Chemical Modifications of L-Asparaginase to Improve Acute Lymphatic Leukemia Therapy

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY INDORE
MAY,2022

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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree

of

Master of Science

by

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INDIAN INSTITUTE OF TECHNOLOGY INDORE

MAY,2022

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Chemical modifications of L-Asparaginase to Improve Acute Lymphoblastic Leukemia"** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from August 2021 to May 2022 under the supervision of Prof. Avinash Sonawane, Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Signature of the student
PIYUSH GOEL

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Signature of the Supervisor
Prof. Avinash Sonawane

Piyush Goel has successfully given his/her M.Sc. Oral Examination held on **5th May, 2022**.

Signature of Supervisor of MSc thesis
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Convener, DPGC
Prof. Prashant Kodgire

Signature of PSPC Member
Dr. Hem Chandra Jha

Signature of PSPC Member
Dr. Parimal Kar

ACKNOWLEDGEMENTS

"No one can whistle a symphony. It takes a whole orchestra to play it." This thesis has become a reality due to the collective efforts of many people in terms of advice, helps, suggestions and blessings. It gives me immense pleasure to express my gratitude, regards, and acknowledgment to them.

First and foremost, I would like to offer all glory and remembrance to almighty God for providing me the strength to complete my thesis despite many problems. With ever-failing experiments, I sometimes got too disheartened. As days passed, I got strength and endurance to cope with the negative results. With repeating experiments, and positive results, my enthusiasm and confidence got up a boost. My research interest increased to several folds with every new investigation. The completion of this present thesis work inculcated many unique qualities in me. I learned to face new challenges, troubleshoot problems, and work with a new vigor every day. Believe that every day is a new day, and so will be for my experiments. This won't be possible without the consistent suggestions of many people who played a vital role in completing this present thesis work.

I want to express my gratitude to my thesis supervisor **Prof. Avinash Sonawane**, for letting me do my master's project in his lab. I am fortunate enough to have his unwavering support, guidance, and insight throughout this research project. I want to thank him for all the data discussions, motivations, and wisdom he provided regarding my experiments. Without his continuous support, it would not have been possible to complete my thesis.

I would also like to thank the **Director, Prof. Suhas Joshi,** for letting me be a part of such a prestigious institute. Also, I want to thank my PSPC

committee members, **Dr. Hem Chandra Jha and Dr. Parimal Kar,** for their constant suggestions and advice. I want to thank **Prof. Amit Kumar (Head, Department of Biosciences and Biomedical Engineering), Prof. Prashant Kodgire (Convener, DPGC),** and **Dr. Parimal Kar (M.Sc. Coordinator)** for their suggestions throughout my M.Sc. journey.

I would also like to express my humble thanks to all the **faculty members of BSBE** who taught me various courses during my coursework and constantly motivated me to bring better in me. I also like to thank the **Department of Biosciences and Biomedical Engineering (BSBE), the Indian Institute of Technology Indore,** for providing a shear platform for me to pursue my research interest without any difficulties and for aiding in every possible way during this process.

My special thanks to my lab member **Mr. Mainak Biswas** for guiding and helping me out during the experiments initially. I want to thank him for his guidance at every point of time when I required some experienced insights from him.

I would like to express my deep gratitude to **Ms. Bhagyashri Soumya Nayak** for helping me with AKTA purification.

I want to express my sincere thanks to all other lab members, Dr. Jhasketan Badhai, Ananyaashree Behera, Soumika Sengupta, Suchi Chaturvedi, Satyam Singh, and Priya Ghosh, who are a source of learning and constant motivation during my project. I also want to thank each of them for providing a lab environment that taught me a lot. It will surely help me in my future endeavors.

I would also like to pay my sincere respect to my father, **Rajender Kumar Goel,** and my brother, **Prateek Goel,** for constantly encouraging me and their unconditional support, love, and care. Without them, I would not be here, where I am today.

Last but not least, I would like to acknowledge my friends **Vedant Dushant Salve, Akshay Mehta** and other batchmates who consistently motivated me and supported me during this project. I am indebted to everyone who made the smooth functioning possible.

Piyush Goel

DEDICATION

I would like to dedicate my

M. Sc. thesis to all my loved ones,

most notably my dad and brother.

Abstract

Acute Lymphoblastic Leukemia (ALL) is one of the usual cancers in children below ten years. ALL is characterized by the production of many immature lymphoblasts in the bone marrow. The treatment of ALL majorly includes chemotherapy. Chemotherapeutic treatment is a phased process and involves many drugs, mainly given in concoction with the drug Asparaginase (ASNase). ASNase works by depleting the serum asparagine required by blast cells to form proteins. ASNase-based therapies have shown promising effects in the treatment of ALL. However, the current ASNase enzyme used for the treatment of ALL is of bacterial origin. Owing to its bacterial origin, it has many side effects, including hypersensitivity, pancreatitis, hyperglycemia, thromboembolism, etc. Also, the ASNase enzyme has a presence of secondary glutaminase activity. Due to glutaminase activity, there are several ill effects, including hepatotoxicity and neurotoxicity. There is a significant drawback of the ASNase therapy. The silent inactivation of ASNase is due to the formation of anti-drug antibodies. These ill effects were counter-managed by forming a novel mutant asparaginase drug-using protein engineering approaches. This novel mutant has shown better outcomes in terms of stability and lesser toxicity. However, the mutant drug is susceptible to the serum proteases such as endopeptidase and cathepsin. This causes a reduction in the half-life of the mutant drug due to proteolytic degradation. To enhance the pharmacokinetic properties of this drug, further chemical modification using Methoxy-Poly-ethylene glycol N-Hydroxysuccinimide esters (mPEG-NHS) is performed. Random chemical modification using mPEG-NHS was performed with the mutant ASNase. Chemical modification maintains the stealth effect and reduces the number of infusions by increasing the enzyme's half-life. Chemically modified ASNase has been tested for its stability, activity, and other parameters. It is also hypothesized that there will be lesser proteolytic degradation of these chemically modified

ASNase. The chemically modified ASNase is a subject of testing anti-leukemic activities in-vitro and in-vivo leukemic animal models. We are incredibly hopeful for producing an indigenous chemically modified ASNase that can be a better potential candidate for the treatment of ALL.

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LIST OF ABBREVIATIONS

PTAD	4-phenyl-3H-1,2,4-triazoline-3,5(4H)-dione
6MP	6-mercaptopurine
ALL	Acute Lymphoblastic Leukemia
AML	Acute myelogenous leukemia
AA	Amino acids
ADA	Anti-drug antibodies
ASNase	Asparaginase
ASNS	Asparagine synthetase
BSA	Bovine Serum Albumin
CNS	Central nervous system
CLL	Chronic lymphoblastic leukemia
CML	Chronic myelogenous leukemia
CV	Column volume
Dox	Doxorubicin
EcA-II	*E. coli* ASNase-II
Etbr	Ethidium bromide
FAB	French-American-British
IPTG	Isopropyl β-d-1-thiogalactopyranoside

AHA	L-Aspartic acid hydroxamate
LB	Luria Bertani
Mtx	Methotrexate
NTA	Ni–nitrilotriacetic acid
PEG	Poly-ethylene glycol
TCR	T-cell receptor
TCA	Tri-chloro acetic acid
Vcr	Vincristine
WBC	White blood-cells
WHO	World Health Organization
mPEG	Methoxy-Poly-ethylene glycol
NHS	N-Hydroxysuccinimide esters

1. Chapter 1

1.1. Introduction

1.1.1 Leukemia

Leukemia is a malignant disorder of blood and bone marrow. It is an uncontrolled growth of leucocytes **(Figure 1.1)** in bone marrow or lymph nodes [1]. It is divided into different types based on the development of the cells. The primary classification of leukemia is acute versus chronic [2]. In acute leukemia, the abnormal blood cells known as blasts remain immature, and as a result, they cannot carry out their normal function [3]. In acute leukemia, blast cells rapidly increase in number, thereby worsening the disease [4]. On the contrary, in chronic leukemia, blast cells are somewhat mature and can carry out certain functions [5], [6]. Also, in chronic leukemia, there is a slow increase in the number of blast cells; therefore, the disease progresses less rapidly than acute leukemia [5].

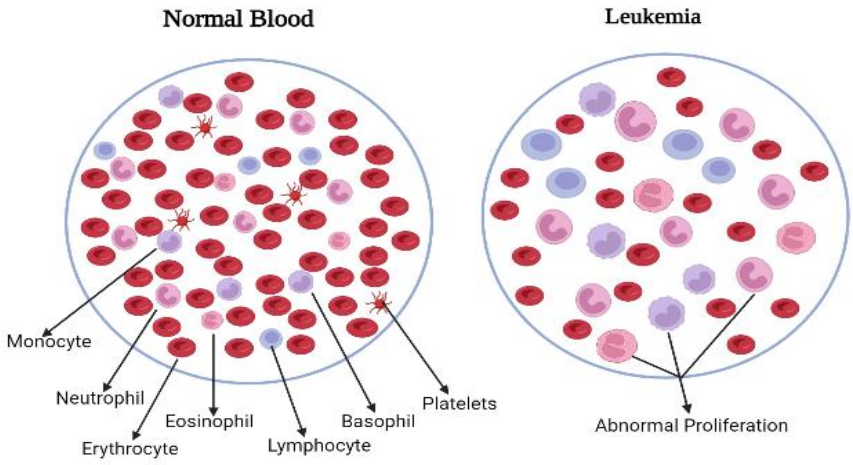
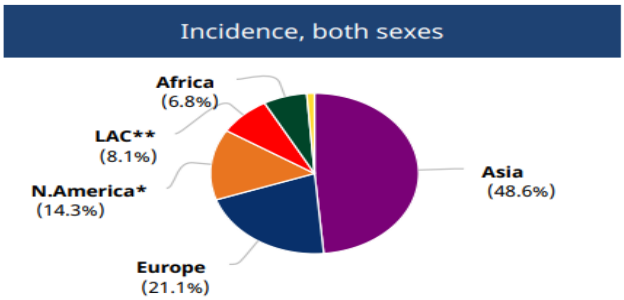


Figure 1.1: Difference in morphology in normal blood cells and leukemic cells

In general, overcrowding of immature white blood cells interferes with the process of hematopoiesis, resulting in a decrease in normal and healthy blood cells. Consequently, the body suffers from a lack of oxygen supply due to a depleted number of red blood cells [2]. The symptoms of leukemia include persistent fatigue, frequent or severe infections, fever or chills, weakness, swollen lymph nodes, easy bleeding or bruising, enlarged liver or spleen, weight loss, etc. [7].

Leukemia is one of the most dreaded diseases in the world. According to the reports published by the World health organization and GLOBOCAN, there was an increase in 4,74,519 cases of leukemia worldwide in 2020 **(Figure 1.2)**. The mortality rate of leukemia is significantly high. About 3,11,594 people lost their lives to this deadly disease in 2020 only. Leukemia accounts for 30% of all cancer occurring in the world. The relative survival rate for this disease is about 70% because many people die from leukemia every year worldwide. The maximum number of leukemic cases arises in Asia (48.6%), followed by Europe (21.1%) and other countries [8]. India accounts for up to 68.5% of all the leukemia cases reported in South-central Asia [8]. It has been estimated that a total of 48,419 cases of leukemia will be notified in India in 2020 **(Figure 1.3)**.

There are four significant types of leukemia which include acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphoblastic leukemia (CLL), chronic myelogenous leukemia (CML) [9]. Acute lymphoblastic leukemia is found in lymphoid cells, and it proliferates quickly. The incidence of acute lymphoblastic leukemia is prevalent in children mainly. Acute myelogenous leukemia is found in myeloid cells and is fast growing in nature. It is common in both adults



Population	Number
Asia	230 650
Europe	100 020
*Northern America	67 784
**Latin America and the Caribbean	38 256
Africa	32 138
Oceania	5 671
Total	474 519

Figure 1.2: Incidences of Leukemia worldwide

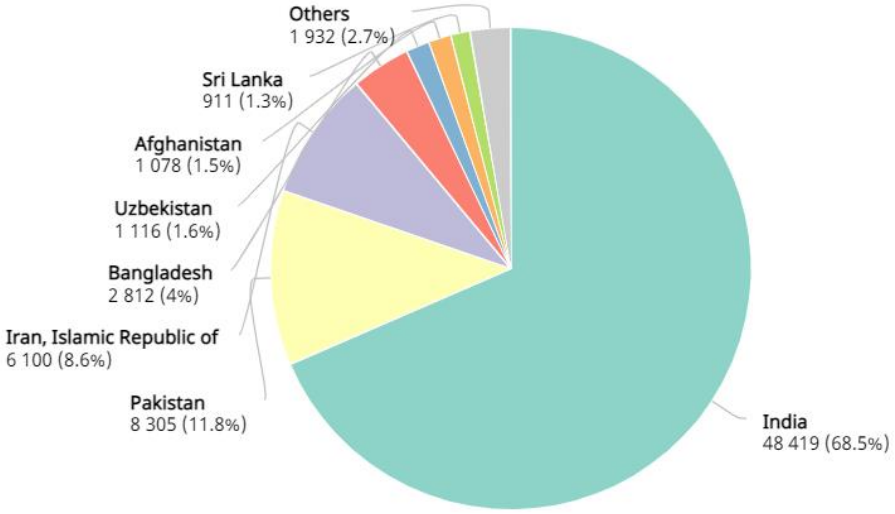
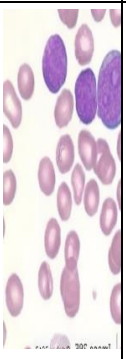
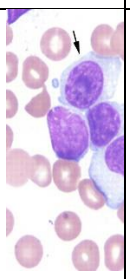
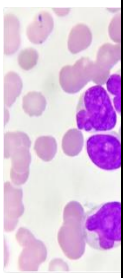
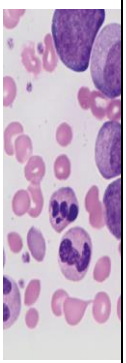


Figure 1.3: Estimated number of new cases of Leukemia in South central Asia *[8]*

and children [10]. Chronic lymphoblastic leukemia is slow-growing leukemia found in lymphoid cells. It is more common in the adult population exceeding 55 years. Chronic myeloid leukemia is also found in myeloid cells and grows slowly. It is common in adults [11].

Table 1.1: Characteristics of different types of leukemia

Leukemia type	Cellular Image	Cells involved	Clinical presentation	CBC Results	Demographics	References
Acute Lymphocytic Leukemia (ALL)		Immature B or T cells (marrow cells)	Bone pain, CNS manifestation, depressed marrow function	Anemia, thrombocytopenia, variable WBC's, >30% lymphoblasts	Children	[12]
Chronic Lymphocytic Leukemia (CLL)		Peripheral B or T cells (lymph nodes)	Asymptomatic, LAD, Hepatosplenomegaly	Lymphocytosis >5000/µl, low platelets	Most common leukemia in adult males	[13]
Acute Myelogenous Leukemia (AML)		Immature myeloid lineage cells (marrow cells)	Anemia, bleeding, petechiae	Anemia, neutropenia, thrombocytopenia, >30% myeloblast	Adults	[14]
Chronic Myeloid Leukemia (CML)		Pleuripotent hematopoetic stem cells (marrow cells)	Insidious onset, mild anemic symptoms, splenomegaly,	Leucocytes>200,000 – 1,000,000, increased eosinophils and basophils	Ages 20-50 years, Rare in children	[15]

1.1.2 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a significant proliferation of lymphoid cells (blasts) blocked at an early stage of their differentiation. These blasts can invade the bone marrow, blood, and extramedullary sites [16]. ALL is believed to occur due to various genetic lesions in hematopoietic progenitor cells committed to differentiate into T-cells or B-cells. The ALL is further divided into T-cell and B-cell ALL, depending on the cell- lineage. Chromosomal translocation is the most common type of chromosomal aberration found in ALL. B-cell ALL is found to be associated with t(12;21) (p13;q22) chromosomal translocation [17], while T-cell ALL results from the reciprocal translocation, which disrupts specific developmental genes. The rearrangement of the loci containing T-cell receptor (TCR) genes, most commonly TCRa (14q11.2) and TCRb (7q35), is quite frequent in T-cell ALL [18]. A blast count of >25% indicates ALL. In 2018, it was observed that around 103,536 people were living with acute lymphocytic leukemia in the United States [19]. It was also estimated that ALL comprises 60-80% of all leukemia occurring in India. ALL shows poor prognostic factors. It is common in children but can also affect adolescents and adults. It is prevalent in the male sex and characterized by high white blood cells (WBC) count of >50 x 10^9/liters. The relative survival rate for this disease is 70% from 2011-to 17. ALL accounts for up to 80% of all pediatric hematopoietic malignancies [16].

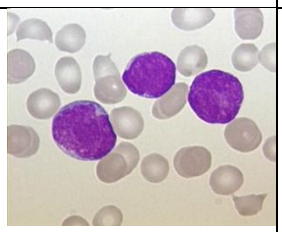
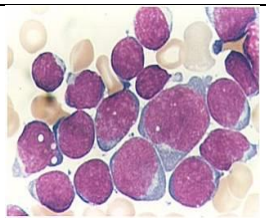
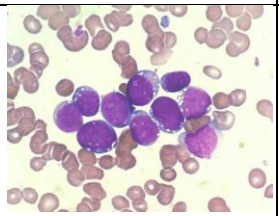
There is a sharp incidence peak of ALL cases in early childhood. It is also observed that there is also a sudden increase in the cases of ALL in the later stage of life, from 70-85 years of age. The clinical symptoms include cytopenia, fever, fatigue, bone pain, pallor, etc. [20]. Physicians use some clinical features to assess the risk stratification of ALL. According to the biological characteristics, ALL patients have been put under various categories, including standard, moderate, high-risk, and

relapsed. The initial risk stratification methodology is based on the age and WBC count of the patient. Depending on these factors, the children in the age group of 1 year to 9.99 years and having a WBC count <50,000/µl are regarded as standard-risk patients [21]. These patients have a good survival outcome. The patients aged>35 years and with an elevated WBC count fall under a moderate risk category. While patients >35 years with a WBC count >50,000/µl are put in the high-risk category. These patients have suffered from remissions after the treatment.

Among those who are successfully treated for ALL, about 20-30% of the patients suffer from relapsed ALL [22], [23]. The relapse is conditional and can be sighted at bone-marrow or some extramedullary sites [24]. The leading cause of failure of the treatment of ALL is due to the relapse of ALL. Bone marrow transplantation is a better form of treatment for the relapsed ALL than conventional chemotherapies [22]. But finding a suitable allogenic donor remains a problem worldwide.

ALL was further divided into three different subtypes according to French-American-British (FAB) groups [25]. These include L1, L2, and L3 types of acute lymphoblastic leukemia [26]. A homogeneous structure characterizes L1 type leukemia, and it accounts for up to 80% of the cases in children and 25-30% in adults. L2 type has a varied nuclear structure, and it has the accountability of 70% of cases in adults. L3 type is also known as Burkitt's Leukemia. It is a rare subtype that accounts for less than 1% of the total ALL cases **(Table 1.2).**

Table 1.2: FAB Classification of Acute lymphoblastic leukemia [11]

FAB-Type	Cellular Image	Frequency	Morphology
L1		25-30% cases in adults, 85% in children	Homogeneous blasts, regular nucleus, small or no nucleoli, scanty cytoplasm
L2		70% cases in adults, 14% in children	Irregular nucleus, Heterogeneous chromatin, prominent nucleoli
L3		Rare subtype, less than 1-2% case	Large blasts, prominent nucleoli, stippled homogeneous chromatin, abundant cytoplasm, and vacuolation

World Health Organization (WHO) classifies ALL into other subtypes [26], [27]. These include Pre-B ALL, T- cell ALL, and Mature B cell ALL **(Table 1.3)**. Pre-B ALL accounts for up to 75% of all the ALL cases, while T cell ALL and mature B cell ALL account for 20% and 5%, respectively. Some cytogenetic abnormalities are seen in all types of ALL. Pre-B ALL can be identified by medium to small-sized blasts with less cytoplasm. Mostly Pre-B ALL shots express CD10 and CD24 [28]. T cell ALL is the extensive growth of lymphoblasts of T cell origin. They usually express CD1, CD2, CD3, CD4, CD5, and CD7 genetic markers [29]. Burkitt's lymphoma is the mature B cell lymphoma or leukemia [21]. It usually expresses CD19, CD22, and CD7 as the genetic markers.

Table 1.3: WHO Classification of Acute lymphoblastic leukemia [21]

Immunologic Subtype	% Of cases	FAB Subtype	Cytogenetic abnormalities
Pre-B ALL	75	L1, L2	+(9;22), +(4;11), +(1;19)
T-cell ALL	20	L1, L2	14q11 or 7q34
Mature B cell ALL (Burkitt's leukemia)	5	L3	+(8;14)

1.1.3 Treatment of ALL

Treatment majorly involves chemotherapy which extends for about 2-3 years [30]. This usually depends on the risk stratification of the patient. Treatment of ALL is a phased process. The phases involved in the treatment are induction, consolidation, and maintenance [20], [21]. These days a new phase of treatment called delayed intensification or reinduction has been introduced, given to ALL patients under the high-risk category. Several chemotherapeutic drugs like vincristine (Vcr), pegaspargase, methotrexate (Mtx), Capizzi, 6-mercaptopurine (6MP), cyclophosphamide, cytarabine, doxorubicin (Dox) are given in concoction with steroids at different phases [20]. The maintenance phase is unique in the treatment of ALL due to the supply of drugs like methotrexate and 6-mercaptopurine. Central nervous system (CNS) prophylaxis is observed in high-risk patients where blasts cross the blood-brain barrier and can generate tumors in the brain [31]. Induction therapy is used to restore average blood cell production. Asparaginase, vincristine,

and steroids are given to the patient in the induction phase. About 95% of the patients exhibit remission after the induction phase of treatment. The induction phase stretches up to 3-4 weeks. The consolidation phase involves using cytotoxic drugs like methotrexate at high doses to remove the residual leukemic cells. Maintenance therapies are the last phase of chemotherapeutic treatment [32]. This involves the administration of 6-mercaptopurine and methotrexate for a period of 1-2 years.

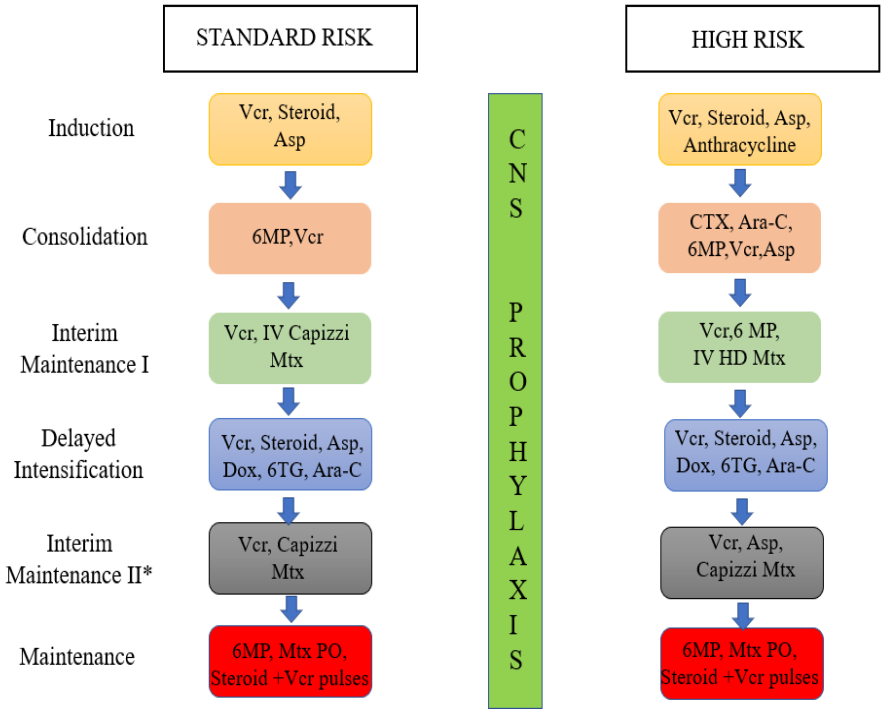


Figure 1.4: Phases of Therapy in Pediatric Acute Lymphoblastic Leukemia

Asparaginase (ASNase) is one of the essential drugs in the treatment of ALL. Administration with ASNase showed promising results and better efficacy ASNase is one of the most widely studied drugs by researchers worldwide. Lang first observed ASNase in 1904 [33]. Later, Broome 1961 showed its neoplastic activity using substrate specificity in the guinea pig. ASNase isolated and purified from the guinea pig serum, and E.

coli was profoundly used to treat ALL [34]. Two isozymes of L-ASNase were discovered, namely type-I and type-II, by Ohuma in 1967 [35]. Both these isozymes have shown their effect on L-asparagine and L-glutamine. However, type-II isozyme is used mainly due to its better neoplastic activity and higher specificity against serum asparagine. *E. coli* ASNase-II (EcA-II) is a homo-tetramer and has a high molecular weight of 138 kDa, and it has four subunits of ~34.6 KDa. It has a three-dimensional structure with two tetramers of four identical monomers each (**Figure1.5**). The structure is correctly regarded as a dimer of dimers. The active site of these three hundred twenty-seven amino acid long enzymes lies between these dimers [36]. Each active site is shaped by the haulage of amino acids arranged in two adjacent monomers.

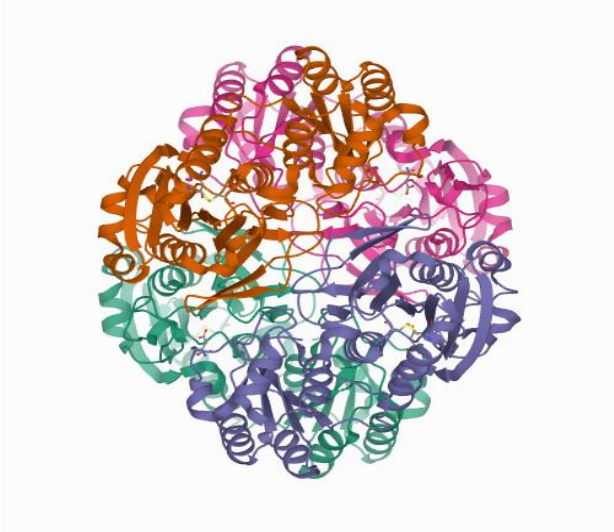


Figure 1.5: Structure of *E.coli* ASNase (PDB Entry: 3ECA)

ASNase works by the metabolic shutdown of the neoplastic leukemic cells [37]. The system of action of ASNase involves the depletion of amino acid asparagine from blood. Cancerous cells have a mutation in asparagine synthetase

(ASNS), and hence they cannot produce their asparagine. Therefore, leukemic cells are entirely dependent on the circulating blood- asparagine, forming other amino acids by transamination. This hinders the protein synthesis of blast cells (**Figure 1.6**). The absence of asparagine in the blood due to asparaginase-induced hydrolysis causes metabolic shutdown in the cancerous blast cells and forces them to undergo apoptosis [38]. At present, numerous clinical data strongly support the use of asparaginase for the therapy of pediatric ALL. The intensive asparaginase treatment is more beneficial than the less intensive one. ASNase has been profoundly used due to its efficacy in converting almost complete asparagine to ammonia and aspartate.

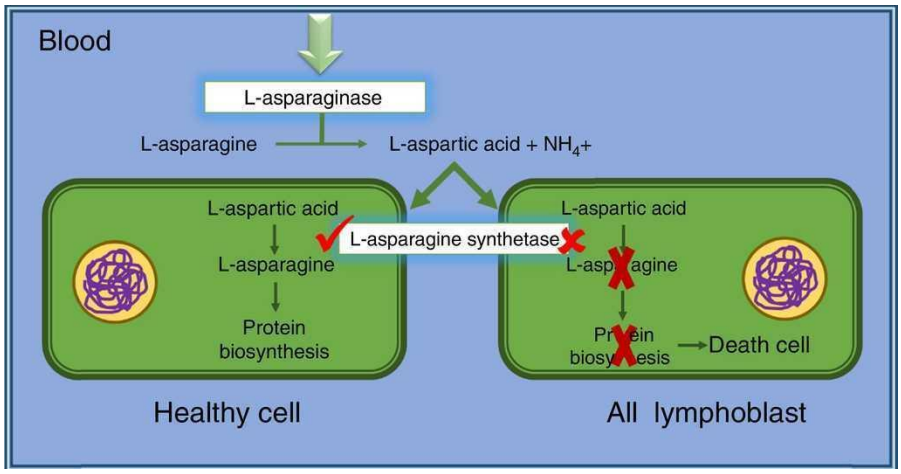


Figure 1.6: Anti-neoplastic mechanism of L-Asparaginase [39]

ASNase has been isolated from plants, fungus, bacteria, guinea pigs, and other mammals. However, currently, three ASNase enzyme preparations are commercially used. These are native ASNase isolated from *Escherichia coli* [38], ASNase obtained from *Erwinia chrysanthemi* known as *Erwinia* ASNase [40], and pegylated form of native *E. coli* ASNase [41]. *E. coli* ASNase has major medical applications and is used almost in

every formulation of ASNase provided to the patient. ASNase derived from Erwinia species is only provided when a patient has developed hypersensitivity towards *E. coli* ASNase and Peg-asparaginase [42].

E. coli-derived native ASNase is a bacterial preparation and is immunogenic to humans [43]. ASNase formulations may result, to some extent, in allergic reactions and hypersensitivity. Almost 30-70% of the patients had developed an immune response to *E. coli* ASNase. This leads to the discontinuation of the ASNase therapy, which reflects an adverse outcome in survival [44]. The addition of such an ASNase can result in allergic effects and anti-drug antibodies (ADAs) formation. Patients with ASNase therapy have developed reduced ASNase activity levels over time [45]. ADAs are formed when the ASNase is first administered to the patients **(Figure 1.7)**. On subsequent exposure to ASNase, these ADAs neutralize the drug's effect and reduce its activity and half-life in the blood. This hinders the purpose of administration of the drug at regular intervals. This is referred to as silent inactivation [32]. These ADAs formed can also be attributed to hypersensitivity reactions occurring in some patients provided with ASNase therapy.

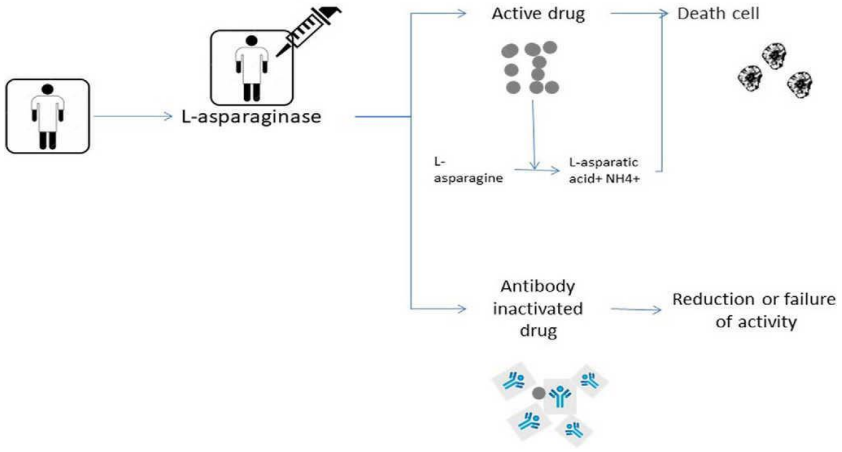


Figure 1.7: Pathophysiology of the mechanism of action of ASNase [44]

Numerous other side-effects are using *E. coli*-derived ASNase. L-ASNase can also degrade L-glutamine along with L-asparagine. Due to this glutaminase ability of ASNase, it results in several side effects, including neurotoxicity and hepatotoxicity. There are other ill effects of using ASNase therapy. Some ASNase therapy patients have shown hyperglycemia, hypoalbuminemia, anaphylaxis, pancreatitis, bronchospasm, angioedema, etc. [36], [45]. Due to these adverse effects of using ASNase, there is a vigor need to have an ASNase-based therapy with lesser side effects and better efficacy.

1.2 Aim, Scope, and Novelty of our study

According to previous studies in our lab, the rational protein engineering approach produced novel *E. coli* ASNase variants with the desired properties [46]. Specific Amino acids (AA) for particular side effects were substituted with other Amino Acids by Polymerase Chain Reaction-based site-directed mutagenesis [46]. These novel *E. coli* ASNase variants show high enzymatic activity, high stability in human serum, and negligible

glutaminase activity. These variants also show low immunogenicity *in vivo* in BALB/c mice and ALL patients. There is also less binding to pre-existing antibodies present in ALL patients receiving asparaginase chemotherapy, thus preventing silent inactivation [47]. All the EcA variants also showed significantly improved efficacy in leukemia animal models and in vivo pharmacokinetics. None of them were found to be toxic *in vivo*.

However, some proteolytic serum proteases like acetyl endopeptidase and cathepsin cleave these variants reducing their *in vivo* half-life and activity. Hence, further modifications are required to reduce the number of dosages of ASNase to get optimum efficacy. Due to various side-effects of E. coli native ASNase and to improve the dosage interval and number, some chemical modifications in the structure of ASNase must be done [48]. PEGylation can do this by adding random poly-ethylene glycol to already prepared mutants. It has been shown that PEGylation of asparaginase improves its therapeutic efficacy with a costly treatment protocol. The proposed project aims to further improve the therapeutic efficacy of these novel asparaginase variants by chemical modifications followed by the evaluation of their antileukemic and immunogenic properties using ALL cells, and lymphoblasts isolated from ALL patients in BALB/c and xenograft mice models. After their pre-clinical verification is complete, it will be subjected for future clinical trials to produce a more advanced chemotherapeutic drug to treat childhood ALL.

1.3 Chemical modification by PEGylation

One of the critical limitations behind the therapeutic usage of asparaginase is its proteolytic degradation. These result in rapid

serum clearance, significantly reducing the drug's half-life. To improve asparaginase's stability in human serum, researchers have tried to modify the enzyme with various forms of polyethylene glycol (PEG). PEGylation exerts the beneficial effect by two different mechanisms: (i) improves the half-life of the enzymes by increasing the size to retard glomerular filtration, and [49] (ii) protecting the enzymes from degradation via its known stealth effect [50].

Several enzymes have been PEGylated to obtain desired effects. PEG moiety offers a variety of advantages and serves as a mechanism to improve protein half-life in blood [51]. PEG has been used due to its flexibility, low toxicity, and increased hydrophilicity. The PEG molecule has a variable size and can easily bind to the protein motifs [49]. Several proteins, including ASNase, have already been modified using PEG and have shown promising results. PEGylation doesn't hinder the protein activity and retains the pharmaco-kinetic properties of the protein. Most of the therapeutic proteins are prepared using non-specific PEGylation techniques. However, two approaches are popular where PEG molecule has been introduced at a particular site and a random PEGylation mechanism [52].

Site-specific protein PEGylation can be performed via chemical modifications of different amino acids, including cysteine, tyrosine, serine, threonine, histidine, etc. [49], [53]. PEGylation is generally performed on the disulfide bridges of the cysteine residues by first reducing the bridge and then reacting it with the PEG molecule **(Figure 1.8)**. Tyrosine residues are preferably reacted with 4-phenyl-3H-1,2,4-triazoline-3,5(4H)-dione (PTAD) to form a covalent bond between the protein molecule and the PEG molecule [54]. N-terminal residues of serine and threonine are also utilized for the PEGylation

mechanism. Histidine tag can also target the PEG molecule with the protein. The protein is added with a polyhistidine tag [41], [49]. The tagged protein is incubated with Ni–nitrilotriacetic acid (NTA)–PEG reagent, resulting in PEGylation of protein.

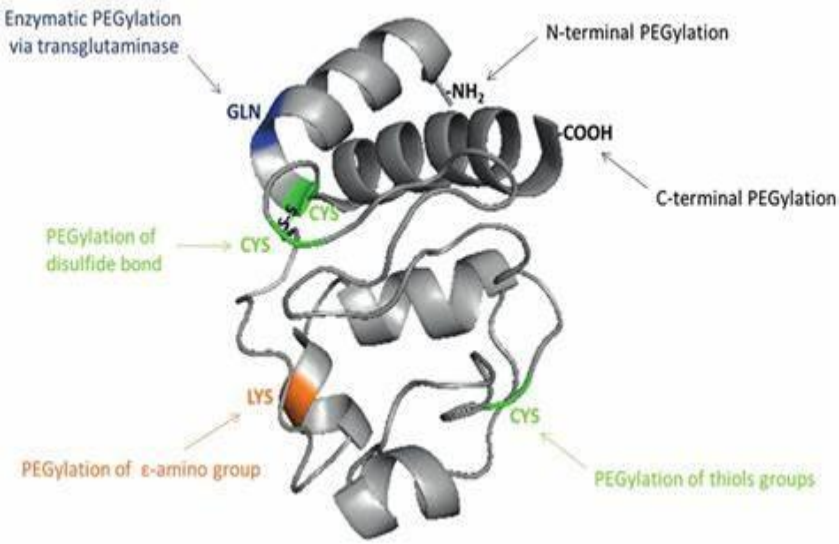


Figure 1.8: Different mechanisms of site-specific PEGylation in proteins [49]

PEGylated ASNases is poly-ethylene glycol conjugated ASNase produced to reduce the immunogenicity compared to native ASNase and reduce the number of infusions by increasing the half-life of ASNase inside the patient's body [41], [42]. Several reports state that even the pegylated forms of native E. coli ASNase can cause hypersensitivity reactions in the patient's body [50], [55]. However, this has been seen in patients pre-provided with E. coli native ASNase and then later shifted to pegylated forms. PEGylation maintains the stealth effect of the drug and reduces immunogenicity *in vivo* [55]. The addition of PEG to the already prepared *E. coli* ASNase variants might result

in reduced hypersensitivity, better stability, and efficacy.

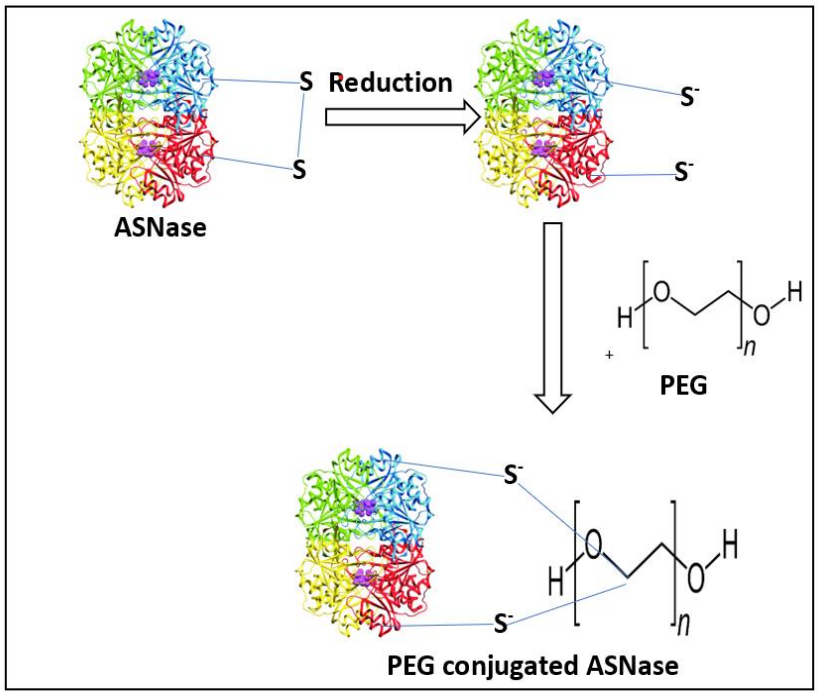


Figure 1.9: PEGylation of ASNase at disulphide bridges of cysteine residues

2. Chapter 2

2.1. Experimental Work Flow

It is evident from the literature that the efficacy of L-asparaginase therapy can be significantly enhanced by reducing its antigenicity and improving stability and activity with the help of site-directed mutagenesis followed by chemical modification. Various studies have shown that mutation at certain positions affects L-asparaginase's stability, activity, and antigenicity. However, further changes to some of those mutants are yet to be explored. This project aims to chemically modify the *E. coli* ASNase variants through PEGylation to improve the efficacy and stability of *E. coli* ASNase variants produced in our lab previously.

The course of action during the project involved the following sub-objectives-:

a) Transformation of Mutant-A-pET-28a-DH5α and W-pET-28a-DH5α plasmids into BL21(DE3) competent cells.
b) Isolation of crude wild-type and mutant-A protein from the transformed *E.coli* BL21(DE3) colonies.
c) To study the enzyme kinetics- and yield of the crude enzyme.
d) Purification of wild-type and mutant protein.
e) Characterization of the purified ASNase, including enzyme kinetics, asparaginase activity, pH, and thermal stability profile.
f) Chemical modification by PEGylation.

g) Characterization of purified enzyme for its thermal stability, enzyme kinetics, activity, antigenicity, and the comparison with the unmodified enzyme.

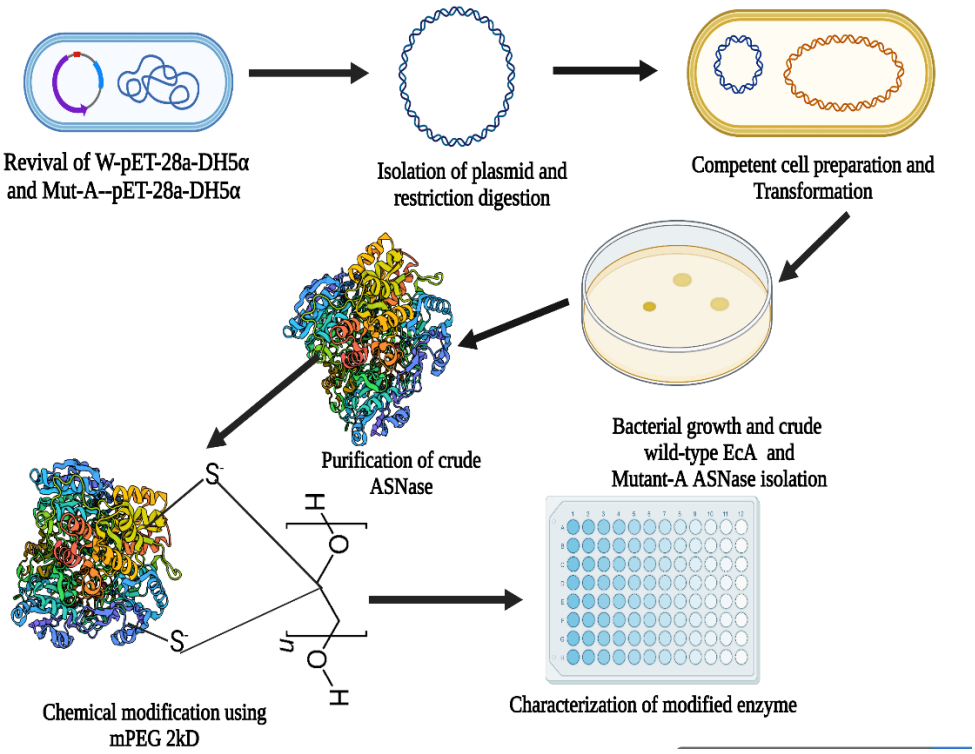


Figure 2.1: Schematic representation of the project objectives

The present project aims to develop a novel PEG-modified ASNase mutant, which can be beneficial in treating primary and relapse ALL.

3. Chapter 3

3.1. Materials and Methods

3.1.1. Materials

3.1.1.1. Strains

E. coli DH5α (Kindly provided by Dr. Klaus Roehm, University of Marburg, Germany), E. coli BL21 (DE3) (ATCC) are used throughout the study.

3.1.1.2. Chemicals

All chemicals used in the present study were of reagent grade and were purchased either from Merck (Darmstadt, Germany), Sigma Aldrich (New Jersey, USA). The plasmid isolation kit was from BioHelix (Taipei, Taiwan). All the reagents and solvents used in the project, SDS PAGE, were purchased from Sigma (New Jersey, USA), SRL (Mumbai, India), and Hi-Media (Mumbai, India). The chemicals were used as it is without any purifications or modifications. The antibiotics used for growing cultures were purchased from Sigma Aldrich (New Jersey, USA), and the column used for Anion exchange chromatography is the Hi-trap Q-HP Sepharose column from Cytiva (Marlborough, USA)

3.1.2. Methods

3.1.1.1 Bacterial growth condition

E. coli strains were grown ready to use Luria Bertani (LB) media from HiMedia (India), pH 7.2-7.4 at 37^0C and 160 r.p.m, supplemented with Kanamycin/Ampicillin and Tetracycline/ Chloramphenicol antibiotics.

3.1.1.2 Storage and Revival of bacterial cultures

Glycerol stocks of bacterial culture were revived by inoculating 30 µl of frozen cells into 3 ml of LB medium followed by overnight incubation at 37°C, 160 r.p.m. A loopful of overnight culture was streaked on LB agar plate supplemented with appropriate antibiotics and incubated overnight at 37°C. A single colony obtained on the plate was used for further experiments. Plates were stored at 4°C for up to one month.

3.1.1.3 Plasmid isolation

E. coli cell having the required plasmid was grown in Luria Bertani broth (from HiMedia) containing antibiotic kanamycin and tetracycline (from HiMedia). The plasmid was isolated from E. coli cells using the Bio-Helix mini prep plasmid extraction kit user manual. Briefly, the overnight grown bacterial culture was centrifuged at 11000 x g for 1 min to pellet the cells. It was added with resuspension, lysis, and neutralization buffer. Lastly, it was washed and eluted in a fresh microcentrifuge tube.

3.1.1.4 Restriction Digestion

The restriction digestion of the isolated plasmids of Mutant-A and Wild-type was performed using NEB (Ipswich, Australia) buffers and restriction enzymes. The isolated plasmids were added with the Cut Smart restriction buffer with EcoRI and HindIII restriction enzymes. The mixture was incubated at 37º C for 90 minutes, and the resultant was run on 1% Agarose gel to obtain the band of the insert on the gel.

3.1.1.5 Agarose gel electrophoresis

DNA, RNA can be separated in an agarose matrix by applying electric fields. The shorter the molecule travels, the more considerable distance in the agarose gel, and the larger molecule travels the shortest distance. For preparing wells into the gel, molten agarose with a fluorescent dye Ethidium bromide (EtBr) for staining the sample was poured into the casting tray of the electrophoresis unit and allowed to solidify. EtBr intercalates into the rings of DNA, which can then be seen under UV. The electrophoresis unit is filled with the 1X TAE buffer, and the samples were mixed into the loading dye and then loaded into the wells. The loading dye contains glycerol to give density to the sample and bromophenol blue to track the movement of the sample into the gel. Electric fields were then applied to separate the sample of different sizes from the mixture. The run gel was then analyzed under a UV transilluminator.

3.1.1.6 Competent Cell preparation

Competent cells were prepared using the Calcium chloride ($CaCl_2$) and Magnesium chloride ($MgCl_2$) method. *E. coli*

BL21(DE3) Cells were grown overnight in Luria Bertani broth supplemented with Chloramphenicol (Himedia) antibiotic. 1% of the overnight grown bacterial culture is again inoculated for secondary culture into the LB broth and incubated at 37° C, 160 r.p.m, till the culture's OD reaches 0.6 at 600 nm. All the subsequent steps were then done on the ice. Cells were first arrested -by incubating on ice for 10 minutes and then centrifuged at 4° C for 10 minutes to pellet down the cells at 3500 rpm. Cells were then re-suspended in chilled $CaCl_2$ and $MgCl_2$ (80mM $CaCl_2$ and 50mM $MgCl_2$). 10ml of the solution was added to the pellet obtained from 40ml of culture and was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was discarded, and 10 ml of the mixture of $CaCl_2$ and $MgCl_2$ were added to the pellets and kept on ice for 2 hours. Repeat the centrifuge at 3500 rpm for 10 mins at four 4°C, and the supernatant was discarded. Pellet was then finally suspended with 80µl of solution of Glycerol and $CaCl_2$ before storing it at -80ºC.

3.1.1.7 Bacterial transformation

The transformation was done by the heat shock method. *E. coli* BL21(DE3) ultra-competent cells were kept on ice for 15 minutes. The competent cells (80 µl) were added with 7µl of plasmid DNA and mixed gently. The mixture was kept on ice for 30 minutes. Immediately, the microcentrifuge tubes were kept at 37ºC thermomixer and held there for 90 seconds. After that, it was immediately followed by transferring the tubes back to the ice for 10 minutes. LB broth was added to each tube and incubated for 60 minutes at 37° C, 160 r.p.m. Transformed cells were then spread onto the LB antibiotic resistance plate. The appearance of single colonies in the following days confirms successful transformation.

3.1.1.8 Recombinant protein isolation

A single colony of transformed cells was inoculated into LB broth containing 50 µg/ml kanamycin and chloramphenicol and grown overnight at 37° C. The secondary culture was prepared in new LB broth supplemented with appropriate antibiotics, and cells were kept on an incubator shaker at 37° C, 160 r.p.m, till 0.6 OD was attained. The secondary culture was induced with 0.5mM IPTG and incubated at 37° C for 4 hours in the incubator (160 rpm). After centrifuging the culture at 10,000 rpm for 3 minutes at 4° C, the cells were suspended in a specific hypertonic buffer solution. The mixture was vortexed and kept on ice for 10 minutes. The mixture was centrifuged at 13,000 rpm for 20 minutes at 4° C. 500µl of chilled Mili-Q water was added to the tube and vortexed vigorously. It was incubated in ice for 5 mins and again centrifuged at 13,000 rpm for 25 mins at 4° C. Supernatant was collected and stored at 4° C. SDS-PAGE result shows asparaginase yield after that.

3.1.1.9 Determination of protein concentration (Bradford et al., 1976)

The protein sample was diluted 100 times in distilled water for quantitative protein estimation. Then 80 µl of protein solution was mixed with 120 µl of Bradford reagent in a microtiter plate and incubated at room temperature for 5 minutes. Absorption was measured at 595 nm against a blank (80µl distilled water + 120 µl Bradford reagent). For the standard curve, increased concentration of 5 sample solutions of diluted Bovine Serum Albumin (BSA) was measured in the same way.

3.1.1.10 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is run to separate the protein onto the gel depending on their size by applying the electric field and discontinuous polyacrylamide gel as the support medium. SDS in the gel acts as a surfactant and imparts a negative charge, thus covering the charge of the protein depending on the charge-to-mass ratio. The basic pH reduces the positive charge of the protein, and the intrinsic charge is also negligible compared to the SDS loaded. In the discontinuous SDS PAGE, first, the protein migrates into the stacking gel of pH 6.8 for proper stacking and then gets separated through the resolving gel of pH 8.8.

The difference in pH leads to the stacking effect at the junction between the stacking gel and the separating gel. The running buffer mainly contains Tris-glycine with SDS. The glycine in pH 6.8 acts as a zwitterion, and at an alkaline pH, it remains deprotonated, slightly negatively charged. The somewhat negative charge Cl- ion moves in front; thus, the protein is sandwiched between the Cl- ion and the positive glycinate ion. In resolving gel, the glycinate exists as negatively charged, thus losing the positive slowing charge and becoming the leading ion that causes the appearance of the bands after staining. When the external electric field is applied, protein migrates towards a positive electrode anode. The gel act as a sieve resulting in the separation of the protein. The minor protein travels a more considerable distance than the more significant protein. The gel is stained in the Coomassie dye at the end of the SDS PAGE electrophoresis. The amount of protein yield can also be estimated by looking at the band intensity. Samples were prepared using 1X loading dye and heating at 95° C for 3 minutes. After

centrifugation at 13000 r.p.m for 5 mins, samples were loaded, and the 12% gel was run in the 1X running buffer. The gel was stained and destained for the required period, and the gel was analyzed.

3.1.1.11 Thermal stability assay

E. coli asparaginase exhibits similar activity against its natural substrate L-asparagine and synthetic substrate hydroxamate (AHA). The assay is based on the reaction of hydroxylamine liberated from AHA with 8-hydroxyquinoline at high pH. The resulting green oxin dye has an absorption coefficient of about $1.75*10^4$ per mol per cm at 705 nm, which is detectable with high sensitivity. Briefly, the enzyme dilutions were incubated at 30ºC for 30 mins at 600 rpm in a thermocycler to obtain optimum temperature. 1mM of AHA was added to each well of 96 healthy plates. The enzymes were set for 5 mins each at 55ºC,60ºC,65ºC, and 70ºC sequentially, and then 10 µl of enzymes were taken and added to the substrate in the plate at the interval of 1 min between the triplicates. The plate was kept for 30 mins allowing the enzyme and substrate to react.10 µl of 12% Trichloroacetic acid (TCA) was added to the enzyme to stop the reaction by decreasing the pH of the response. 200 µl of Oxin solution was added to each well. Absorbance was taken at 705 nm to check for enzyme stability.

3.1.1.12 Protein precipitation using Ammonium sulfate

After isolation, the protein was precipitated with 50% ammonium sulfate for 1 h at 4 °C followed by centrifugation at 16,000 r.p.m. for 15 min at 4 °C. The remaining supernatant was

brought to 95% saturation by further addition of ammonium sulphate and kept at 4 °C. Precipitated proteins were collected by centrifugation 16,000 r.p.m. for 15 min at 4 °C. The pellets were dissolved in 50mM Tris-HCl, pH 8.5 buffer and dialysis was performed using a 15 MWCO dialysis membrane to remove the ammonium sulfate contamination. The dialysed samples were stored for purification by mandatory Anion Exchange Chromatography followed by gel filtration chromatography.

3.1.1.13 Chromatographic Purification

The purification was performed using Anion-exchange chromatography. The same is performed using the AKTA-Pure machine. The column used for Anion exchange chromatography is Hi-trap Q-HP Sepharose + column from Cytiva (Marlborough, USA). It has positively charged resin that binds to charged ASNase and negatively elutes the protein. The buffers used were equilibration buffer (50mM Tris) and elution buffer (50mM Tris, 300mM NaCl). The column volume was 5 cv. The machine was set at method run, and the method was designed by putting 15 column volume (CV) and a flow rate of 0.5 ml/minute

Gel filtration Chromatography was also performed to get 100% pure protein. The column used for the gel filtration chromatography was Hi Prep 16/60 Sephacryl S-200 high resolution (Cytiva, Marlborough, USA). The total column volume was 120 ml. The flow rate was set at 0.5 cv/ min. Each elution was obtained in 2 ml of buffer.

3.1.1.14 Buffer-Exchange

The enzyme is present in Tris-HCl buffer till yet. The modification can be performed by exchanging Tris-HCl buffer for Phosphate buffer. This exchange was performed using Microcon-10 KDa centrifugal filter purchased from Millipore (New Jersey, USA). 500 µl of the protein was added and centrifuged at 11000 g for 30 minutes. The flow-through was collected, and the tube was inverted to contain the retentate concentrated enzyme. Phosphate buffer was added to change the buffer and centrifuged under the same conditions. This was repeated at least 6 times to completely exchange the buffer of the enzyme. The exchanged buffer and the filtrates were quantified using the Bradford assay.

3.1.1.15 Chemical modification

The buffer exchanged enzyme was used for the chemical modification. The molar ratio of 25:1 (PEG: Protein) was used for the modification. 2KDa mPEG-NHS esters were used for the modification. 500mM Phosphate buffer and Milli-Q was added along with buffer exchanged enzyme and mPEG molecules. The mixture was incubated at 30ºC at 300 rpm shaking for 1 hour, 2 hours, 3 hours, and so on till 6 hours. Modified protein is kept after each hour to optimize the yield of the modified protein. The mixture was then quenched using 30µM hydroxylamine solution. The modified enzyme was checked on 12% SDS-PAGE

3.1.1.16 Asparaginase Activity

The usage of commercially available L-asparagine has prepared the calibration requirements. The enzymes were diluted at a ratio of 1:100, and the diluted enzyme was added to 10 mM

of AHA. The mixture was incubated at 37ºC for a minute. Autoclave Milli-Q water was added to the reaction mixture. Oxin dye was used to obtain the green color. Absorbance was taken at 710 nm. The obtained readings were analyzed using my-curve fit software.

3.1.1.17 Enzyme Kinetics Assay

The enzyme kinetics study was based on the varying concentrations of substrate. Different concentrations of substrate, AHA, were prepared. The enzyme was incubated with each substrate for 30 minutes. The reaction was halted using 12% Tri-chloroacetic acid (TCA). Oxin dye was used to obtain the green color. The absorbance was recorded at 705 nm. The velocity of the reaction was calculated using the formula-:

$$Velocity = \frac{OD\ Difference}{Absorption\ coefficient\ of\ green\ oxindol\ dye\ \times\ time}$$

The absorption coefficient of green oxindole dye is $1.75 \times 10^9\ M^{-1}\ cm^{-1}$. The reciprocal was taken to obtain $\frac{1}{v}$ and $\frac{1}{s}$ Value. The graph was plotted for the reciprocal values, and the calculation of maximum reaction velocity (V_{max}), Michaelis constant (K_m), and turnover number (K_{cat}) were performed using the Lineweaver-Burk plot equation.

$$\frac{1}{V} = \frac{K_m + [S]}{V_{\max}[S]} = \frac{K_m}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}}$$

3.1.1.18 Ammonia Release Assay

L-Asparagine (Sigma Aldrich, New Jersey, USA) was used as the natural substrate dissolved in 50Mm Tris-HCl buffer. The diluted enzyme was added, and the mixture was incubated at 37ºC for 30 minutes. The reaction was stopped using 1.5 M TCA. The

mixture was centrifuged to remove the precipitated proteins. Nessler's reagent was added, and the mixture was incubated at room temperature for 30 minutes. Absorbance was taken at 480 nm, and calculations were performed.

3.1.1.19 Antigenicity Assay

The antigenicity of the modified enzyme vs. the unmodified enzyme was evaluated using an indirect enzyme-linked immune sorbent assay (ELISA). The initial enzyme concentration was 1µg/ml, and 100µl of each enzyme was loaded on the well. Overnight incubation was provided so that efficient coating could occur. Blocking with 1% BSA (Sigma Aldrich, New Jersey, USA) was done, and the same was kept for incubation for 8 hours at room temperature. Washing was performed using the washing buffer, 1X Phosphate-Buffered Saline, 0.1% Tween 20 Detergent (PBST). Primary anti-asparaginase antibody was added and left overnight. Washing was performed using PBST and secondary antibody was added and kept for 2 hours at room temperature. Excess antibody binding was removed by washing. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added and kept in dark. Reaction was stopped using sulphuric acid and absorbance was taken at 450 nm.

4. Chapter 4

4.1. Results

4.1.1 Plasmid isolation and restriction digestion of wild-type ASNase and Mutant-A

The plasmids were isolated from *E. coli*-pET-28a-DH5α using Bio-helix mini-prep plasmid isolation kit. The plasmids were isolated from transforming the plasmid of *E. coli*-pET-28a-DH5α in the expression vector *E. coli* BL21(DE3) strain. Spectrophotometric analysis shows that the plasmids isolated were of high-grade purity, and the concentration obtained was also satisfactory **(Table 4.1)**. Hence it can be used to perform further transformation experiments. Agarose gel electrophoresis was also performed to confirm the presence of plasmid qualitatively **(Figure 4.1).** Since the band for the desired plasmid is not observed in the expected range, restriction digestion has been performed. The restriction digestion confirmed the insert size of 1.5 Kbp **(Figure 4.2)**

Table 4.1: The purity and concentration of isolated plasmids

S.No.	Plasmid	Absorbance (260/280)	Concentration (ng/µl)
1.	Wild-type ASNase	1.79	132.1
2.	Mutant-A ASNase	1.81	94.9

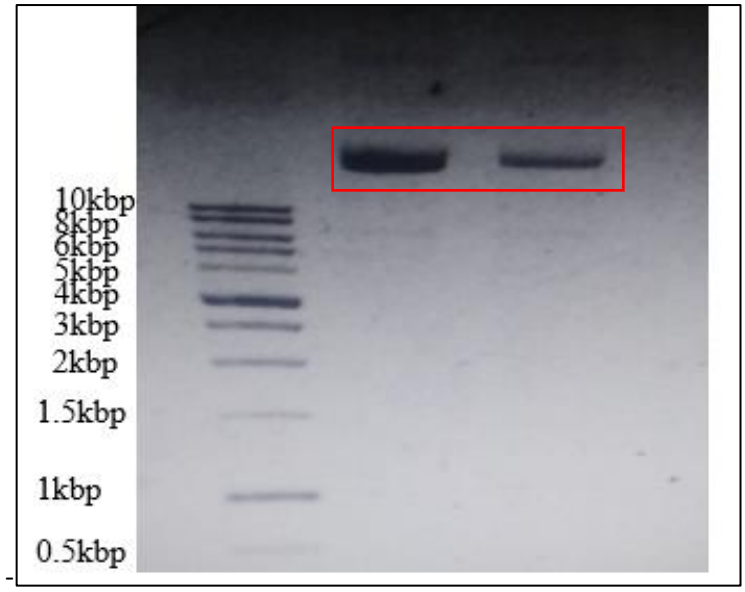


Figure 4.1: Agarose gel electrophoresis of isolated plasmids **lane 1**: ladder, **lane 2:** Mutant-A plasmid, lane 3: Wild-type plasmid

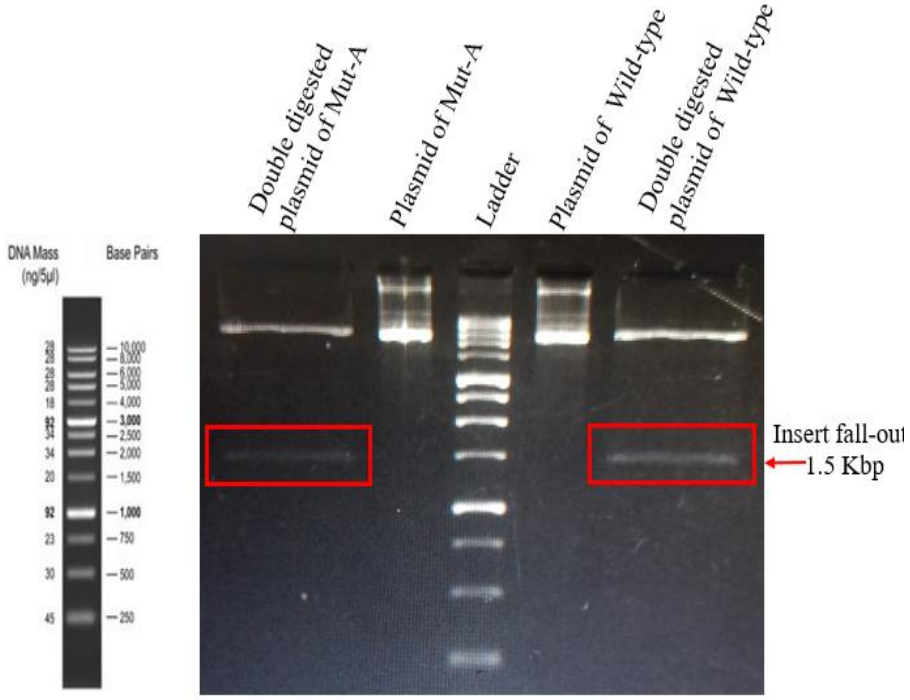


Figure 4.2: Restriction digestion of the isolated plasmids

4.1.2 Transformation of Mutant-A-pET-28a-DH5α in *E.coli* BL21 (DE3)

The isolated plasmids were incorporated in *E.coli* BL21(DE3) competent cells, spread on antibiotic-containing plates, and incubated overnight at 37ºC. The colonies were observed (**Figure 4.2** and **Figure 4.3)**. Single colonies were marked and used for the other protein isolation process. Both wild type and mutant-A have a good number of single transformed colonies. Obtaining single transparent colonies on antibiotic selection plate confirms successful transformation of the *E. coli*-pET-28a plasmid.

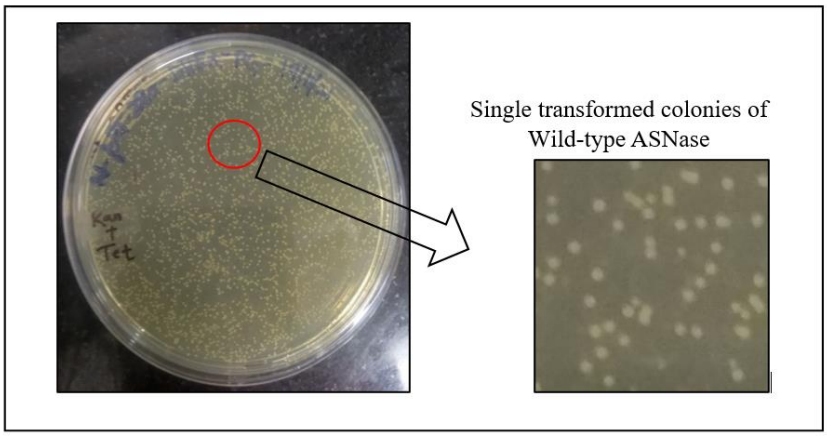


Figure 4.3: Transformed colonies of wild-type E. coli pET-28a BL21(DE3) construct. Single colonies are marked in red.

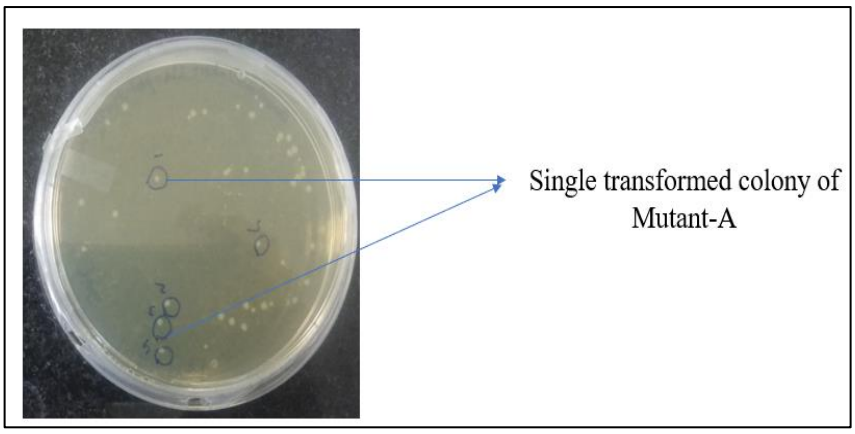
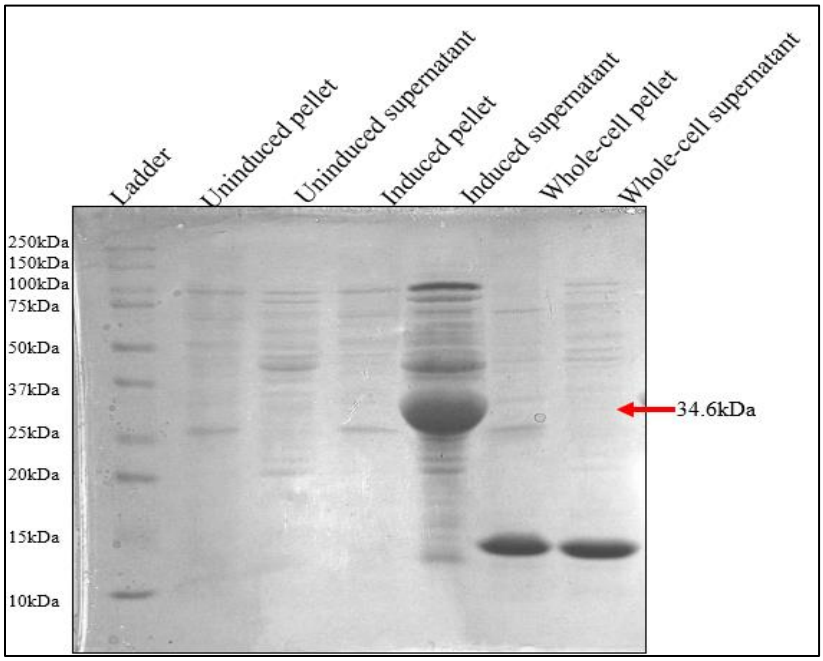


Figure 4.4: Transformed colonies of mutant-A E. coli pET-28a BL21(DE3). Single colonies are marked in blue.

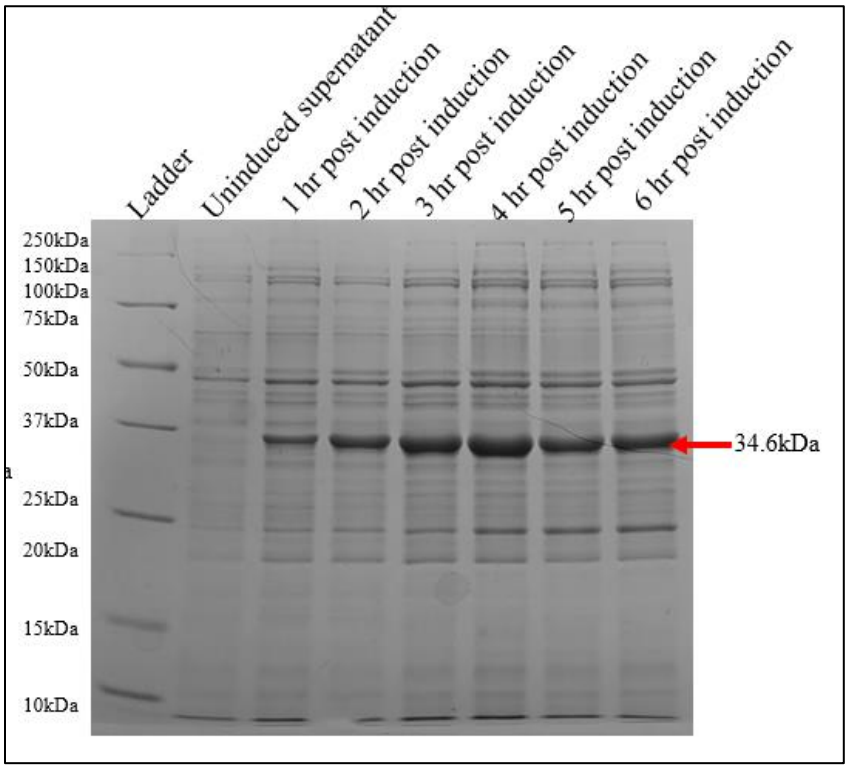
4.1.3 Isolation of Recombinant protein

The transformed single colonies of Mutant-A and wild-type were used to isolate the recombinant protein. 0.5mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) induction was provided in *E.coli* BL21(DE3) and protein was isolated using osmotic shock method. **Figure 4.5a** and **Figure 4.6a** shows that there is the presence of protein of interest in the supernatant pool after the induction for the wild-type and mutant-A respectively. The induction was checked for subsequent 4 hours to optimize the better yield conditions for the protein. **Figure 4.5b** and shows that there is a high expression of wild-type after 4 hours of induction by IPTG. The single transformed colonies were selected on random from the transformed plates of both wild-type and mutant-A. **Figure 4.5c** indicates there is a high expression of wild-type enzyme from the colony-1 taken from the plate while **Figure 4.6c** indicated that all the six colonies selected yielded good amount of ASNase. However, further characterization was performed to select the final colonies for protein isolation for further experiments.

a.


Ladder
Uninduced pellet
Uninduced supernatant
Induced pellet
Induced supernatant
Whole-cell pellet
Whole-cell supernatant
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
10kDa
34.6kDa

b.


Ladder
Uninduced supernatant
1 hr post induction
2 hr post induction
3 hr post induction
4 hr post induction
5 hr post induction
6 hr post induction
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
10kDa
34.6kDa

c.

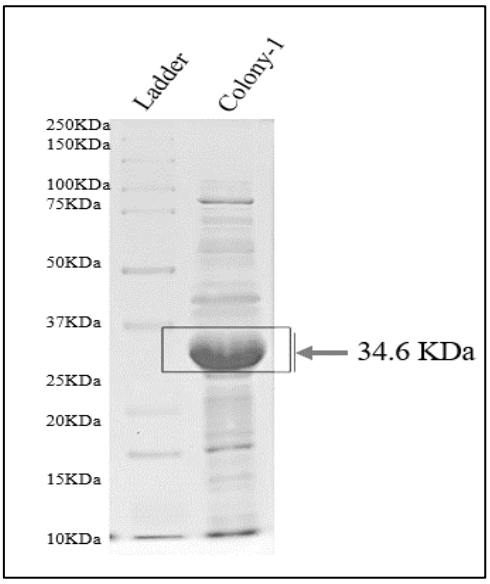
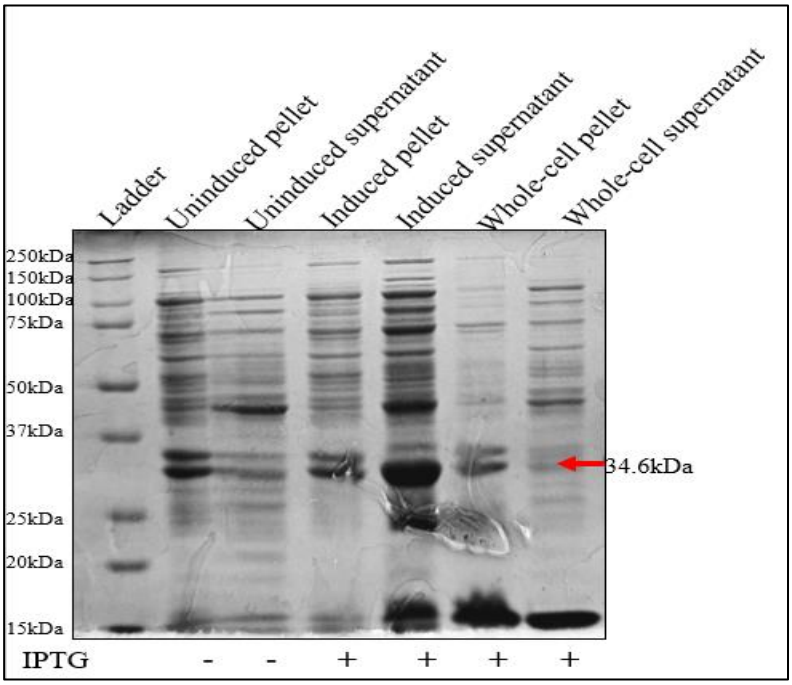


Figure 4.5: 12% Gel Images (a) Induction of wild-type ASNase (b) Induction of wild-type after 1-hour, 2-hour, 3 hours and 4 hours (c) Wild-type ASNase isolated from colony 1

a.



b.

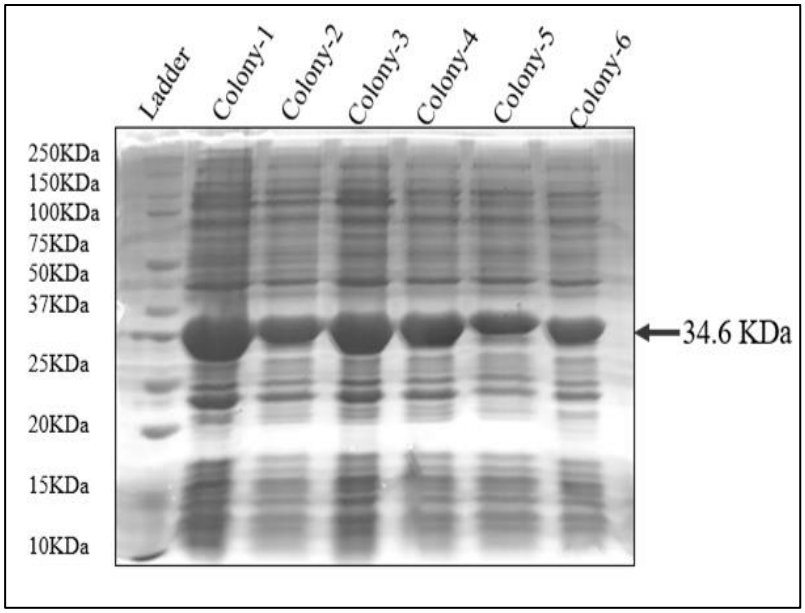


Figure 4.6: 12% Gel Images (a) Induction of mutant-A ASNase (b) Mutant-A ASNase isolated from colony-1,2,3,4,5,6 respectively

4.1.4 Thermal Stability Profile for isolated enzymes

Thermal stability profile was checked for the isolated wild-type and mutant-A colonies. Each colony of mutant-A was tested for its thermal stability. It was also found that mutant-A is significantly more stable than wild-type at higher temperatures. This shows that the conformation of the mutant-A was intact at high temperatures while the wild-type has shown a considerable decrease in the thermal stability. **Figure 4.7** shows that mutant-A, colony-1 was highly thermally stable. Hence, Mutant-A Colony-1 was selected for further experimental procedures.

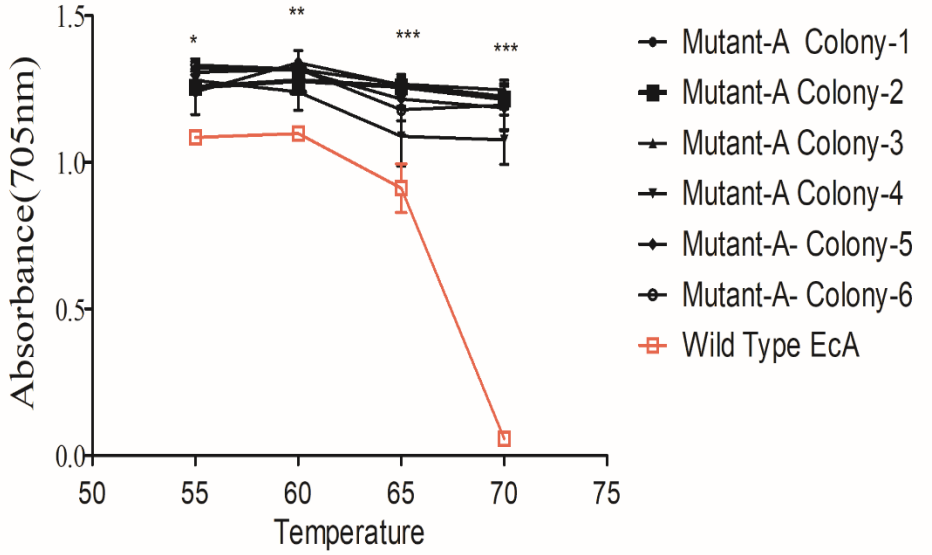


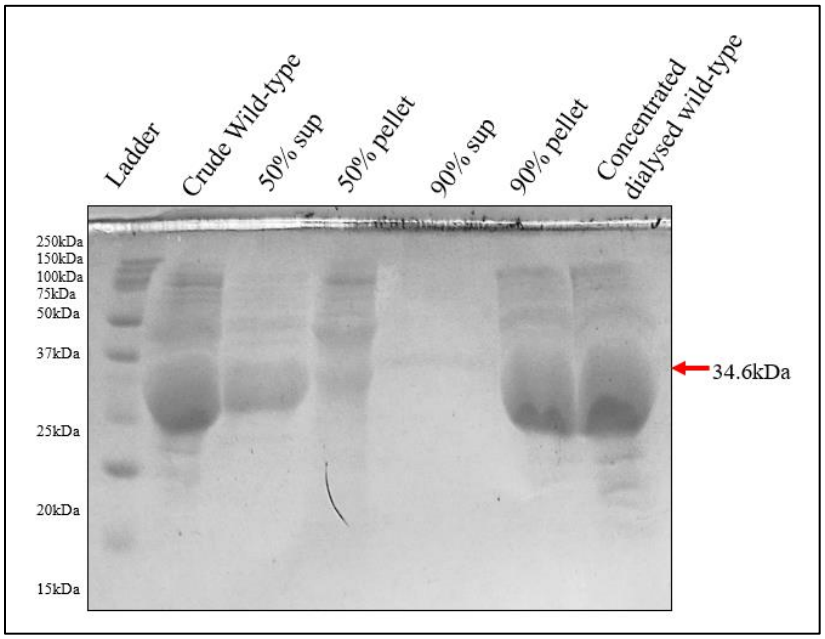
Figure 4.7: Thermal stability profile of crude wild-type and mutant-A ASNase. Colony-1 of Mutant-A was selected as a best fit due to its high thermal stability.

4.1.5 Purification of wild-type and mutant-A ASNase

The crude asparaginase is contaminated with other periplasmic proteins. Of note, amalgamation of asparaginase by both glutaminase and urease give rise to many unwanted side-effects clinically. It is reported in many literatures about the low bioavailability of commercial generic asparaginases and their low clinical effectivity. Therefore, asparaginase with high purity is desirable and this can be achieved by multi-step purification process. Here, the isolated crude wild-type and mutant-A enzymes were precipitated **(Figure 4.8a** and **Figure 4.8b),** dialysed and then purified with anion-exchange chromatography using the Hi-trap Q-Sepharose strong anion exchange column in AKTA Pure machine. The elution fraction, which showed peaks were collected. The chromatogram was obtained for both wild-type and

mutant-A for anion exchange chromatography (**Figure 4.9a** and **Figure 4.11a**), and the 12% SDS PAGE was run to check for the yield (**Figure 4.9b** and **Figure 4.11b**). Since, the gel image of wild-type enzyme does not confirms very high purity of the enzyme, we had performed gel filtration chromatography later. The chromatogram was obtained showing a single peak confirming the purity of the enzyme (**Figure 4.10a**). The eluted fractions from size exclusion chromatography were run on 12% SDS-PAGE. The gel image also confirms that the wild-type enzyme had been completely purified (**Figure 4.10b**).

a.



b.

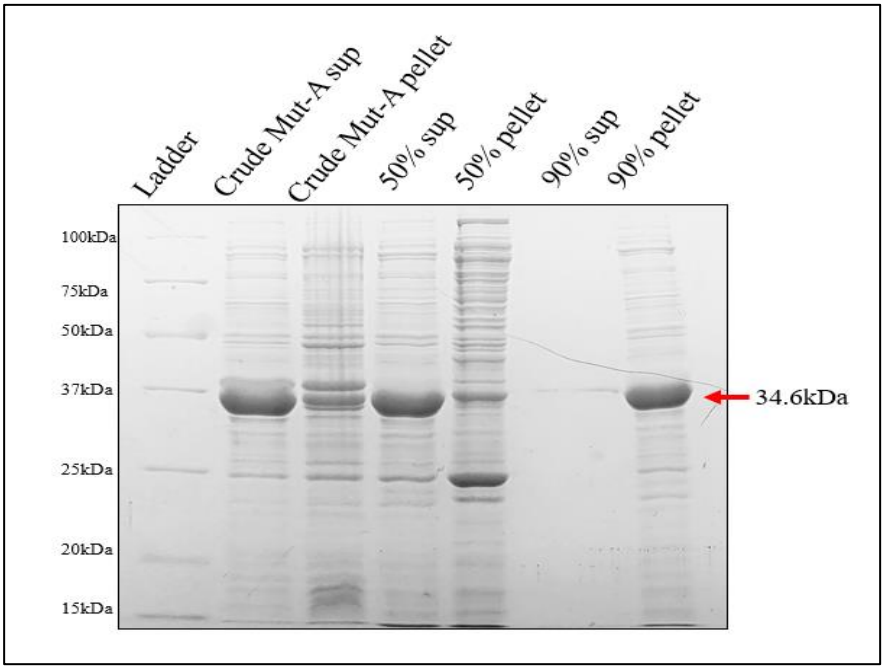


Figure 4.8: (a) SDS-PAGE analysis of wild-type EcA purification by ammonium sulphate. (b) SDS-PAGE analysis of mutant-A ASNase purification by ammonium sulphate.

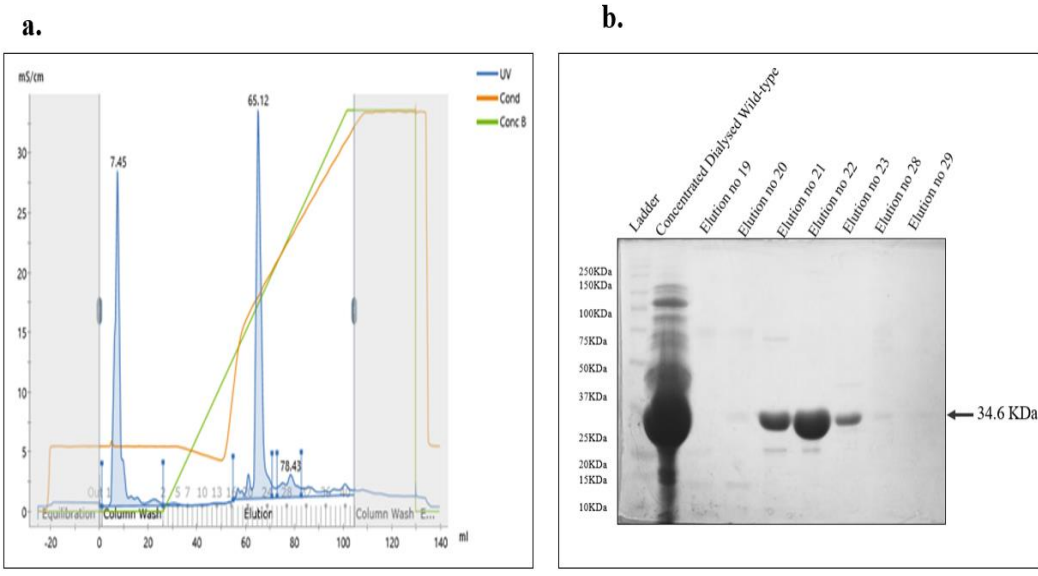


Figure 4.9: Anion exchange chromatography of wild-type ASNase (a) Chromatogram obtained for wild-type anion-exchange

chromatography. Elution fractions are shown. (b) 12% Gel image of the eluted fractions showing about 80% purified ASNase

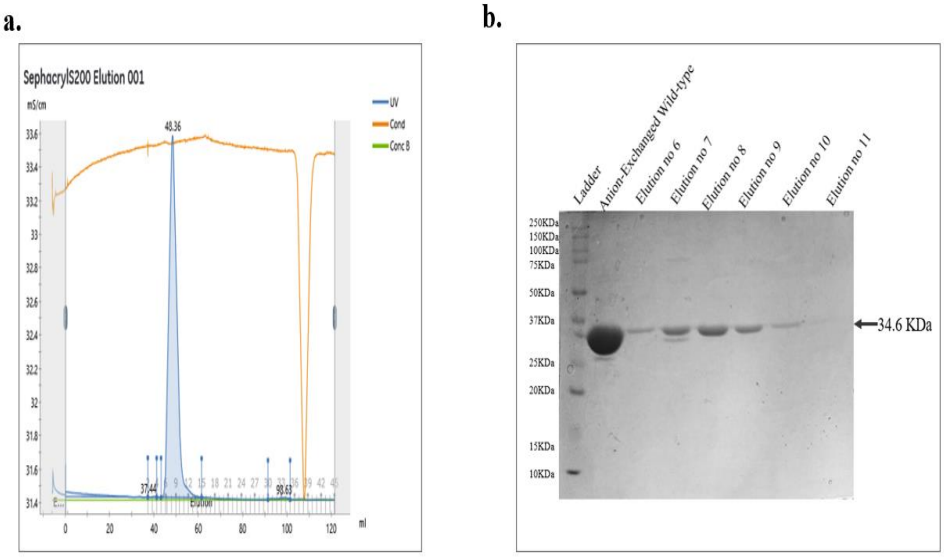


Figure 4.10: Size exclusion chromatography of wild-type ASNase (a) Chromatogram obtained for wild-type size-exclusion chromatography. Elution fractions are shown. (b) 12% Gel image of the eluted fractions showing 100% purified ASNase

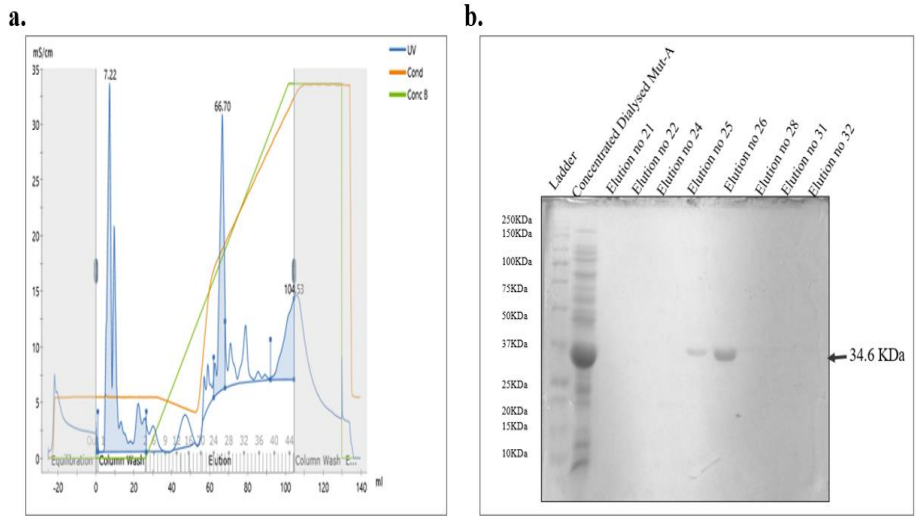


Figure 4.11: Anion exchange chromatography of Mutant-A ASNase (a) Chromatogram obtained for wild-type anion-exchange chromatography. Elution fractions are shown. (b) 12%

Gel image of the eluted fractions showing about 100% purified mutant-A ASNase

4.1.6 Chemical Modification

Chemical modification was performed using 2 KDa mPEG-NHS esters. The molar ratio of PEG: Protein was fixed at 25:1. It was observed that the PEG molecule binds to the protein present in the mixture. The modification was standardized and checked for every hour upto 6 hours. However, no significant difference was observed in the rate of modification even after 5 hours. Both wild-type and mutant-A were modified (**Figure 4.12** and **Figure 4.13**). But, the yield of the modified protein was not much. It has to be standardized further to increase the yield of the modified protein.

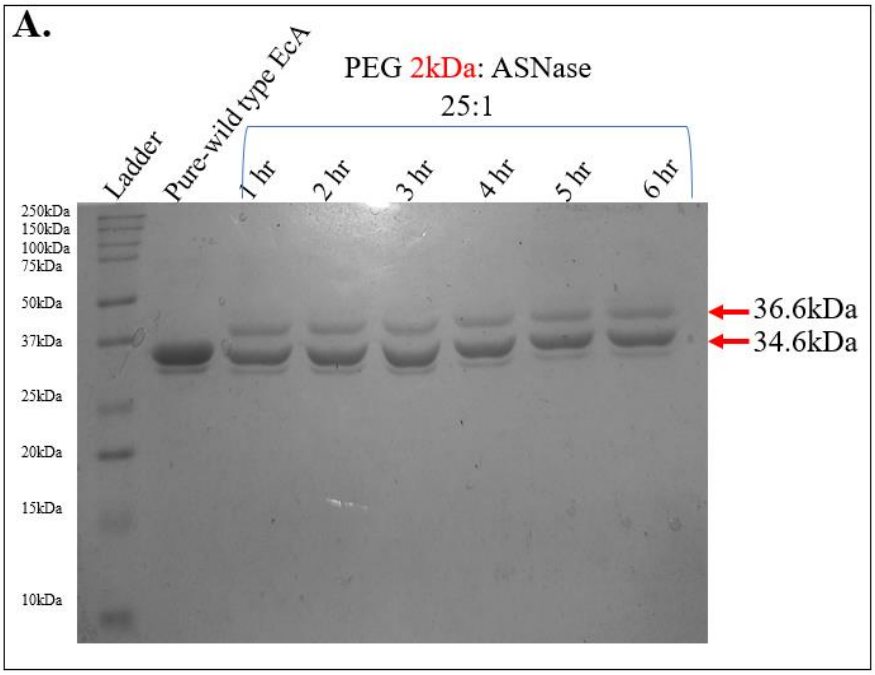
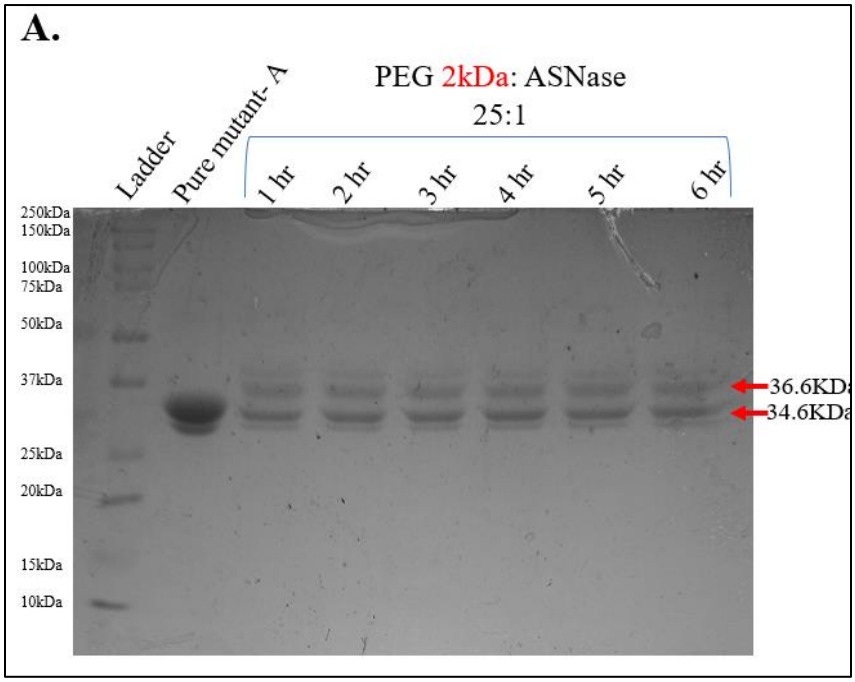


Figure 4.12: A. Time-dependent chemical modification of wild-type ASNase using mPEG NHS ester-2kDa



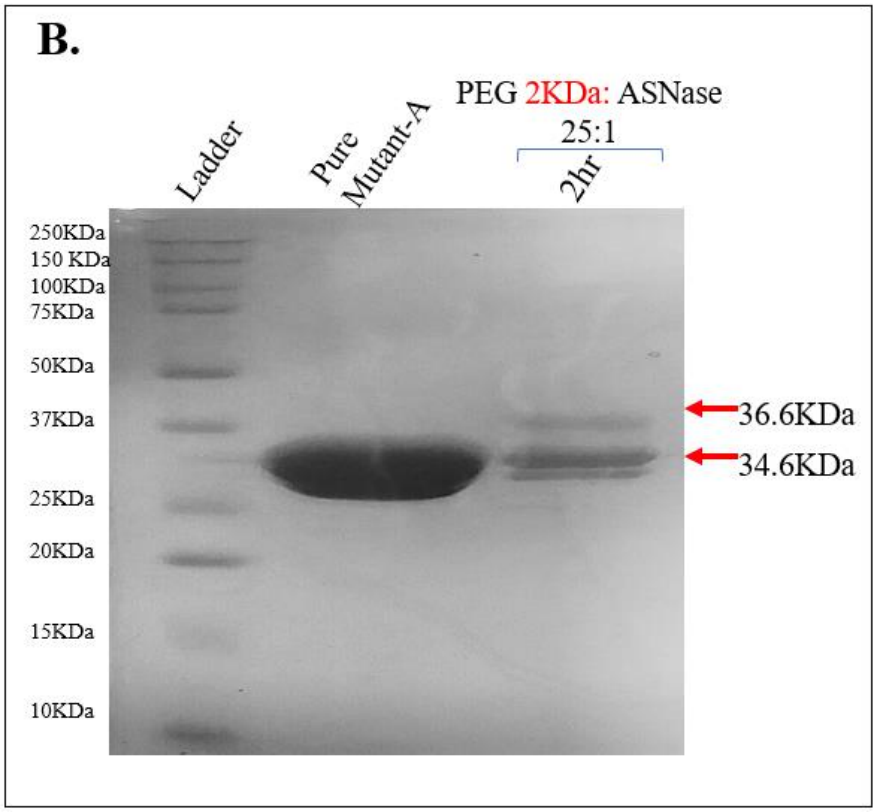


Figure 4.13: A. Time-dependent chemical modification of Mutant-A ASNase using mPEG NHS ester-2KDa **B.** Chemical modification of Mutant-A ASNase using mPEG NHS ester-2KDa

4.1.7 Thermal stability profile of modified crude enzyme

The thermal stability profile of modified crude enzyme was tested at different temperatures. 2kD PEG Mutant-A have shown significant thermal stability than the wild EcA at higher temperatures **(Figure 4.14).** This shows that the modified Mutant-A is much more stable and would not get degraded at even higher temperatures as compared to wild-type. This could result in improved pharmaco-kinetic properties of the enzyme. The significance was calculated using Two-way ANOVA in GraphPad Prism 5 software.

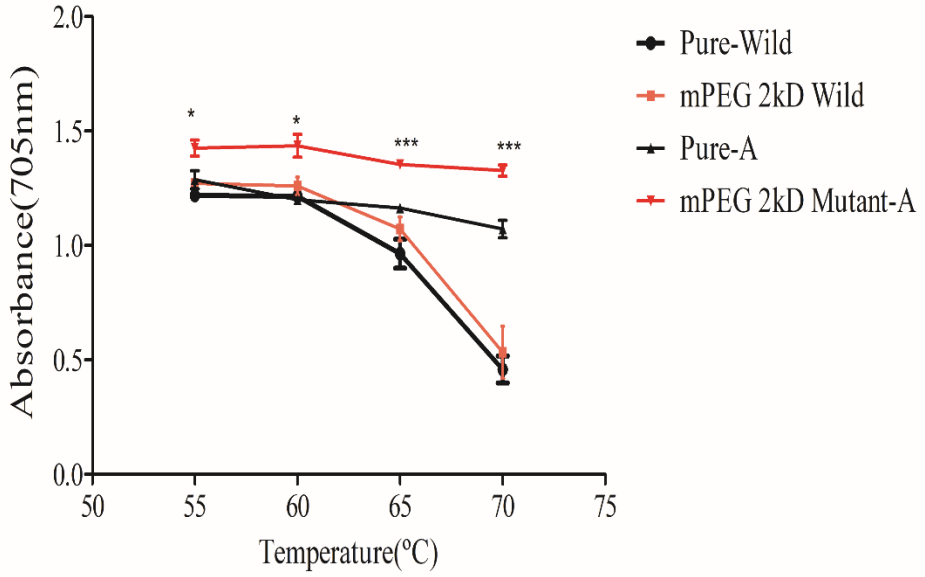
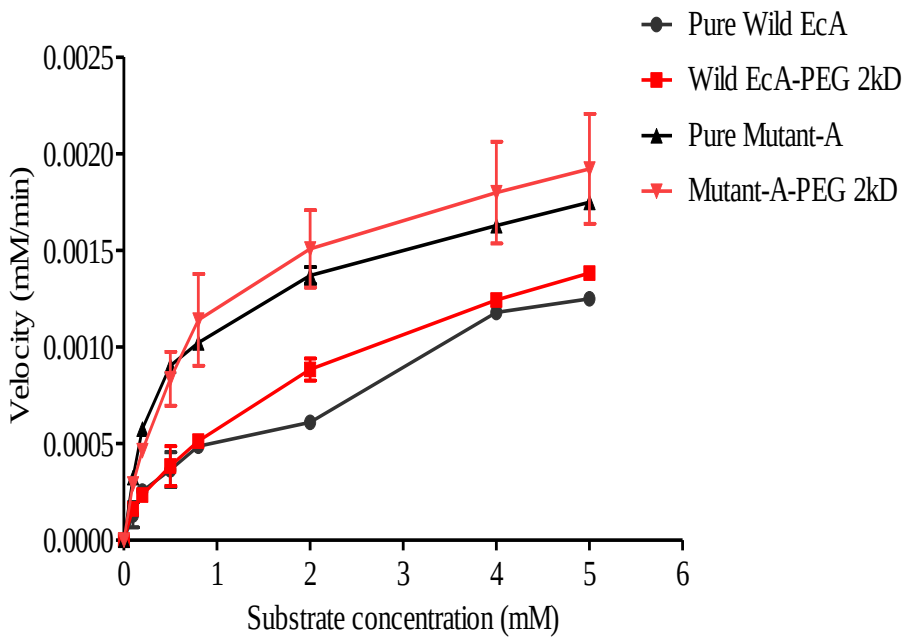


Figure 4.14: Thermal stability profile for mPEG 2kD Wild and mPEG 2kD Mutant-A v/s Pure wild and Pure A. This experiment was performed in biological replicate. Mean ± SEM are shown here. * Significance of the experiment were calculated by Dunnett's multiple comparison test of one-way ANOVA P value<0.05 using GraphPad Prism 5 software.

4.1.8 Enzyme kinetics study of modified enzyme

The kinetic parameters of an enzyme are essential to predict the enzyme activity. The enzyme kinetics were studied using the synthetic substrate AHA of ASNase. This study was carried out at physiological temperature (37 ºC) and 62 ºC. It was observed that mPEG 2kD Mutant-A retained its native asparaginase activity at both 37 ºC and 62 ºC. A curve was plotted against velocity and substrate concentration for both the temperatures. **(Figure 4.15a and 4.15 b)**. This curve was used to obtain Lineweaver burk plot. With the help of the Lineweaver-burk plot all other kinetic parameters were obtained **(Table 4.2 and Table 4.3)**. % Kcat was calculated and a bar plot was obtained. **(Figure 4.16 a** and **Figure 4.16 b)**

a.



b.

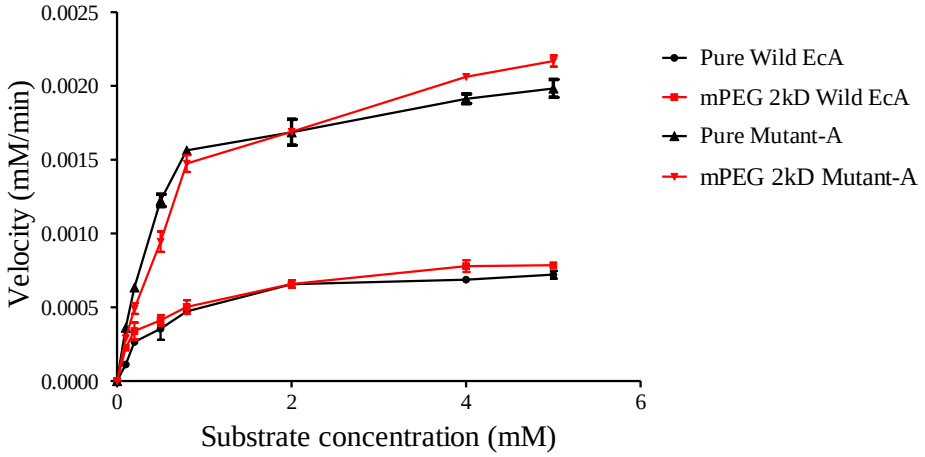


Figure 4.15: a. Velocity v/s substrate concentration plot for each enzyme at 37 ºC **b.** Velocity v/s substrate concentration plot for each enzyme at 62 ºC

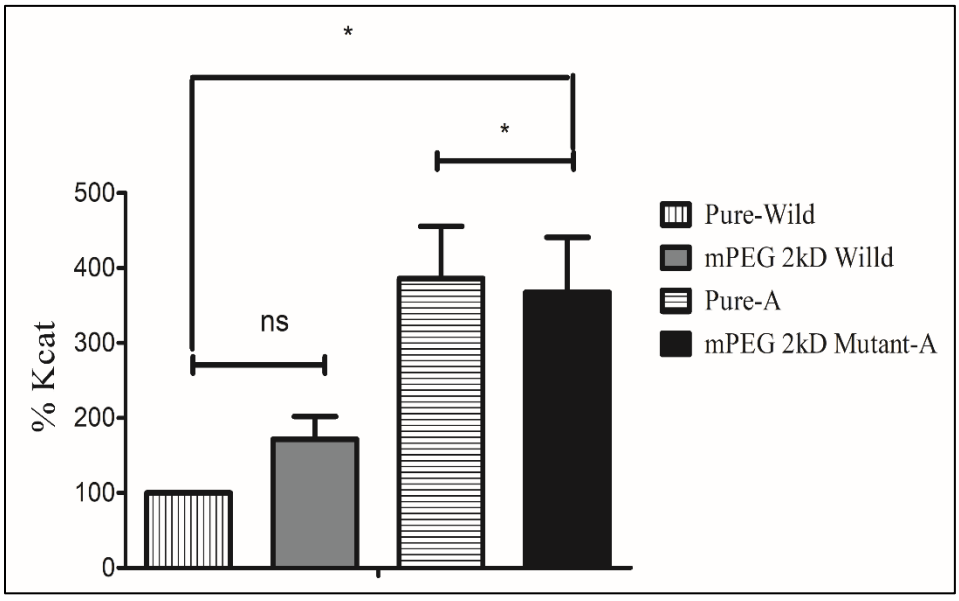
Table 4.2: Table showing all the kinetic parameters for each enzyme at 37 ºC

Enzyme	**K_m/V_{max}**	**$1/V_{max}$**	**V_{max}**	**K_m**	**K_{cat}**	**$\%K_{cat}$**
Pure-Wild	878.075	769.05	0.003715	2.083374	0.001532	99.99999
mPEG 2kD Wild	548.8	1045.075	0.001951	0.540868	0.001832	154.8021
Pure-A	247.225	581.785	0.003439	0.425709	0.004058	346.0995
mPEG 2kD Mutant-A	272.62	686.645	0.002915	0.397148	0.003669	325.2544

Table 4.3: Table showing all the kinetic parameters for each enzyme at 62 ºC

Enzyme	**K_m/V_{max}**	**$1/V_{max}$**	**V_{max}**	**K_m**	**K_{cat}**	**$\%K_{cat}$**
Pure-Wild	732.94	1154.565	0.000883	0.654754	0.001374	99.99999
mPEG 2kD Wild	311.125	1432.3	0.000702	0.217488	0.003223	237.2954
Pure-A	231.065	425.405	0.002353	0.543459	0.004329	317.6525
mPEG 2kD Mutant-A	308.085	408.35	0.002451	0.756379	0.003253	237.5073

a.



b.

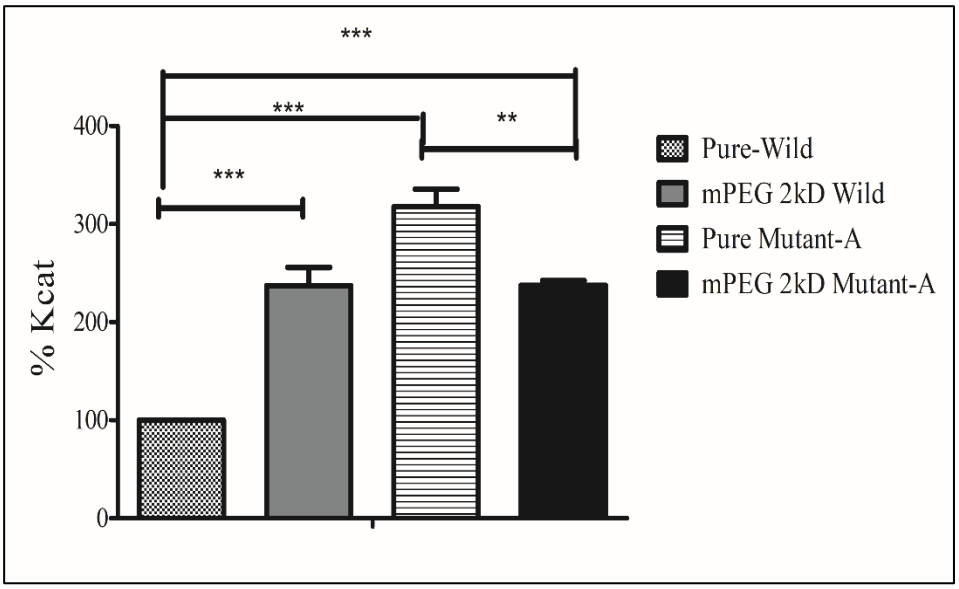


Figure 4.16: **a.** % Kcat plotted for each enzyme at 37ºC **b.** % K_{cat} plotted for each enzyme at 62ºC

4.1.9 Ammonia release assay of modified enzyme

Ammonia release assay was performed to check the activity of the modified crude enzyme towards the natural substrate and to compare its activity with the wild-type. It was observed that mPEG 2kD Mutant-A retained its native asparaginase activity **(Figure 4.17)**. This shows that the modified enzyme is comparable to the wild-type in degrading asparagine. The absorbance was detected at 480nm when nessler's reagent was added to the mixture solution **(Figure 4.18).**

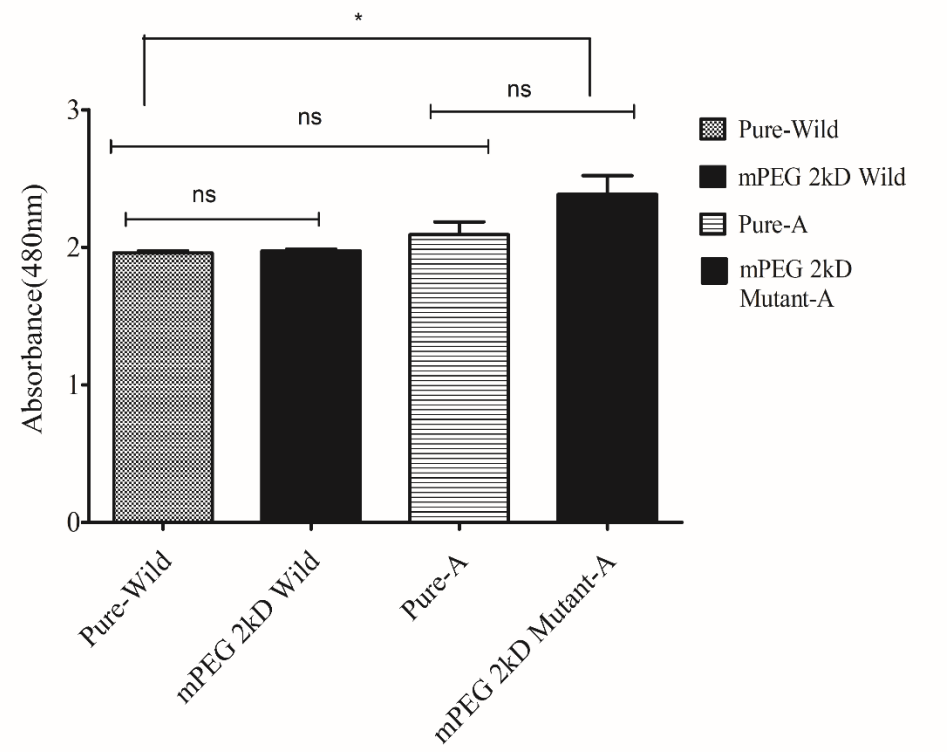


Figure 4.17: Asparaginase activity of wild-type EcA, mutant-A and mPEG 2kD wild and mutant-A at 37 ℃ using natural substrate.

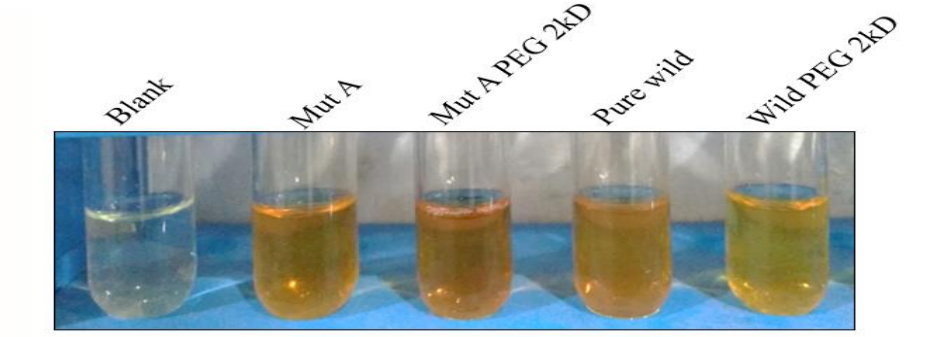


Figure 4.18: Experimental image showing change in colour after adding Nessler's reagent.

4.1.10 Asparaginase assay of modified enzyme

Asparaginase assay was performed to assess the ability of the enzyme to degrade its synthetic substrate AHA. The standards were prepared using commercially available L-asparagine using my-curve fit

software. The four parametric logistic curve was used to plot the graph and obtain the standard values **(Figure 4.19)**. These values were used to calculate the activity of the modified enzymes. It was observed that mPEG 2kD Mutant-A retained its native asparaginase activity **(Figure 4.20).** It co-relates with the results of Ammonia release assay. The enzyme activity is measured in International units per ml (IU/ml). One unit of activity is defined as the amount of enzyme that liberates 1.0 mol of NH_2OH from AHA per min at 37 °C. The absorbance was taken after the addition of oxin dye and obtained change in colour **(Figure 4.21)**. The final obtained units of enzyme activity are tabulated **(Table 4.4).** This shows that the modification with PEG does not hinder the enzyme activity.

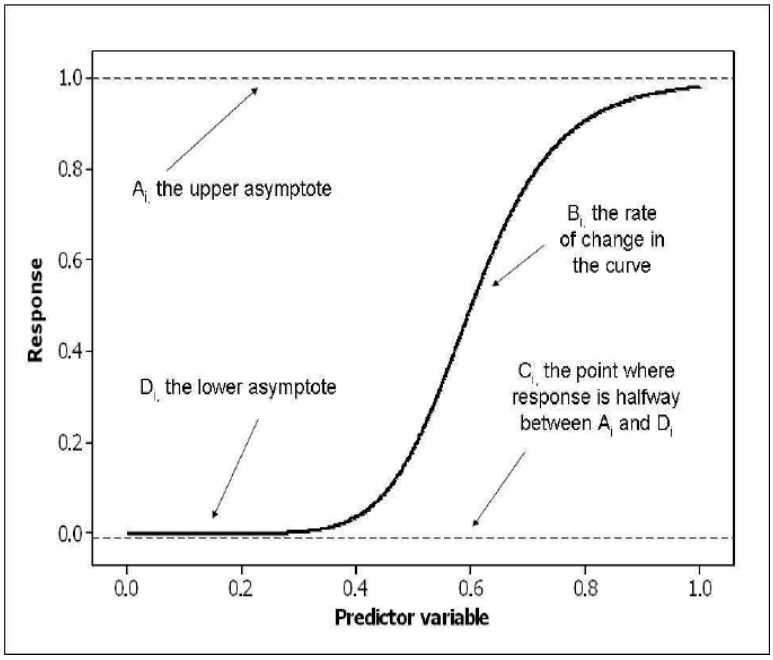


Figure 4.19: Four-parametric logistic curve used for analyzing the results.

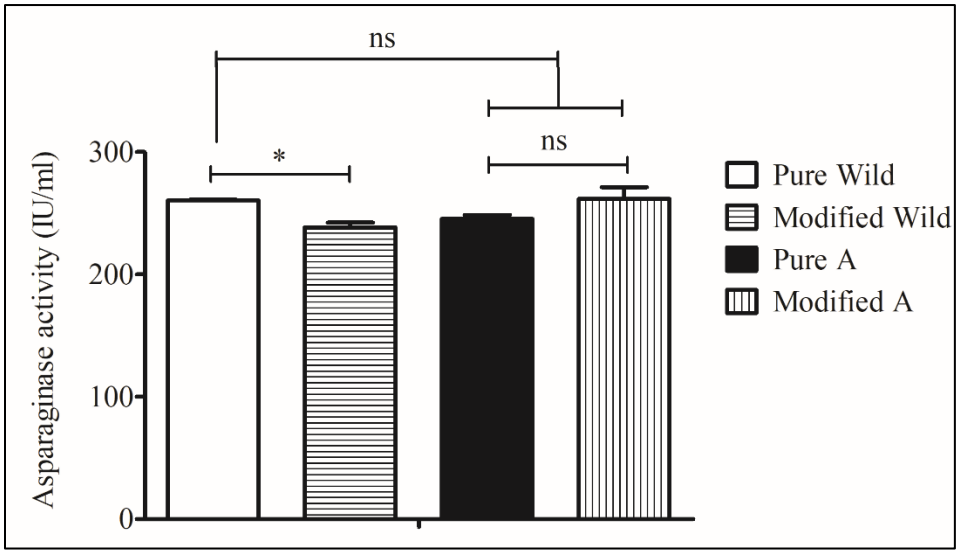


Figure 4.20: Asparaginase activity of wild-type EcA, mutant-A and mPEG 2kD wild and mutant-A at 37 ℃ using synthetic substrate.

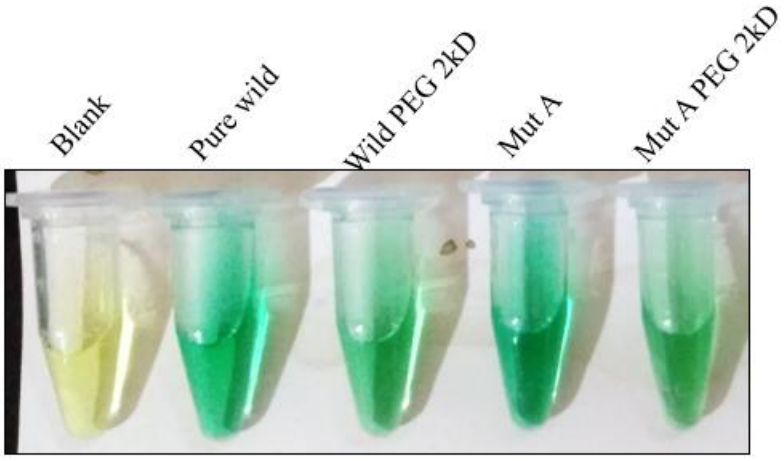


Figure 4.21: Experimental image showing change in colour after adding oxindole dye.

4.1.11 Antigenicity Assay by indirect ELISA

Indirect ELISA was performed to check the antigenicity of the modified enzymes. It was speculated that the PEGylation would decrease the antigenicity of the enzyme. The observed results are in co-relation with the expected results. The modified 2kD PEG Mutant-A has significantly lower antigenicity than the wild-type **(Figure 4.22)**. This

can be a promising result since, this can pave a way for future developments of the novel PEGylated drug to improve ASNase therapy.

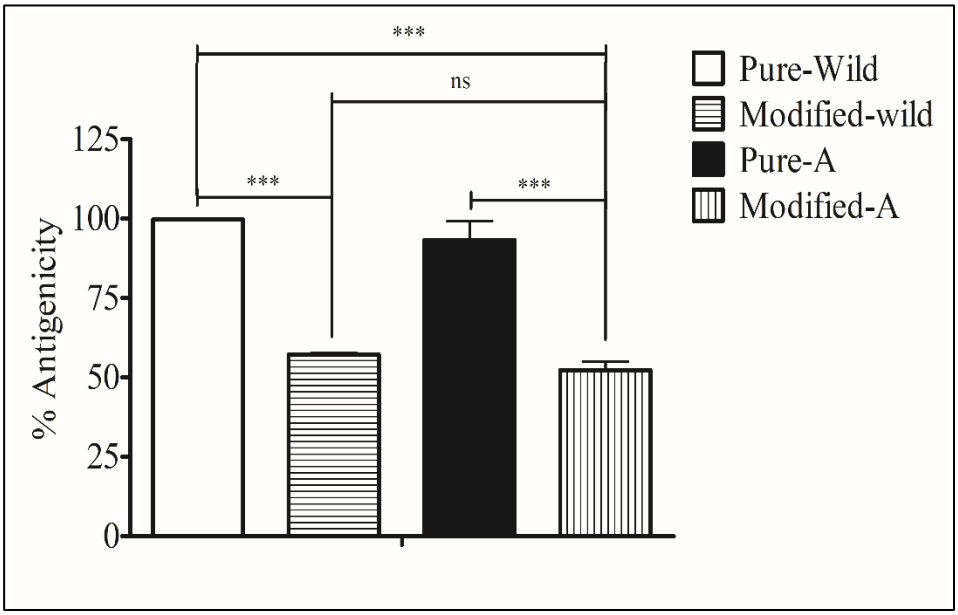


Figure 4.22: The in-vitro antigenicity of modified v/s unmodified ASNase's were determined by indirect ELISA.

5. Chapter 5

5.1 Discussion

E. coli ASNase is widely used for the treatment of primary and relapsed ALL. The native ASNase has shown promising outcomes in ALL patients. However, there are remissions of ALL. This is attributed to the fact that the neutralizing antibodies decrease the efficacy of ASNase [44]. The short half-life of ASNase can also be a significant factor for the relapse. Wild-type ASNase has been known to show various side-effects and they are a cause of concern for many who develop hypersensitive responses towards asparaginase therapy. Wild-type ASNase also show glutaminase activity which is responsible for various deleterious effects like nephrotoxicity and neurotoxicity [36]. Although, the EcA mutants which are already prepared have shown improved stability, activity, half-life, immunogenicity, efficacy, reduced silent inactivation and no toxicity *in vivo*, it is still susceptible to proteolytic degradation by serum proteases. To increase the half-life and reduce the enzyme dosage, it is imperative to modify the prepared mutants to obtain better - therapeutic efficiency. PEGylation maintains the stealth effect of the enzyme and does not interfere with sustained activity. PEGylated therapeutics have been known to increase the hydrodynamic volume, reduce proteolytic degradation, and reduce immunogenicity, resulting in increased solubility and increased stability and serum half-life [56]. It also aids in reduced kidney clearance, thereby enhancing the therapeutic efficacy of the enzyme [57]. PEGylation can be mono-PEGylation or poly PEGylation. Several researchers have done PEGylation to native E. coli ASNase and obtained desired results [58].

PEGylation can be N- terminal site-specific or can be random. During our course of studies, *E. coli* asparaginase variants will be subjected to modification *via* thiol end of cysteine side chain. To be specific, we will explore the two Cys present in *E. coli* asparaginase to stall the PEG groups. First, the Cys-Cys disulphide bond will be reduced and then, the disulphide bond will again be formed with the help of a linker molecule that will bear our desired PEG group. The whole process is expected to minimally disturb the tertiary structure of asparaginase and hence, retain the activity. We will be performing several *in vitro* enzyme assays to confirm the desired results for increased stability and therapeutic efficacy. According to the literature, it is anticipated that the PEGylation at specific sites will result in lesser side effects due to decreased hypersensitivity. It is also expected that the modified enzyme will be more thermally stable and can be used in countries with a high temperature goes high up to 65ºC. These altered enzymes have the potential to open up a whole new arena for developing ALL chemotherapies and increasing ALL patient's survival rates in near future.

5.2 Conclusions

In conclusion, we have chemically modified the pure L-ASNase wild type and mutant that were prepared in our lab previously. It has been proved that chemical modification of enzymes improves its therapeutic efficacy, increase stability and half-life in blood. In this work, we performed chemical modification of this novel EcA variant by random chemical modifications such as PEGylation, which are already known to increase its serum stability, half-life and reduce antigenicity using published protocols. Then we have compared its stability, activity properties with the unmodified and wild type asparaginase. We

believe that this application will lead to development of effective EcA based therapy with reduced side effects and increased half-life for the treatment of childhood ALL.

In the present ~~thesis~~ work, Mutant-A and wild-type ASNase has been isolated, purified and chemically modified using 2KDa mPEG-NHS ester molecule. It has been observed that chemically modified 2KDa ASNase shows better thermal stability, enhanced enzyme activity and is believed to also showed lesser side-effects.

Our research can pave a way towards development of chemically modified novel L-Asparaginase drugs show lesser side-effects. These loss of side-effect in the modified novel mutants have the potential to improve the overall treatment outcome and could be a benchmark in asparaginase therapy for treatment of ALL. Thus, it can be a boon to the patients suffering from ALL.

5.3 Future Prospects

We already have the crude modified wild type and mutant-A ASNase. First and the foremost, we have to standardize the separation of modified and unmodified enzyme from the crude modified enzyme. Later on, *in-vitro* characterization of the modified enzyme can be performed on different ALL cell lines to test its anti-leukemic properties. Furthermore, we will be checking its safety and efficacy in pre-clinical leukemic animal model. If the desired results are obtained the modified enzymes can be patented before starting clinical trials with our partner institutions.

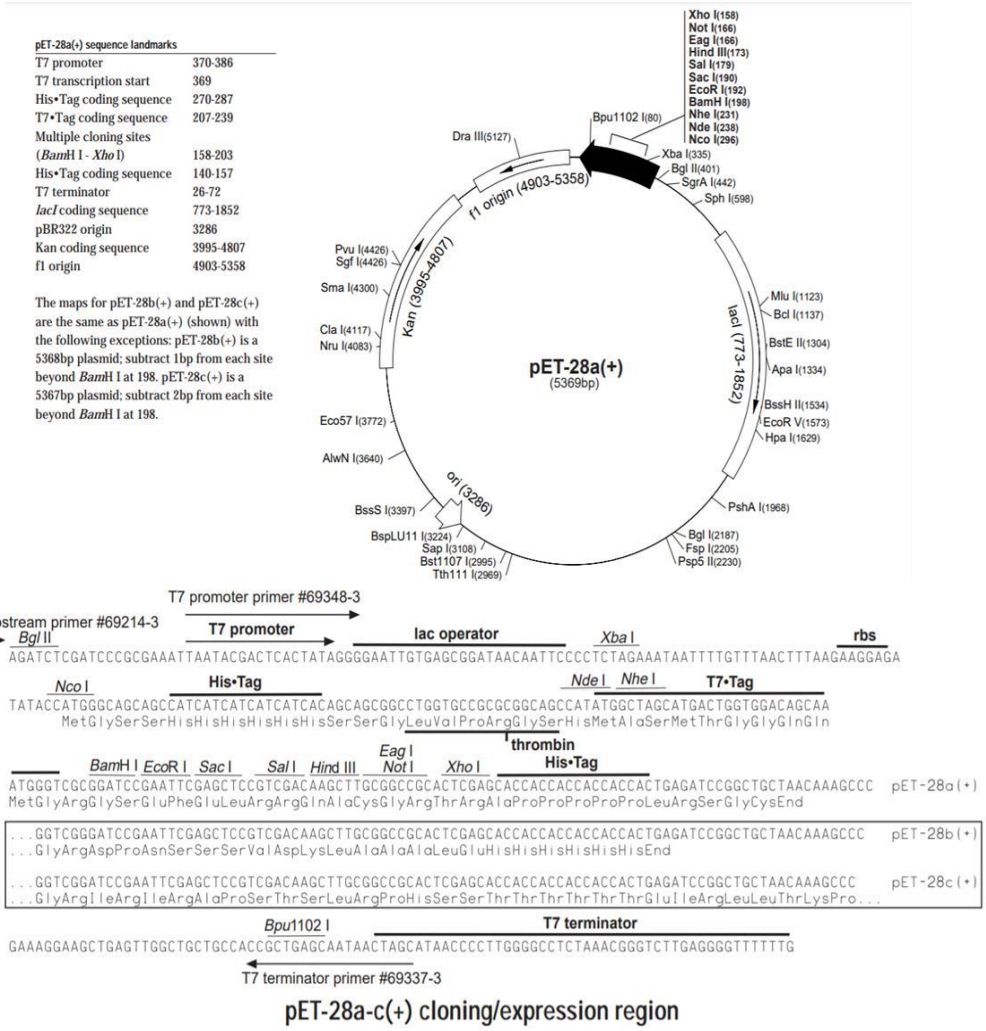
But for this process we need to have separated modified enzyme in bulk. The modification will also be performed using 5kD and 10kD PEG molecules and will be checked and compared with appropriate controls. Those modified with 5kD and 10kD can be further tested on ALL cell lines and patients lymphoblasts. If every results comes out

to be beneficial, then we can put it for clinical trials. We hope to develop an indigenous novel modified asparaginase mutant that is affordable and improves the overall treatment outcome and survival.

6. Chapter 6

6.1 Annexure

6.1.1 Plasmid map of pET-28a



7. Chapter 7

7.1 References

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