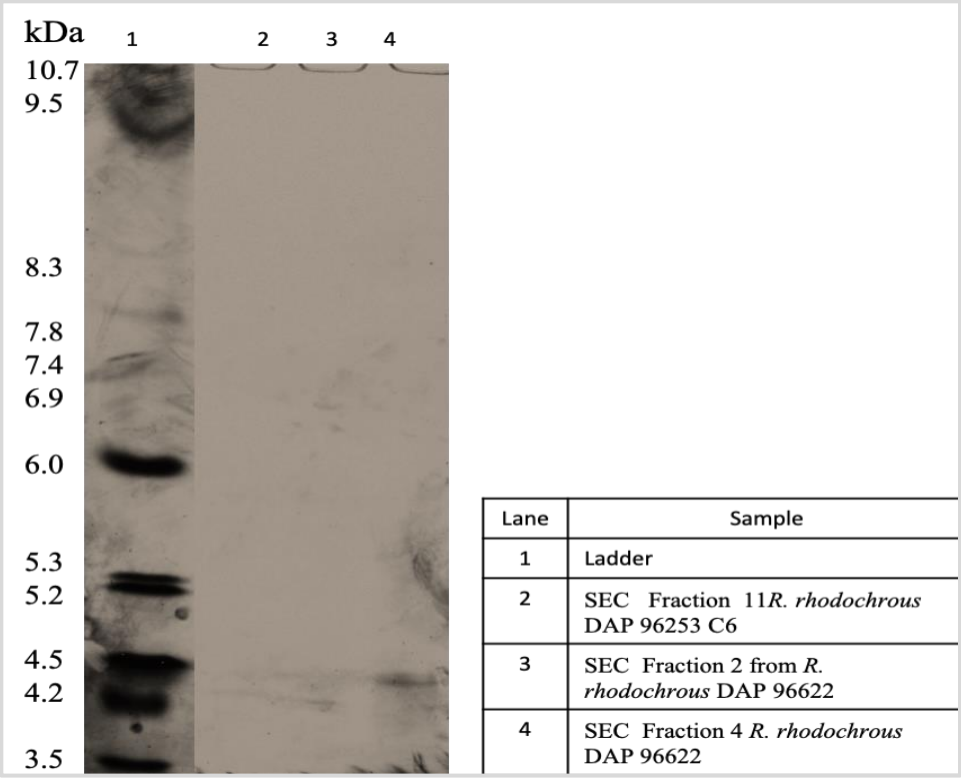


Figure 3.23 SDS- PAGE for SEC Fractions from Induced R. rhodochrous DAP 96253 C6
(A) The gel shows the fraction 0.5 M loaded in SEC and SEC fractions 4 to 13 with ASNase and GLNase activity. NHase activity was only detected in fractions 12 to 14. The gel was stained with Coomassie and protein bands for fractions 4, 12, and 13 were no detected. In figure (B) bands 4, 12 and 13 are shown on a gel stained by silver staining.

3.2.2 Isoelectric point of NHase with ASNase Activity.

As is illustrated in figure 3.24 the NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96253 C6 and *R. rhodochrous* DAP 96622 has an estimated isoelectric point of ~4.5.



Lane	Sample
1	Ladder
2	SEC Fraction 11*R. rhodochrous* DAP 96253 C6
3	SEC Fraction 2 from *R. rhodochrous* DAP 96622
4	SEC Fraction 4 *R. rhodochrous* DAP 96622

Figure 3.24 IEF-Gel for Purified Enzyme from Induced R. rhodochrous DAP 96253 C6 and DAP 96622

3.2.3 K_m of ASNase for ASN and GLN

The K_m and V_{max} of NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96622 were 0.3 mM and 3.4μmoles/min respectively for ASN, while the K_m and V_{max} for GLN was 8.4 mM and 49.3μmoles/min respectively. Figures 3.25 and 3.26 show the Lineweaver-Burk plot used to calculate the Km and Vmax values.

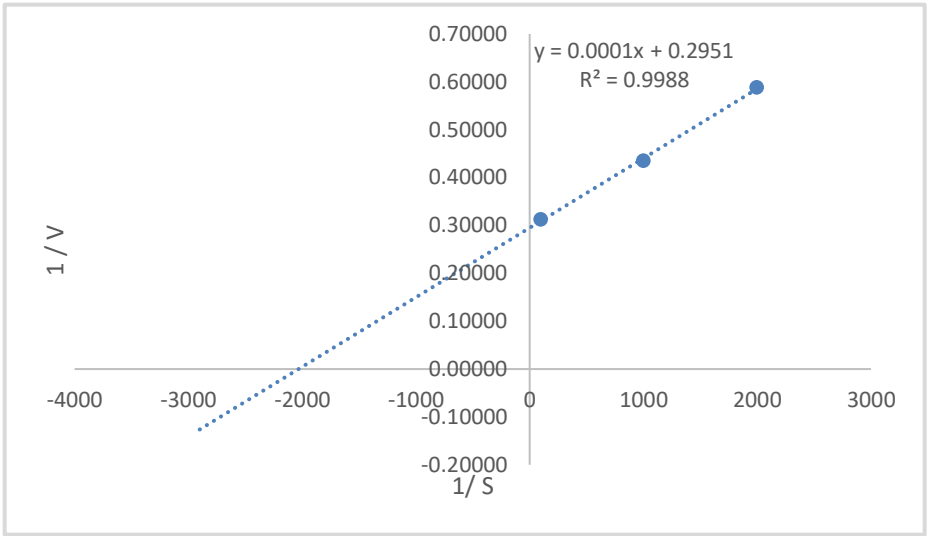


Figure 3.25 Lineweaver-Burk Plot for ASN Km of ASNase from Induced R. rhodochrous DAP 96622

The substrate concentration used was 0.5 to 10 mM ASN. The kinetics evaluation was made after size exclusion chromatography using the fraction 4 which presented a ASNase activity of 8555IU mg^{-1} and GLNase activity of 10106 IU mg^{-1}

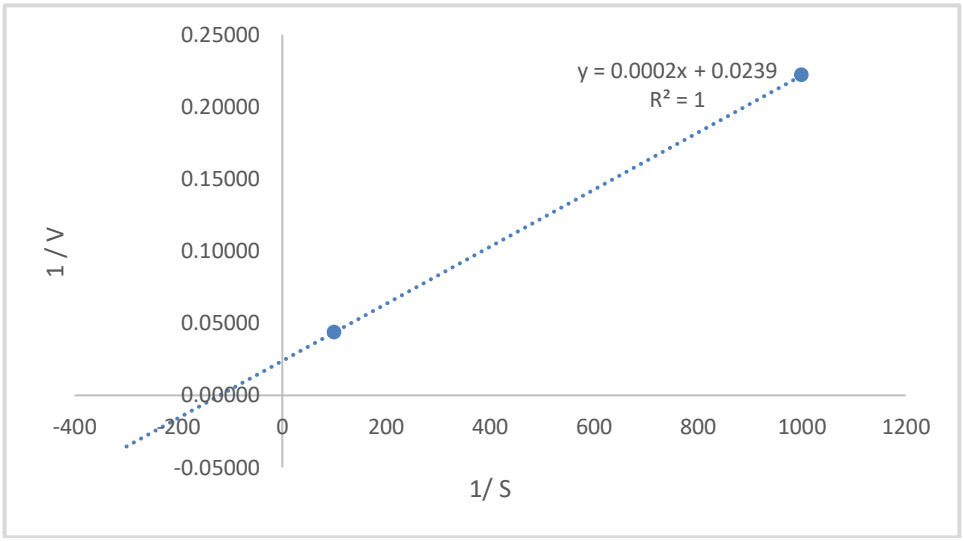


Figure 3.26 Lineweaver-Burk Plot for Km of ASNase from induced R. rhodochrous DAP 96622 using GLN.

The concentrations of substrate tested were 1 to 10mM *GLN*. The kinetics evaluation was made after size exclusion chromatography using the fraction 4 which presented an ASNase activity of 8555IU mg^{-1} and GLNase activity of 10106 IU mg^{-1}

The NHase with ASNase and GLNase activity purified from $GuCo^{2+}$ induced cells of *Rhodococcus rhodochrous DAP 96253* C6 at pH 7.6, presented a K_m for ASN of 0.007 mM and a V_{max} of 3.5 µmoles/min, while for *GLN* the K_m was 6.45 mM with a V_{max} of 90.9 µmoles/min

The Lineweaver- Burk plots used to calculate these values are shown in figures 3.27 and 3.28.

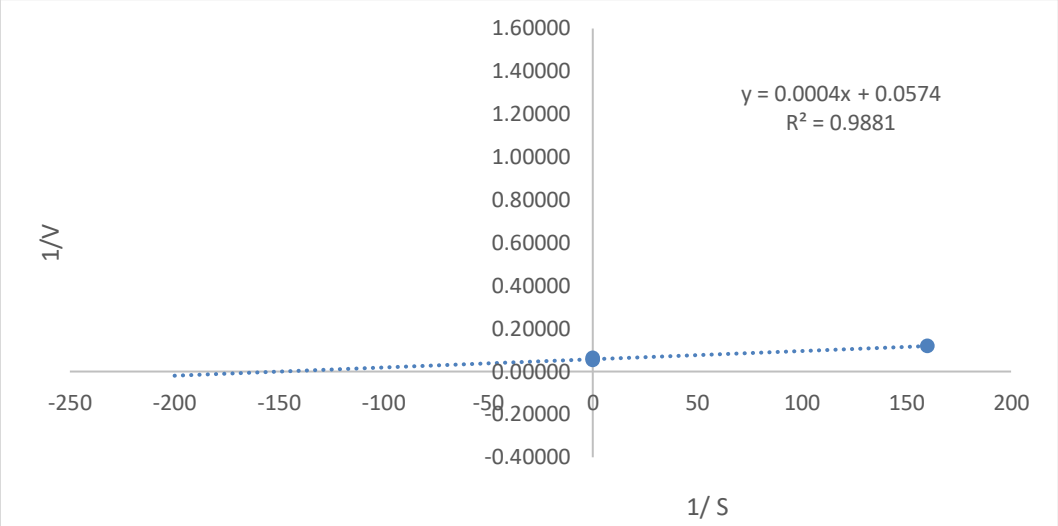


Figure 3.27 Lineweaver-Burk Plot for Km of ASNase from Induced R. rhodochrous DAP 96253 C6 using ASN.

The concentration of substrate used for Km determination was 0.1 mM to 10mM *ASN*

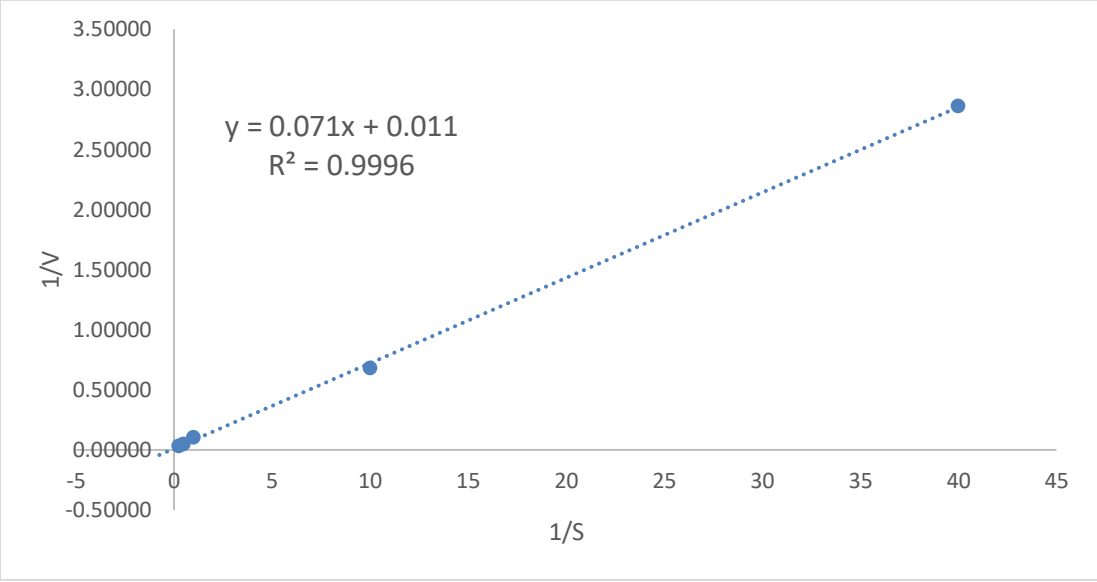


Figure 3.28 Lineweaver-Burk Plot for Km of ASNase from Induced R. rhodochrous DAP 96253 C6 using GLN.

The concentration of substrate used for Km determination was 0.1 mM to 10mM GLN.

The NHase with ASNase and GLNase activity purified from non-induced cells of *R. rhodochrous DAP 96253* C6 at pH 7.6, presented a K_m for ASN of 20.5 mM and a V_{max} of 34 µmoles/min, while for GLN the K_m was 4.80 mM with a V_{max} of 96.15 µmoles/min The Lineweaver- Burk plots used to calculate these values are shown in figures 3.29 and 3.30.

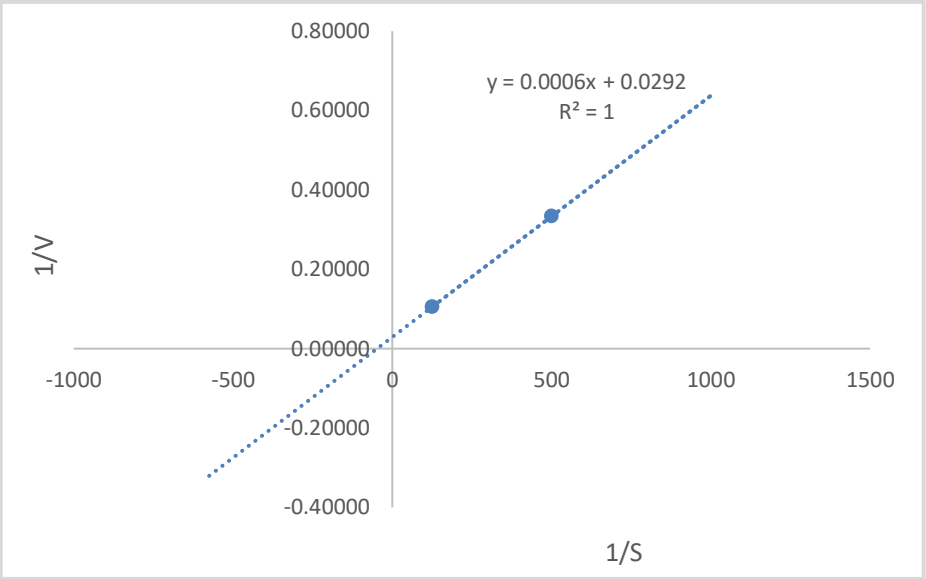


Figure 3.29 Lineweaver-Burk Plot for ASN Km of ASNase from Non -induced Cells of R. rhodochrous DAP 96253 C6.
The concentration of substrate used for Km determination was 0.1 mM to 10mM ASN.

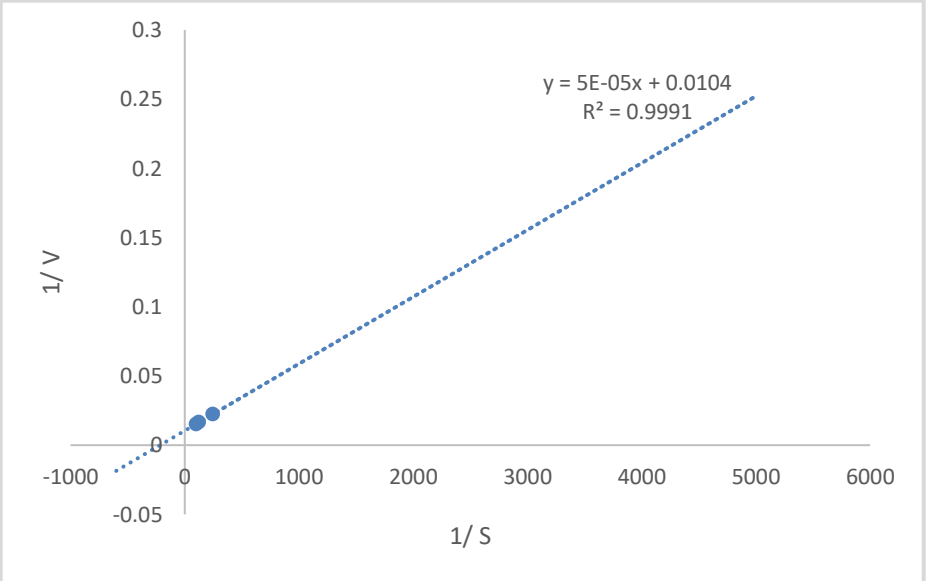


Figure 3.30 Lineweaver-Burk Plot for GLN Km of ASNase from Non -induced cells of R. rhodochrous DAP 96253 C6.
The concentration of substrate used for Km determination was 0.1 mM to 10mM GLN

Table 3.7 shows a comparison between ASNase kinetics obtained from purifications made from cells of *Rhodococcus rhodochrous* DAP 96253 C6 using two different pHs, flow rates and time of purification after harvest. The purified enzyme with ASNase activity had more affinity for the substrate *(Km)* when purification was done at pH 7.6 and flow rate of 0.6ml/min. Contrary, the *Vmax* of the enzyme was higher at pH 8 and flow rate of 1ml/min. The velocity of the enzyme decreased in function of time.

Table 3.7 Comparison of ASNase Kinetics Vs Purification Variables.
The *Km* and *Vmax* values were calculated for fractions purified from induced *R. rhodochrous* DAP 96253 C6 using ASN as substrate.

ASNase kinetics	Purification variables					
	pH		Flow rate		Time after harvest	
	7.6	8	0.6ml/min	1ml/min	10 days	45 days
Km (mM)	1.8	3.3	3	30	1.8	0.7
Vmax	9.1	55.5	43.4	52.9	9.1	3.5

3.2.4 Effect of Temperature on ASNase Stability

As is illustrated in figure 3.31, the enzyme with ASNase and GLNase activities purified from *R. rhodochrous* DAP 96622 and DAP 96253 C6 has thermal stability at 37 °C with a loss of the initial ASNase activity of 8% and 11% respectively.

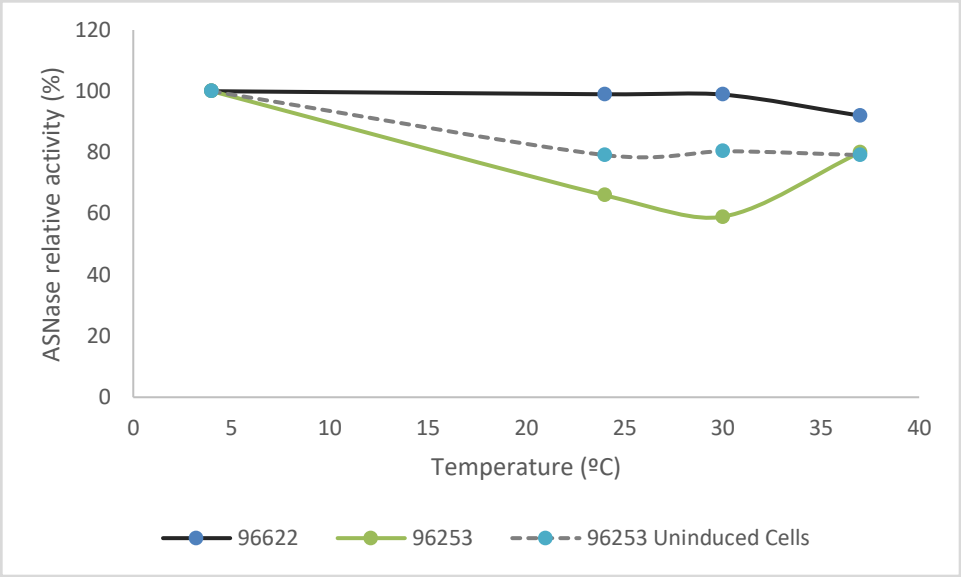


Figure 3.31 Temperature Stability of ASNase from R. rhodochrous DAP 96253 C6 and DAP 96622

The enzyme was incubated at 24, 30 and 37 ºC for 15 minutes and ASNase activity was measured as is described in section 2.3. Continuous lines indicate the ASNase activity for purified fractions from induced cells.

3.2.5 Effect of pH on ASNase Stability

As is depicted in figure 3.32 and 3.33, the purified enzyme presented stability when was incubated at physiological pH retaining activity between 77 and 79%. The enzyme with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96622 presented stability between pH 7.2 to 8 showing a retention of activity between 77% ad 94%. Although this enzyme was stable at slightly alkaline pH, the enzyme purified from Gu Co^{+2} induced cells of *R. rhodochrous* DAP 96253 C6 lost activity after incubation for 24 hours at pHs above 8. The ASNase purified from uninduced cells of *R. rhodochrous* DAP 96253 C6 has stability at 7.6, but at pH of 7.4 the enzyme presented 78% of the initial activity (Figure3.34)

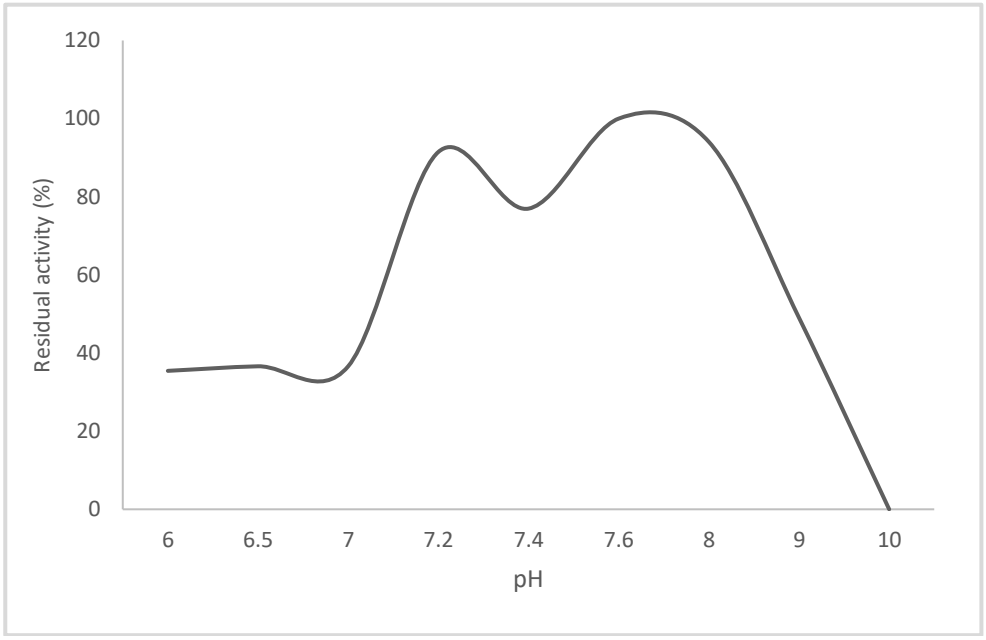


Figure 3.32 pH Stability of ASNase from Induced cells of R. rhodochrous DAP 96622.
The NHase with ASNase and GLNase activity was purified at pH 7.6 by AEX using a flow rate of 0.6ml/min and followed by SEC. The enzyme assay was performed at pH 7.6.at 25ºC.

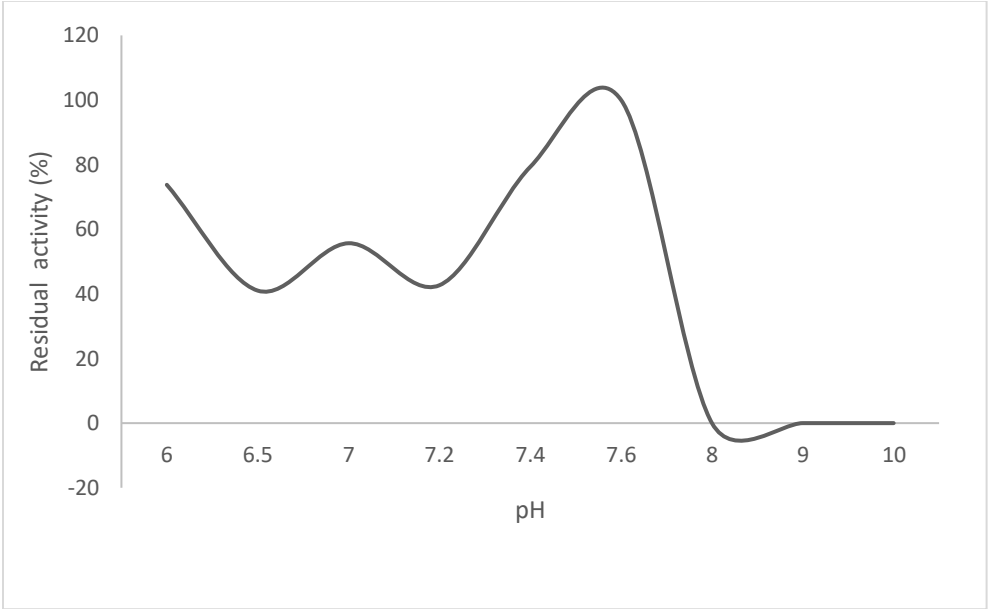


Figure 3.33 pH Stability of ASNase from Induced Cells of R. rhodochrous DAP 96253-C6
The enzyme assay was performed at pH 7.6.at 25ºC.

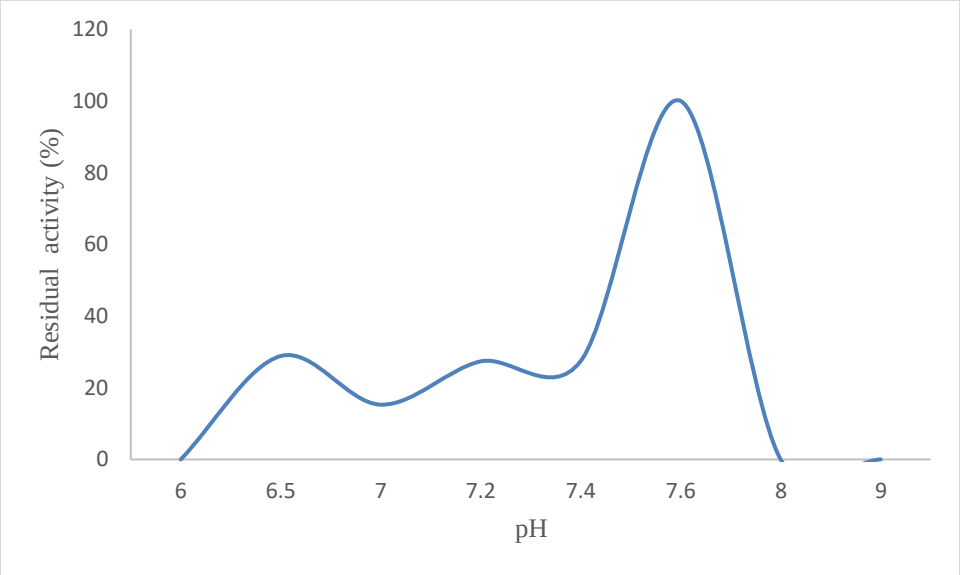


Figure 3.34 pH Stability of ASNase Purified from Uninduced cells of R. rhodochrous DAP 96253-C6
The enzyme assay was performed at pH 7.6.at 25ºC.

3.2.6 Half-life of ASNase Activity

The trypsin half-life of the purified enzymes from *R. rhodochrous* DAP 96622 is illustrated in figure 3.35. The purified enzyme with ASNase and GLNase activity presented a reduction of initial ASNase activity of 50% after approximately 33 minutes of incubation at 37°C, while purified enzyme with only ASNase activity exhibited better trypsin resistance with 50% of reduction of ASNase initial activity after 43 minutes of incubation. The trypsin half-life for the enzyme with ASNase and GLNase activity purified from $GuCo^{+2}$ *R. rhodochrous DAP 962*53-C6 was approximately 29 minutes showing less trypsin resistance than the ASNase from *R. rhodochrous* DAP 96622 (Figures 3.35and 3.36). However, the enzyme purified from uninduced cells of from *R. rhodochrous DAP 962*53-C6 was around 39 minutes showing the longest trypsin half-life. (Figure 3.37).

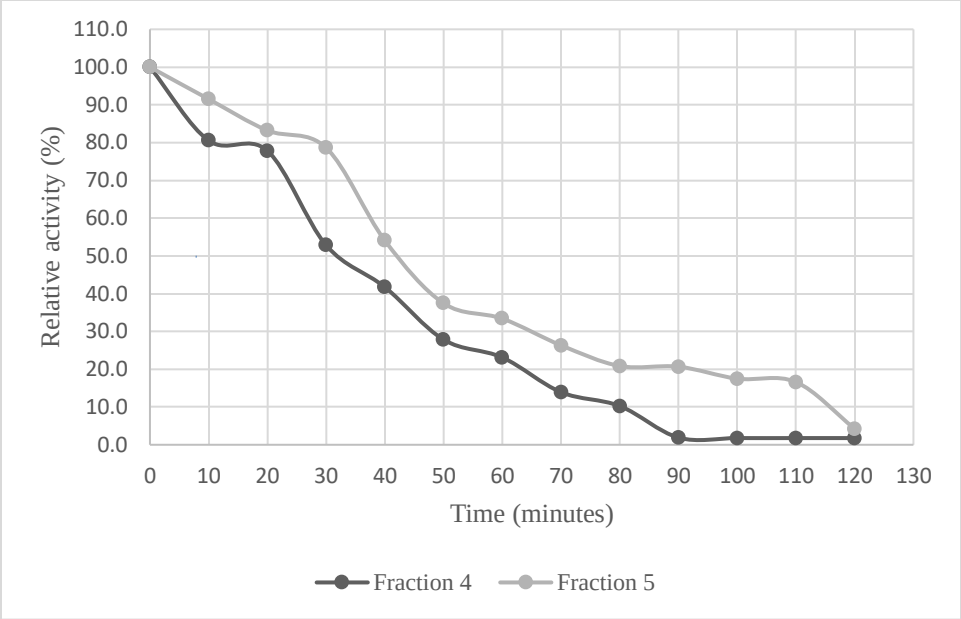


Figure 3.35 Trypsin Half Life of ASNase from R. rhodochrous DAP 96622
The trypsin half-life was measured for fraction 4 and 5 after SEC. For fraction 4 ASNase and GLNase activity were present and NHase activity was undetectable, while in fraction 5 only ASNase was detectable.

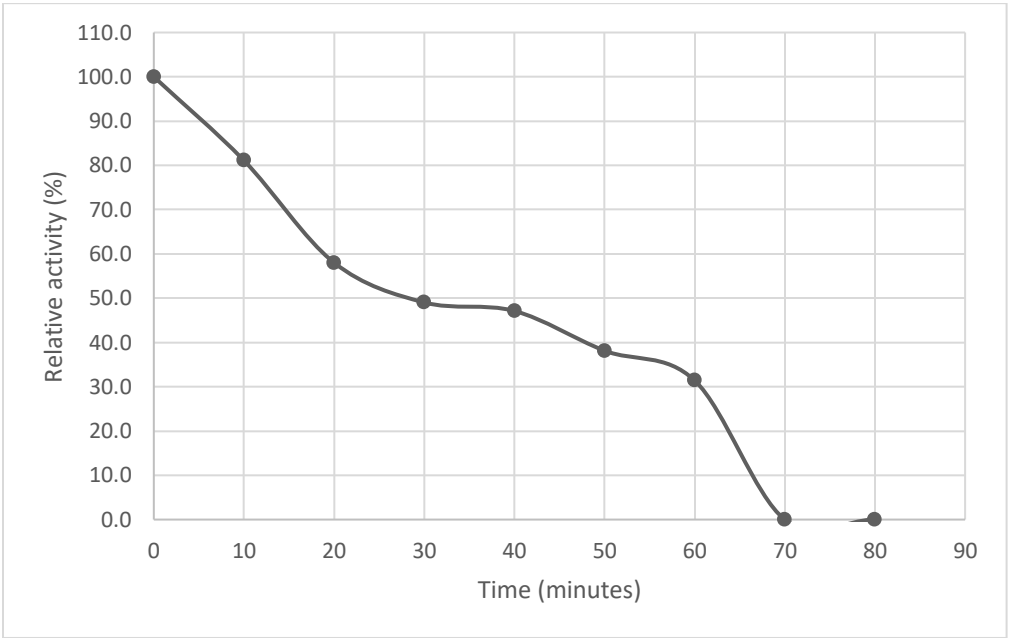


Figure 3.36 Trypsin Half Life of ASNase from R. rhodochrous DAP 96253 C6.
The purified enzyme used for trypsin half-life evaluation showed ASNase and GLNase activity, but NHase was undetectable.

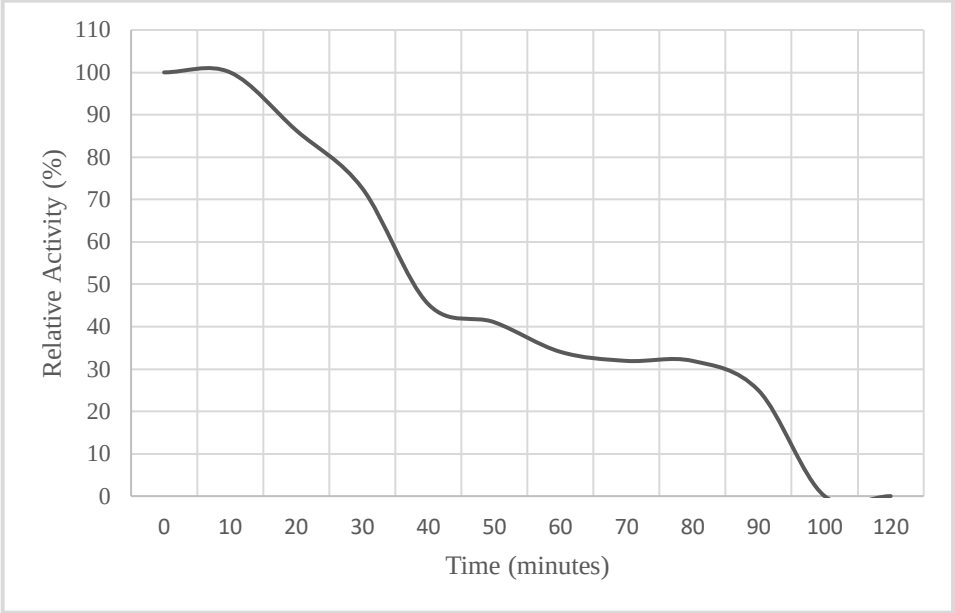


Figure 3.37 Trypsin Half Life of ASNase from uninduced cells of R. rhodochrous DAP 96253 C6.

The purified enzyme with ASNase and GLNase isolated from *R. rhodochrous* DAP 96622 activity presented a reduction of initial ASNase activity of 50% after 8 hours of incubation with human serum at 37 ºC and a reduction of 96% after 53 hours, while the enzyme from $GuCo^{+2}$ *R. rhodochrous DAP 962*53 C6 with ASNase and GLNase activity exhibited a 50% reduction of initial ASNase activity after 50 hours of incubation and a reduction of 53% after 53 hours. The serum half-life for ASNase purified from uninduced cells of R. *rhodochrous DAP 962*53 was 29 hours (Figure 3.38).

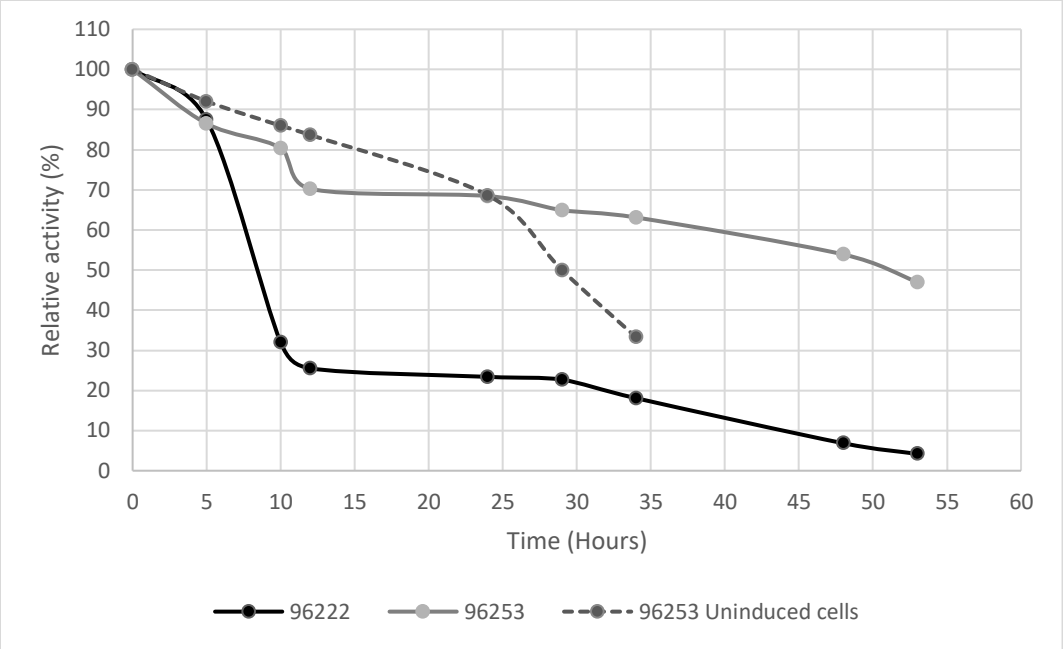


Figure 3.38 Human Serum Half Life of ASNase from R. rhodochrous DAP 96622, GuCo^{+2} induced and uninduced cells R. rhodochrous DAP 96253 C6.

3.3 Effect of Storage Time on ASNase and GLNase Activity

Figure 3.39 illustrates the results of ASNase and GLNase activity after storing the SEC fractions at 4°C on ice. Enzyme purified from uninduced cells of *R. rhodochrous* DAP 96253 lost 83% of activity after 6 days of storage. Contrary, the enzyme purified from Gu Co^{2+} induced cells of *R. rhodochrous* DAP 96253 retained between 45 and 93% of the initial ASNase activity. The fraction without GLNase activity, isolated from *R. rhodochrous* DAP 96622 retained the 85.2% of ASNase activity while the fraction without GLNase activity lost the 48% of the initial ASNase activity. GLNase activity showed less stability than ASNase showing a retention of activity around 40%.

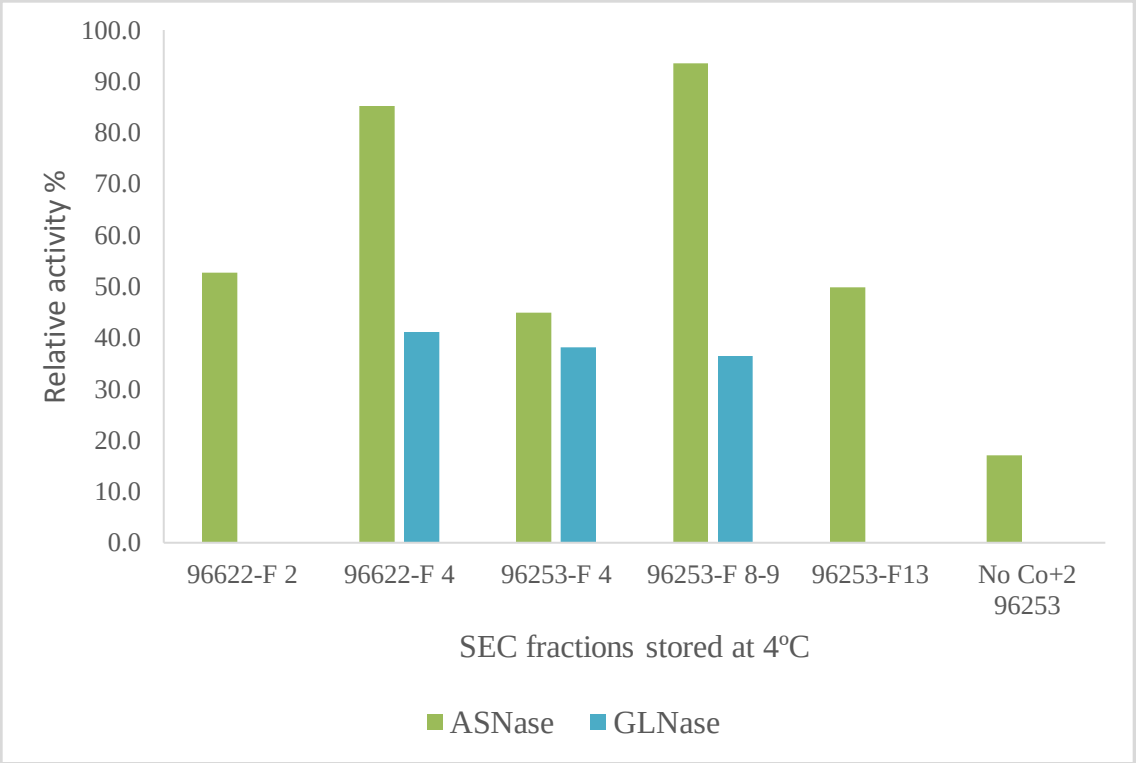


Figure 3.39 Storage Time Stability for ASNase and GLNase.
Purified fractions 96622-F 2 and 96622-F 4 were purified from *GUCo*$^{+2}$ Induced cells of *R. rhodochrous* DAP 96622 and stored at 4°C on ice for 45 days. Fractions 96253-F 4, 96253-F8-9, 96253-F13 and96253-F13 were purified from *GUCo*$^{+2}$ Induced cells of *R. rhodochrous* DAP 96253-C6 and the enzyme activity was measured after 30 days sat 4°C on ice. The fraction No Co^{+2} 96253 was purified from *R. rhodochrous* DAP 96253-C6 and the enzyme activity was measured after 6 days of storage at 4°C on ice.

According to the results summarized in table 3.8, the enzyme purified 23 fold using induced GUCO^{+2}cells of *R. rhodochrous* DAP 96253 C6 presented the highest ASNase activity (3147±111 I IU mg^{-1}) and the longest human serum half-life (50 minutes). However, the enzyme that showed the best affinity for ASN was the NHase with ASNase and GLNase activity purified from induced GUCO^{+2}cells. The enzyme with most ASNase resistant was the NHase with ASNase activity without GLNase activity purified from *R. rhodochrous* DAP 96622.

Table 3.8 Summary of Purification Process and Characterization of NHase with ASNase and GLNase activity from R. rhodochrous

Purified Enzyme	Specific Activity IU/mg			ASNase		GLNase		Km (mM)		Vmax (umol/min)		Molecular Weight (kDa)		Stability		Half-life	
	ASNase	GLNase	NHase	Fold purification	% Yield	Fold purification	% Yield	ASN	GLN	ASN	GLN	Subunits	Native protein	pH	T° (ºC)	Trypsin (min)	Human Serum (hours)
GUCo +2												20					
R. rhodochrous DAP 96622 Free GLNase	2511	< 0.1	< 0.1	6.8	24	0	0	ND				25	25		ND	42	ND
GUCo +2												20			37		
R. rhodochrous DAP 96622	2852	3416 ± 47	< 0.1	136	3.3	255	59	0.3	8.4	3.4	49.3	25	88	7.4-8.0		33	8
GUCo +2												23					
R. rhodochrous DAP 96253C6	3147 ±111	1569 ± 15.8	1850 ± 253	292	23	118	9.2	0.1	6.5	3.5	36.6	28	276	7.4 - 7.6	37	30	50
												72					
Non GUCo +2												23					
R. rhodochrous DAP 96253 C6	233± 59	36±5	875±73	87	68	31	24	21	4.8	34	96.2	48	242	7.6	37	39	29
												72					

4 DISCUSSION

4.1 Effect of Buffer Composition and pH on ASNase Activity

It is known that pH has an important function in anion exchange process because, the protein charge is dependent s on the pH. If protein is an environment in which the pH is higher than the isoelectric point (p*I*) of the protein, the protein is going to have a negative charge, on the contrary if the protein is in an environment with a lower pH than the p*I* the protein is going to have a positive charge (Roe, 2001). The "environment" surrounding protein is important from the moment the protein is removed from the cytoplasm at the beginning of purification. Cell lysis was the first step in the purification of NHase exhibiting ASNase and GLNase activity. It was readily apparent than ASNase activities were higher when lysis was performed above 5.8 pH

(Figures 3.1 and 3.2). When lysis was performed using 20 mM PB at pH 5.8, while the activity of NHase and GLNase were present, ASNase activity was undetectable. As high-mass, Co-NHase exists as a multimeric heteromer (4mer to 4mer), the influence of buffer composition and pH may influence the quaternary structure of the high-mas, Co-NHase. It is suggested that the structure of NHase can be affected by the pH of 5.8 providing a better binding to acrylonitrile than ASN. Contrary, when lysis process is done at pH 7.2 the structural conformation of active site of NHase might change improving its ASN affinity.

In addition to the pH of the lysis buffer, the effect of incorporating a reducing agent was tested by adding 2- mercaptoethanol, which resulted in an ASNase activity increase (Figures 3.4 and 3.5). The ASNase activity present in purified NHase (obtained from whole cells of *R. rhodochrous* DAP 9653 C6, harvested on 01-18-18) was quite high when the lysis was conducted at pH 7.2 in presence of 5 mM 2-mercaptoethanol suggesting that reducing agent was necessary to protect the ASNase active site. Also, 2-mercaptoethanol has the ability to react with cysteine disulfides stabilizing the protein and its conformation. Another way in which 2-mercaptoethanol can improve the ASNase activity in lysates is for inhibiting of some proteases, released during lysis process. This reducing agent is used in lysis of Gram- negative bacteria to eliminate ribonucleases during RNA extractions (Hermanson, 2013). Suggesting that mercaptoethanol avoid that the enzyme loss the ASNase activity during lysis step.

The NHase purifications made using an additional step of dialysis presented higher ASNase activity (Figure 3.4). The dialysis can remove contaminants such as 2-mercaptoethanol that might interfere with the binding capacity of the column during AEX. Although 2-mercaptoethanol is helpful to avoid damage during lysis of cells, it can affect the efficiency of the anion exchange chromatography. When the dialysis was done, mercaptoethanol was diffused

through the dialysis membrane and as a consequence the 2-mercaptoethanol concentration decreased increasing binding of loaded sample to the column to purify NHase with the ability to use ASNase.

The NHase purifications made using an additional step of dialysis presented ASNase activity while ASNase activity was undetectable in AEX fractions without a previous dialysis step (Figure3.4). The dialysis can remove contaminants such as 2-mercaptoethanol that might interfere with the folding of the protein in a form in which ASNase activity is present or salts that can affect the binding capacity of the column during AEX. Usually dialysis process eliminates molecules with small molecular weight such as reducing agents and salts ("Dialysis Methods for Protein Research | Thermo Fisher Scientific - US," n.d.); (Vallejo & Rinas, 2004). Although 2-mercaptoethanol can avoid structural changes in the protein during lysis, residues of 2-mercaptoethanol during AEX affects the capacity of NHase to use ASN as substrate.

The results of buffer exchange between purification steps show that when 0.05 M TRIS-HCl was used as unique buffer in the AEX- purification process, the specific ASNase activity was lower (29 IU/mg in the fraction 0.5 M NaCl) than the ASNase activity (64 IU/mg in fraction 0.5 M NaCl) obtained from AEX-purification which used 50 mM PB for lysis , 0.05 M TRIS-HCl for dialysis buffer and 10mM sodium butyrate, 25mM NaH_2PO_4 for equilibration and elution buffers (Figures 3.6 and 3.9). However, the purification in which PB was part of the composition of lysis buffer and dialysis buffer presented an ASNase activity of 91 IU/mg which was the highest between this set of purifications (Figure 3.8- 3.9). Thus, the change of buffers between purification steps help to improve the binding of the protein with ASNase activity.

Results of purification made at different pHs demonstrated that when lysis, dialysis, AEX and SEC were conducted at pH 7.6 the ASNase activity for NHase was high and the affinity of

NHase for Acrylonitrile was lower (Figures 3.10 and 3.11). Although the majority of purification process of bacterial ASNase have been done at pH of a range between 8.4 and 8.6 (Husain et al., 2016b) (Amena, Vishalakshi, Prabhakar, Dayanand, & Lingappa, 2010) (Husain, Sharma, Kumar, & Malik, 2016) (El-Naggar, Deraz, El-Ewasy, & Suddek, 2018), the purification approach at 7.6, conducted in this study, might provide better characteristics for therapeutic uses because this pH is closer to the physiological pH .

4.2 Effect of AEX Flow Rate on ASNase Activity

The results of the set of purifications made to test the flow rate using during AEX (0.3, 0.6 and 1 ml/ min) demonstrated that a flow rate of 0.6 ml/min provide better ASNase activity for NHase purified from *R rhodochrous* DAP9653-C6 than flow rates of 0.3 ml/min and 1 ml /min (figure 3.3). Nonetheless, future work should be conducted to test the flow rate of 1 ml/min because it can save time and increase percentage of yield during purification. Husain et al, 2016 achieved an overall recovery of 53% using a flow rate of 1 ml/min during AEX compared to the range of 41 to 43% of the purifications obtained in this study (Tables 3.3- 3.6).

4.3 ASNase Activity Vs *R. rhodochrous* Cells Type

High-mass, Co-NHase with ASNase and GLNase activity can be purified from $GUCo^{+2}$ induced cells from either *R. rhodochrous* (DAP 96622) or *R. rhodochrous* (DAP 96253 C6) and noninduced $GUCo^{+2}$ cells of *R. rhodochrous* DAP 96253. However, enzymes purified from $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96253 C6 showed higher specific activity (8279 and 3036 IU mg^{-1}) and fold purification between 839 and 292. The fold purification of 839 was obtained in the SEC fraction 4, purified from a CFL exhibiting 10.4 IU mg^{-1} ASNase activity producing a final ASNase activity of 8279 IU mg^{-1}. The SEC fraction 13 was purified from the

same CFL, but the final ASNase activity was 3036 IU mg^{-1} resulting in a fold purification of 291 (Table 3.6).

Although the overall recovery for the purification made using cells of *R. rhodochrous* DAP 96253 C6 fermented in media with glucose, urea and cobalt was 68% which was higher compared with the percentages obtained in the purifications made with the other cells mentioned before, the specific NHase activity (809 IU mg-1)obtained in this purification was 4.6 fold higher than ASNase activity (174 IU mg-1). Contrary, in purifications using $GUCo^{+2}$ induced cells of *R. rhodochrous* fractions with undetectable or lower NHase activity compared with ASNase activity were obtained. In addition, induced cells of *R. rhodochrous* DAP 96622 allowed the purification of a 136-fold purified protein with a specific activity of 2851 IU mg-1 ASNase, 3369 IU mg-1 GLNase and undetectable NHase (Table 3.4). Also, purifications from induced cells of *R. rhodochrous* DAP 96622 provided a 23.9-fold purified enzyme with ASNase activity of 2511 IU mg-1 and undetectable NHase and GLNase activity (Table 3.5). The ASNase specific activity for this GLNase free enzyme is higher than other enzymes that were purified or modified in order to obtain low or undetectable GLNase activity. For example, a GLNase-free asparaginases purified from *Streptomyces brollosae* NEAE-115 showed an specific activity of 76.671 IU mg-1(El-Naggar et al., 2018), another ASNase without GLNase activity from *Pseudomonas otitis* exhibited an ASNase activity of 107 IU mg-1, ~23 fold lower than ASNase specific activity obtained from R. rhodochrous DAP 96622(Husain et al., 2016b), and the ASNase variant of *Erwinia chrysanthemi* ErA TM2 which presented an ASNase activity of 443.5±4.8 IU mg-1 and GLNase activity of 1.53±0.02 IU mg-1(Nguyen et al., 2016). Thus, although the purification process in which a 23.9 fold purified free GLNase ASNase was isolated from *R. rhodochrous* DAP 96622 the overall recovery was only 6.8%, the high specific

ASNase activity made of this enzyme a competitive option in ALL treatments based on ASNase depletion in ASN-dependent leukemia cell lines (Parmentier et al., 2015).

Fractions without NHase activity should provide much useful information about ASNase stability and more adequate characteristics in ALL trials. Even so, future research has to be done in order to test the effect of NHase activity in ASN depletion or cytotoxicity in trials conducted with NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96253-C6.

The approach purification followed in this study using $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 C6 purified an enzyme with ASNase activity higher than others purified from other microorganisms such as *Pseudomonas spp.* (Manna, Sinha, Sadhukhan, & Chakrabarty, 1995),(Husain et al., 2016b) *Enterobacter cloacae*(Husain et al., 2016a), *Thermus thermophilus*(Pritsa & Kyriakidis, 2001), *Streptomyces spp*. (Reda, 2015)(Amena et al., 2010);(El-Naggar et al., 2018) (Hungund & Desai, 2018), *Bacillus spp.* (Mahajan, Saran, Kameswaran, Kumar, & Saxena, 2012);(Alrumman et al., 2019) and in fact this approach allowed higher ASNase activity than the specific ASNase activity reported for the commercial ASNase from *Erwinia chrysanthemi* which has 353.2 ± 6.1 IU mg^{-1} and ASNase *E. coli* with 76.32 ± 0.5 IU mg^{-1} (Nguyen et al., 2016)

Despite the high ASNase specific activity of purified fractions from *R. rhodochrous* DAP 96253 C6, the co-existing GLNase, also of high activity presented in these fractions might be an issue in treatments of children with ALL because the side effects associated to the intrinsic GLNase activity. According to this statement and based on purification results, purified fractions which more advantages for ALL therapy can be fractions as fraction 13 which presented ASNase activity of 3147 ± 911 IU mg^{-1}, GLNase activity of 1569± 152 IU mg^{-1} and NHase activity of 1850 ± 253 IU mg^{-1} (Figure 3.16). Although Parmentier et al., 2015r found that

GLNase activity is important for ASN depletion in GLN dependent ALL cell lines, the high GLNase activity founded in the purifications made in this study can be an uncompetitive characteristic for chemotherapy in ALL therapy. As a result, improves to the purification approach from *R. rhodochrous* DAP 96253-C6 have to be done in order to reduce GLNase activity maintaining the high ASNase activity. Nevertheless, additional studies should be conducted to investigate the value of ASNase/GLNase preparation in treating ASNase resistant ALL cells (glutamine synthetase cells).

4.3.1 Molecular Weight of NHase with ASNase and GLNase Activity

The native protein, purified from *R. rhodochrous* DAP 96622 using a dialysis process followed by AEX and SEC, presented an estimated molecular weight of ~ 88 kDa. This estimation comes from the sum of the sizes of the prominent band at 66 kDa and the slightly less prominent band of ~ 22 kDa observed on Native-PAGE (figure 3.21). As a result, the protein can be a heterotetramer composed of 2 subunits of ~20 and 2 subunits of ~25 (figure 3.20). Although native gels show the molecular size of the protein in denaturing conditions, it is possible that the pH provided during the gel running has been affected causing denaturation of the protein (Swamy, Siegers, Minguet, Wollscheid, & Schamel, 2006)

The molecular mass of ~ 88 kDa might correspond to L- NHase produced by *R. rhodochrous* J1, reported as an enzyme of ~ 94 kDa composed of α- and β- subunits of 26 kDa and 29 kDa respectively(Yamada & Kobayashi, 1996); (Lan et al., 2017). Similarly, Du (2013) purified a NHase α- subunit of 22.8 kDa and a β- subunit of 26.3 kDa from *R. rhodochrous* DAP 96622 and 96253cultured on Co/urea supplemented YEMEA. In the same study, a fragment of NHase β- subunit of ~20 kDa (Du, 2013) was identified. Thus, the ~20 and ~25 kDa subunits on SDS-PAGE (Figure 3.20) might belong to NHase. Also, might be possible

that the ~ ~25 and ~66 bands showed in native gel, belong to two different proteins. In this case the ~25 kDa protein can be a dimer of 2 subunits of ~12. kDa.

Based on enzyme activity (Figure 3.13) and molecular weight results (Figures 3.19 and 3.20), it is suggested that we were able to purify a NHase protein with ASNase, and GLNase activities from induced cells of *R. rhodochrous* DAP 96622.

In addition to the purified NHase with ASNase and GLNase activities, a GLNase-free enzyme with ASNase activity was purified in fractions 2 and 5. The enzyme purified in fraction 5 presented a slightly native band protein of ~ 21 KDa (In figure 3.21) and in SDS-PAGE a single band of ~ 20 KDa indicating that it might be a monomeric protein (figure 3.20).

This monomeric enzyme of ~ 20 KDa might be a novel protein with ASNase activity or can be an ASNase itself synthetized by *R. rhodochrous* DAP 96622. However, the structure of Gram-negative bacteria asparaginases have been reported as a homotetramer with subunits between 33 and 36.5 and native conformation of ~ 146 kDa (Manna et al., 1995);(Pritsa & Kyriakidis, 2001) and molecular mass for asparaginases subunits purified from Gram-positive bacteria has been reported around 34 to85 kDa (Amena et al., 2010). In addition, Husain *et al*., 2016 reported a 205 kDa hexameric form of ASNase (GLNase free ASNase) from *Pseudomonas otitidis* composed of subunits of 34 k. Other authors purified a homotetramer GLNase - free ASNase from *Pectobacterium carotovorum* MTCC 1428 with a molecular mass of 144.4 kDa (Kumar, Dasu, & Pakshirajan, 2011). These molecular mass and structures are different to the native protein size of ~ 22 kDa estimated for GLNase free enzyme with ASNase activity purified from *R. rhodochrous* DAP 96622 (Figure 3.20).

According to figure 3.20 and 3.21 the protein structure proposed of GLNase free enzyme with ASNase activity from *R. rhodochrous* DAP 96622 was a monomeric form which is

uncommon in ASNase. However a similar monomeric structure was identified in a crystalized form of ASNase from *E. coli* instead of a homo-tetramer form suggesting that structural changes can be caused by the way in which the enzyme is handled (Verma, Kumar, Kaur, & Anand, 2007). If this is approach is true, the estimated molecular weight of NHase with ASNase and GLNase activity will be ~ 66 kDa suggesting that the ~ 22 kDa protein band showed on Native gel belongs to a different protein which provides the ASNase activity. In this case, the protein responsible of the hydrolysis of ASN and GLN can be a protein complex composed of NHase (~ 66 kDa) and the protein of 25 KDa. Du 2014, reported a hypothetical glutamine synthetase- NHase protein complex supporting the idea that NHase subunits are capable of form complex with another proteins.

Another possibility about the identity of the GLNase free enzyme with ASNase activity with ~ 22 kDa purified from *R. rhodochrous* DAP 96622 is that the ~ 22 kDa band presented in Native gel be a subunit of NHase, and it might be showed in native gel because some denaturing conditions presented during the running of the native gel.

For the protein purification from induced *R. rhodochrous* DAP 96253 C6 two proteins with native molecular weights of ~ 210 and ~ 66 kDa were purified in fractions 11 to 14 (Figure 3.22). While the 66 kDa can be a dimer composed of subunits of ~ 25 and ~ 29 that correspond to the molecular mass of α- and β- NHase subunits from *R. rhodochrous* reported by Yamada & Kobayashi, 1996 and Du, 2013, the ~ 210 kDa protein might form a dimer from ~ 72 kDa subunits (Figure 3.23). In addition, the 66 kDa protein observed on the native gel might be a fragment from the 210 kDa protein. Thus, the NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96253, might be a protein complex composed of α - β NHase subunits and a novel dimeric protein of ~ 210.

As is depicted in figures 3.17 and 3.23, a ~ 72 kDa subunit is present in purified fractions from both induced and uninduced cells of *R. rhodochrous* DAP 96253 C6, but this subunit is absent in purified fractions from induced cells of *R. rhodochrous* DAP 96622 suggesting that the ~ 72 kDa subunit associated to samples with ASNase activity is a property of the strain DAP 96253 C6.

The molecular weight (~ 210 kDa) of the NHase with ASNase and GLNase activity purified from induced cells of *R. rhodochrous* DAP 96253 C6 is out of the fractionation range of the HiPrep 16/60 Sephacryl S-100 SEC column which is between 10 and 100 kDa. It can indicate that the protein preparation loaded in the HiPrep 16/60 Sephacryl S-100 column previously changed its conformation during lysis, dialysis or AEX steps resulting in the diffusion into the gel beads of the column achievement the separation of the protein. According to the molecular weight results and the capacity (10-100 kDa) of the SEC column used in this study, further SEC using a major molecular weight capacity column (100- 300 kDa) should be conducted to improve purification process and determine the native molecular size of the protein purified by SEC. Thus, SEC might be used to polish the AEX fractions and determine the native molecular weight of NHase with ASNase activity purified by SEC. GF or SEC commonly are used to identify the native molecular weight of the proteins(GE healthcare, 2010).

On the other hand, the molecular mass of ~ 210 and ~ 66 kDa found in the same SEC fraction are molecular size dissimilar. It can suggest that the separation based on molecular size was ineffective. Even though these proteins were shown as different on native gel, these might belong to unique protein of ~ 276 kDa that did not diffuse into the gel moving through the column in the flowing eluent and was partially dissociated to monomers or small oligomers by conditions provided during native- PAGE. Future aminoacid sequencing of these subunits of ~

210 and ~ 66 kDa should be performed to verify the structure similarity between these protein fragments.

According to the results showed in this study, a NHase with ASNase, GLNase and undetectable NHase activity with ~66 kDa molecular was purified from both *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 maintaining pH at 7.6 during the purification process, but using similar purification conditions GLNase free enzyme with ASNase activity was purified only from *R. rhodochrous* DAP 96622 . However, additional research about molecular mass amino acid sequencing has to be done in order to identify with accuracy the identity of the protein or proteins which are able to use ASN and/or GLN as a substrate.

4.3.2 Km and Vmax

The ASN *Km* values for NHase with ASNase and GLNase activity isolated from $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 C6 (0.3 and 0.007 mM respectively) are ~ 9 to~ 40 fold lower than the ASN *Km* of 2.8 mM reported for an ASNase isolated from *Thermus thermophilus* (Pritsa & Kyriakidis, 2001). The ASN *Km* value for the enzyme purified from *R. rhodochrous* DAP 96622 is similar to the *Km* value of recombinant L- Asparaginases from *E. coli* (*Km* = 0.3mM) (Zhang, Li, & Li, 2017) and from *Lactobacillus reuteri* (*Km* = 0.33mM) (Zhang, Li, & Li, 2017). Although the ASN *Km* value (0.3 mM) of the enzymes from $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96622 is considerable higher than ASN *Km* reported for the commercial asparaginases, the ASN *Km* (7μM) for the enzyme purified from *R. rhodochrous* DAP 96253 C6 is ~ 2 fold lower than *Erwinia Chrysanthemi* (*Km* = 15 μM), ~ 7 fold lower than *Escherichia coli* (*Km* = 48 μM) and ~ 1.7and 5.7 fold low of the PEGylated form of E. coli (*Km* = 12- 40μM).In order to use ASNase in cancer trials, ASN *km* values have to be in a molecular range similar or lower than

50 μM which approximately is the concentration of ASN in human blood (Nguyen et al., 2016). Thus, the kinetics of the NHase with ASNase and GLNase activity isolated from. *rhodochrous* DAP 96253 C6 is a promissory enzyme for ASN depletion in ALL therapy.

The *Vmax,* 3.4 and 3.5 μmoles/min obtained for the NHase exhibiting ASNase activity was similar to the *Vmax* of 2.36 μmoles/min which was reported for a novel site specific PEGylated ASNase with a ASN *km* of 34.67μM (Meneguetti et al., 2019)

The GLN *Km* values of NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96622 (*Km*= 8.4) and $GUCo^{+2}$ induced cells *R. rhodochrous* DAP 96253 (*Km*=6.45 mM) are ~ 20 fold higher than GLN *Km* value (0.36 mM) of the *Erwinia Chrysanthemi* ASNase which is the commercial enzyme with much intrinsic GLNase activity. Several engineering enzyme efforts have been done in order to decrease the GLNase activity of ASNase because the adverse effects of clinically used asparaginases have been attributed to the depletion of GLN which has a concentration range of 0.4 to 0.65 mM in blood(Nguyen et al., 2016). On the other hand, a study conducted using eleven ALL cell lines and two patient-derived samples demonstrated that the GLNase activity is necessary to achieve the ASNase cytotoxicity in ALL cell lines with sensitivity principally related to Glutamine synthase activity (Parmentier et al., 2015). Thus, the NHase with ASNase and GLNase activity purified from *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 C6 is a competitive source of ASNase with high ASN affinity and lower affinity for GLN improving the ASNase cytotoxicity in cells that exhibit sensitivity to GLNase activity and diminishing the side effects caused for low GLN *km* values.

A purified fraction with ASNase activity and undetectable GLNase activity obtained from *R. rhodochrous* DAP 96622 suggests that this enzyme preparation might have a potential

use in clinical trials in which the ALL cells clearly are totally ASNase dependent such as, Sup-B15, RS4-11 and MOLT-4 (Parmentier et al., 2015)

NHase with ASNase and GLNase activity purified from induced *R. rhodochrous* DAP 96253-C6 might have the characteristics to be a clinically relevant enzyme in ALL trials associated with glutamine synthase inducible cells. This enzyme might deplete the ASN in blood because the ASN *Km* value (7 μM) is lower than ASN concentration in blood (50μM) reducing the side effects attributed to the GLN affinity of asparaginases used in clinical trials because the GLN *Km* of the purified fractions is 6.45mM while asparaginases from *Erwinia chrysanthemi* and *E.coli* have GLN km values between 0.36 and 1.38 mM respectively.

The results of *Km* determination suggest that the $GuCo^{+2}$ induction during fermentation results in improved kinetics of the purified enzymes to be used as a potential leukemia treatment. While fractions purified from induced cells of *R. rhodochrous* strains presented ASN Km values between 0.3 and 0.007 mM, the NHAse with ASNase and GLNase activity purified from uninduced cells of *R. rhodochrous* DAP 96253-C6 showed an ASN km of 21mM which can difficult the ASN depletion in blood.

4.3.3 Thermal and pH Stability

The NHase with ASNase activity purified from *R. rhodochrous* DAP 96622 showed a retention of activity of 92 % after 15 minutes of incubation at 37ºC compared with a retention of 81 % for R. *rhodochrous* DAP 96253 C6 (Figure 3.31). Similar results were observed in a GLNase free ASNase isolated from *Pseudomonas otitidis* which presented a retention of ~ 90 after 15 minutes of incubation in a range of 35 to 40 ºC(Kumar et al., 2011).
Regarding pH stability, the enzyme from *R. rhodochrous* DAP 96622 exhibited more than 77 % of activity retention after 24 hours of storage over a pH range of 7.4 to 9, whereas the pH

stability of the protein from *R. rhodochrous* DAP 96253 C6 presented between 79 and 100% activity retention for pHs 7.4 and 7.6 (Figures 3.32 and 3.33).

Although the pH stability above 9 found for both purified enzymes was lower than reported for an ASNase from *Streptomyces gulbargensis* which retained 80% of activity in this range (Amena et al., 2010), the enzyme from induced cells of *R. rhodochrous* DAP 96253 retain 79% of ASNase activity at pH 7.4. However, the enzyme purified from uninduced cells of *R. rhodochrous* DAP 96253 C6 lost 72.6% of the initial ASNase activity after 24 hours of storage at pH 7.4. Thus, the NHase with ASNase and GLNase activity purified from $GuCo^{+2}$ induced cells has more potential for leukemia treatments because its stability at physiological pH (Figures 3.33 and 3.34).

4.3.4 Trypsin and Human Serum Half-life of ASNase

As seen in figures 3.35 to 3.37, the trypsin resistance of NHase with ASNase and GLNase activity isolated from $GUCo^{+2}$ induced cells from both *R. rhodochrous* DAP 96253 and *R. rhodochrous* DAP 96622 was similar. Purified fractions from these strains presented a T 1/2 between 30 and 36 min. It was shorter than the trypsin half- life reported in the GLNase -free ASNase isolated from *Pseudomonas otitidis* (Husain et al., 2016b). However, a longer trypsin resistance was observed for the GLNase free NHase with ASNase activity purified from *R. rhodochrous* DAP 96622 which showed a reduction of 50% of ASNase initial activity after 43 min. This enzyme might be resistant to the proteases circling in human blood. This characteristic added to the undetectable GLNase activity are helpful to achieve ALL treatments with reduced side effects caused by GLN depletion.

According to figure 3.38, the human serum half-life of the ASNase purified from *R. rhodochrous* DAP 96622 was considerable lower (8 hours) than the enzyme from *R.*

rhodochrous DAP 96253 (29 – 50 hours), and ASNase isolated from *Pseudomonas otitidis* (Husain et al., 2016b)

The human serum half-life of the ASNase purified from *R. rhodochrous* DAP 96622 was considerable lower (8 hours) to the enzyme from *R. rhodochrous* DAP 96253 (29 – 50 Hours) and ASNase isolated from *Pseudomonas otitidis* (Husain et al., 2016b)

The effectiveness of ASNase depletion in ALL treatment is conditioned by the ASNase half-life in serum and tolerance to proteases. When the effective period of depletion is short, larger and/or more frequent doses must be administrated influencing the risk of antibody formation(Asselin & Rizzari, 2015). Therefore, the NHase with ASNase and GLNase activity purified from $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96253 C6 which has a trypsin half-life of 30 minutes and a human serum half-life of 50 hours exhibit better characteristics to maintain a constant ASNase depletion in the ALL patients. The T ½ of 50 hours suggests that a longer ASN depletion can be achieved by using the enzyme purified from induced *R. rhodochrous* DAP 96253 C6. However, additional in-vitro and physiological tests are required.

Although he NHase with ASNase and GLNase activity purified from uninduced cells of *R. rhodochrous* DAP 96253 C6 presented a longer trypsin half-life of 39 minutes than the enzyme purified from $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96253 C6, characteristics such as the human serum half-life of 29 minutes and the ASN *km* of 21mM confer disadvantages to use this enzyme in ASN depletion treatments (Table 3.8) .

According to Rutter and Wade, the isoelectric point of ASNase influences its half -life in blood. ASNase half-life increases from five minutes at pI of 3.3, to ~ 19 hours at pI 6 , but then decreases to 228 min. at pI 9.7(Rutter & Wade, 1971) The pI of ~4.5 found in NHase with ASNase activity purified from *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 C6

is lower than the pI of 8.6 reported for Erwinaze® or Erwinase®, the commercial versions of ASNase from *Erwinia chrysanthemi* L- ASNase (Gervais et al., 2017) and similar to the pI of 4.67 ASNAse from *E.coli* which approximately has a higher half-life (8 to 49 hours) than *Erwinia chrysanthemi* L- ASNase with 6 to 16 hours depending the injection via ("Asparaginase Escherichia coli - DrugBank," n.d.);("Asparaginase Erwinia chrysanthemi - DrugBank," n.d.). Based on this data, the pI of the purified enzyme from *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253 C6 pH 4.5 (Figure 3.24) suggests a good persistence of ASNase in human blood. In addition, this isoelectric point which is similar to the isoelectric point of α NHase subunit (4.7) and the band proteins showed in SDS-PAGE for the fractions isolated from *R. rhodochrous* DAP 96622 and *R. rhodochrous* DAP 96253-C6 suggest that the α NHase subunit might have ASNase and GLNase activities.

4.3.5 Effect of Storage Time on ASNase and GLNase activity

The Storage of purified fractions at 4 ºC on ice confers stability to ASNase because after 45 days of storage in these conditions the initial ASNase activity decreased only 15 %. However, the initial ASNase activity for the fraction without GLNase activity exhibited a reduction of 48%. The GLNase activity present in purified fractions was less stable after storage for 31 and 45 days at 4 ºC on ice showed a reduction between 51 and 64% respectively. The GLNase and ASNase activity for purified fractions from uninduced cells of *R. rhodochrous* DAP 96253 C6 was undetectable after 6 days of storage at 4 ºC on ice showing that the enzyme purified from uninduced cells present less time stability (Figure 3.39).

4.4 Conclusion

The purification approach, conducted in this study, demonstrated that at a constant pH of 7.6 using 50 mM PB, supplemented with 50mM PB 2-mercaptoethanol as a lysis buffer , a

dialysis against 50mM PB for 40 hours followed by an AEX at 0.6 ml/min flow rate using an Toyopearl®super Q column and a subsequent Gel filtration with a HiPrep® 16/60 Sephacryl S-100 column resulted in the isolation of a NHase with ASNase and GLNase activity.

Using the purification approach described above and $GUCo^{+2}$ induced cells of *R rhodochrous* DAP9653-C6 as starter material is possible purify a NHase with specific ASNase activity of 3036 IU mg^{-1} , GLNase activity of 1417 IU mg^{-1}, NHase activity of 1597 IU mg^{-1} , ASN *Km* of 0.007 mM, Vmax of 3.5 μmol/min, GLN *Km* of 6.45 mM with Vmax of 90.9 μmol/min, an estimated and suggested native molecular weight of 276 kDa composed of ~ 23 , ~ 28 ,~ 72 kDa subunits, temperature stability at 37ºC, pH stability between 7.4 -7.6, trypsin half-life of 30 minutes and human serum half-life of 50 hours. The characteristics of this purified enzyme confer promising properties for ASN depletion therapy in ALL patients.

In addition, using the same process of purification and $GUCo^{+2}$ induced cells of *R. rhodochrous* DAP 96622 a NHase with ASNase activity of 2852 IU mg^{-1}, GLNase activity of 3369 IU mg^{-1} was purified. The purified fraction presented undetectable NHase activity, ASN *Km* of 0.3 mM, *Vmax* of 3.4 μmol/min, GLN *Km* of 8.4 mM with Vmax of 49.3 μmol/min, an estimated and suggested native molecular weight of ~ 88 kDa composed of ~20 and ~25 kDa, temperature stability at 37ºC, pH stability between 7.4 -8.0, trypsin half-life of 33 minutes and human serum half-life of 8 hours.

The enzyme purified from induced cells of *R. rhodochrous* DAP96622 that has a low ASN *Km* and higher GLN Km, can be appropriate for leukemia treatment, the human serum half-life can provide a short half-life in blood of ALL patients and the GLNase activity higher than ASNase activity can occasion undesirable side effects. However, from the same purification an NHase with only ASNase activity and a trypsin half- life of 43 minutes was purified in a

different fraction of SEC providing a new possibility for ALL therapy in tumor cell lines ASN dependent.

In summary, both *R. rhodochrous* DAP96622 and *R. rhodochrous* DAP96253 can produce a variant of NHase with ASNase and GLNase activity, but purification from *R. rhodochrous* DAP 96622 provides a GLNase free ASNase which can reduce production costs compared to asparaginases with free or reduced GLNase activity that have been modified by genetic engineering. Finally, the protein purification from *R. rhodochrous* DAP96622 and DAP96253 provided a suit of protein preparations exhibiting ASNase activity with a variety of GLNase and NHase activity. These protein preparations should be investigated in order to know how either GLNase or NHase activity affects the stability and cytotoxicity of ASNase in ALL trials.

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APPENDICES

Appendix A: Buffers for Enzyme Purification

Buffers for Purification

Appendix A.1 100 mM Phosphate Buffer

This was made according to the formulation proposed by R, Simon in Protein purification techniques (Roe, 2001) and with the following table.

Stock solution A 0.2 M sodium dihydrogen phosphate (NaH_2PO_4) Stock Solution: Add 27.8 g NaH_2PO_4 in 1 L distilled water (di H_2O)			
Stock solution B 0.2 M Disodium hydrogen phosphate with 7 H_2O ($Na_2HPO_4.7H_2O$) Sock Solution: 53.65g $Na_2HPO_4.7H_2O$ in 1 L (di H_2O)			
Stock Solution A (ml)	**Stock solution B (ml)**	**Di water**	**pH**
87.7	12.3	100 ml	6.0
68.5	31.5		6.5
39.0	61.0		7.0
28.0	72.0		7.2
19.0	81.0		7.4
13.0	87.0		7.6
5.3	94.7		8

*For preparation of 50 mM buffer concentration an additional dilution was made. For example, to prepare 50 mM PB buffer pH 7.6 200 ml of deionized (di H_2O)h water were added to the initial 200ml of 100 mM PB buffer.

Appendix A.2 Lysis Buffer Composition

50 mM Phosphate Buffer pH 7.6

5mM 2- mercaptoethanol

For 15 ml of lysis buffer add 5.25.µl of 2-mercaptoethanol into 15 ml of PB.

2- Mercaptoethanol has to be manipulated in the chemical hood.

Appendix A.3 Dialysis Buffer

Composition	concentration	Amounts for 300 ml of dialysis buffer
Phosphate Buffer (PB)	50 mM	255 ml
Glycerol	1.5 %	45 ml
$MgS0_4$. $7H_2O$	1%	3 g
2-mercaptoethanol,	5mM	105 µl

Appendix A.4 Equilibration Buffer

Composition	concentration	Amounts for 300 ml of dialysis buffer
NaH_2PO_4	25 mM	255 ml
Sodium butyrate	10mM	45 ml

Appendix A.5 <u>Elution Buffer</u>

Prepare a 1 M NaCl solution dissolving 1.461 g of NaCl into 25 ml of Equilibration buffer and make the elution buffers using the volumes and components registered in the following table.

Elution Buffer	Equilibration Buffer (ml)	1 M NaCl Elution Buffer (ml)
0.1 M NaCl	9	1
0.2 M NaCl	8	2
0.5 M NaCl	5	5
1 M NaCl	0	10

Appendix B: Reagents for Enzyme assays

Appendix B.1: 2. 5 % Sodium Phenate

25 g of Phenol

78 ml 4N NaOH

Di H_2O up to 100 ml

Weight and dissolve the phenol in the chemical hood.

Cover the container with aluminum foil and store at 4ºC

Appendix B.2: 0.01% Sodium Nitroprusside

Prepare a stock solution of 1% Sodium Nitroprusside:

Dissolve 1g of Sodium nitroprusside in 100 ml of distilled water (di H_2O).

Cover the container with aluminum foil and store at 4ºC.

Prepare 0.01% sodium Nitroprusside using 1ml of 1% Sodium Nitroprusside per each 99 ml of distilled water.

Appendix B.3 0.15% Sodium hypochlorite

Prepare the solution according to the concentration of sodium hypochlorite of the bleach.

For example, for a bleach with 6% sodium hypochlorite dissolve 2.5 ml of bleach in 97.5 ml of di H_2O

Appendix C: Calculation for Specific activity, total activity and % of yield in enzyme purification

Specific activity =Units of enzyme / mg protein

Total activity= (Specific activity) x (total milligram protein) X 100

% yield =Total activity of the purification step/total activity of starting material.

In this study the free cell lysate was the starting material

Fold Purification: Specific activity of the purified fraction/ specific activity of starting material

Appendix D: Calculation for *Km* and *Vmax*

Lineweaver-Burk Equation:

$$\frac{1}{V} = \frac{Km}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax}$$

Based on the straight line:

$$y = m \times + b$$

$$y = \frac{1}{V}$$

$$m = \frac{KM}{Vmax}$$

$$x = \frac{1}{[S]}$$

$$b = \frac{1}{Vmax}$$

$$Vmax = \frac{1}{b}$$

Km= (Vmax)*(m)