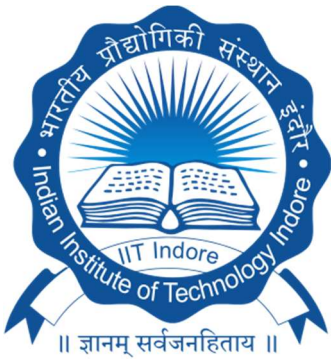
Characterization, Upscaling and Formulation Development of a Recombinant, Engineered Variant of the Anti-leukemic Chemotherapeutic Drug L-Asparaginase

M.Sc. Thesis

By

BIDISHA CHOUDHURY



DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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Characterization, Upscaling and Formulation Development of a Recombinant, Engineered Variant of Anti-leukemic Chemotherapeutic Drug L-Asparaginase

A THESIS

Submitted in partial fulfillment of the
requirements for the award of the degree
of
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by
BIDISHA CHOUDHURY

DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY INDORE
MAY, 2024

INDIAN INSTITUTE OF TECHNOLOGY INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Characterization, Upscaling and Formulation Development of a Recombinant, Engineered Variant of Anti-leukemic Chemotherapeutic drug L-Asparaginase** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE 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 2023 to May 2024 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.

Bidisha Choudhury
24/05/24.

Signature of the student with the date
(Bidisha Choudhury)

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

24.05.2024
Signature of the Supervisor of
M.Sc. thesis (with date)
(Prof. Avinash Sonawane)

Bidisha Choudhury has successfully given his/her M.Sc. Oral Examination was held on 9th May **2024**.

Signature(s) of Supervisor(s) of MSc thesis
Date: 24.05.2024

Convener, DPGC
Date:

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DEDICATION

To everyone I acknowledged

Abstract

According to a SEER (2015) study, 31% of childhood cancers are due to leukemia. One of its subtypes, Acute Lymphoblastic Leukemia, is known to comprise 80-90% of leukemia cases. Of all the therapies available for such patients, chemotherapy has been the most common choice by clinicians. One of the biologics, L-asparaginase has been consistently an important component of the chemotherapeutic regimen administered to such patients. To address some of the limitations/side effects associated with the administration of L-asparaginase, a recombinant enzyme from *E. coli* has been modified through rational protein engineering (Sonawane et.al.) that has shown promising results after in-vitro and in-vivo studies to minimize such side effects/ limitations. After multiple screening, four mutants designated as Mutants A, B, C and D have been evaluated for their therapeutic efficacy at the clinical set-up.

The aim of this thesis is to outline some of the steps in the tedious, lengthy and commercially expensive process of bio similar drug development done industrially. The recombinant, engineered variant of L-asparaginase (Mutant C) selected for the purpose of biosimilar development has the potential to become the first of its kind for the alleviation of ALL. Given this motivation, this thesis has outlined three major steps that need to be developed so that our recombinant variant of L-asparaginase qualifies for clinical trial phase I: Upscaling of fermentation for large-scale production of the enzyme, development of suitable intravenous/ intramuscular formulation and characterization (CQA profiling) of the purified drug.

Upscaling of the fermentation of the *E.coli* BL21 (DE3) strain producing the recombinat engineered protein under constant volumetric oxygen transfer rates was studied. By this method, however, the specific growth rate during exponential feeding, upon scale up was found to be 0.10 h^{-1} less than the target growth rate. Therefore a model has been developed, proposed and discussed in the scope of the theis.

Characterization of the enzyme by biophysical and biochemical methods have been outlined. The first step of characterization involves attaining 99.99% purity of the enzyme extracted for the crude. This has been achieved and discussed in this work. Scope of furthur CQA assessments of the biologic may be undertaken therafter.

The formulations of the recombinant variant have been studied for their ability to effectively protect enzyme activity over a period of 90 days. These formulations are prepared to be capable of parenteral administration. Lyophilized formulations show no significant difference in activity in comparison to a selected Indian reference biosimilar. Lyophilization of the protein with the excipient has shown significant difference ($p<0.05$) percentage of activity recovery in comparison to that without the excipient.

LIST OF PUBLICATIONS

Bidisha Choudhury, Avinash Sonawane "Development of Native MS-aligned techniques: A Critical Assessment in the study of Native Proteomics" (Manuscript under preparation)

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ABBREVIATIONS

6-MP- 6- Methoxy purine

6-TG- 6- thioguanosine

AEC- Anion Exchange Chromatography

ALL- Acute Lymphoblastic Leukemia

Anthr- Anthracycline

ASIR- Age Standardized Incidence Rate

ASDR-Age Standardized Death Rate

ASNS-Asparagine Synthetase

CLL-Chronic Lymphocytic Leukemia

CNS- Central Nervous System

Conc.- Concentration

CPh- Cyclophosphamide

Cpz- Capizzi

CQA-Critical Quality Attributes

Cyt- Cytarabine

Dnr- Danourubicin

Dox- Doxorubicin

EGIL- European Group for the Immunological Characterisation of Leukemias

FAB- French-American-British

FDA- Food and Drug Administration

GLOBOCAN- Global Cancer Observatory

Glucx- Glucocorticoids

HCP- Host Contaminating Proteins

HIC- Hydrophobic Interaction Chromatography

HPLC- High performance (pressure) Liquid Chromatography

I.M.- Intramuscular

IPTG- Isopropyl-β-D-1 thiogalactopyranoside

IU- International Unit

I.V.-Intravenous

mAU- milli Absorbance Unit

mM- Milli molar

LB- Luria-Bertani

LBL-Lymphoblastic Lymphoma

O.D.- Optical Density

NEB- New England Bio Labs

NK-Natural Killer

SEC- Size Exclusion Chromatography

SEER- Surveillance, Epidemiology and End Results

TAE-Tris-base/Acetic Acid/EDTA

TES-Tris-base/EDTA/Sucrose

TFF-Tangential flow filtration

TKI-Tyrosine Kinase Inhibitors

Vcr- Vincristine

WCB- Working Cell Bank

NOMENCLATURE

μ_0: Maximum (Target) Specific growth rate in h^{-1}

μ: Specific growth rate in h^{-1}

F_0: Initial feeding rate in gh^{-1}

F_O': Initial corrected feed rate

$Y_{X/S}$: Yield biomass/substrate in gg^{-1}

$Y_{X/S,abs}$: Asymptotic value of the $Y_{X/S}$ in gg^{-1}

$Y_{X/Smax}$: Maximum yield biomass/ substrate. Reached at unlimited growth from a batch culture in gg^{-1}

F_t: Temporal feeding rate in gh^{-1}

F_t': Final temporal feeding rate in gh^{-1}

X_0: Biomass concentration after inoculation in gL^{-1}

V_0: Volume of cultivation after inoculation in L

m_s: Specific rate of substrate consumption for cell maintenance in $gg^{-1}h^{-1}$

t: Time

k_{La}: Volumetric coefficient of oxygen transfer in h^{-1}

OTR: Oxygen Transfer Rate

C_G^*: Saturation of Dissolved Oxygen

C_L: Instantaneous oxygen concentration

C_{L0}: Initial oxygen concentration

$t_{E:}$ Probe time lag

W_{in}: Substrate concentration in feeding media

Chapter 1

Background

1.1. Leukemia and its sub-type ALL

Leukemia is a type of cancer due to the malignancy of the hematopoietic cells [1]. It may occur as primary or secondary cancer. An interplay of genetic predisposition, carcinogen exposure, infections and lifestyle (occupation, diet) are known to increase the susceptibility of an individual to the disease as a primary cancer [2]. It is one of the most widely prevalent secondary cancers known in patients following radiotherapy and chemotherapy with alkylating agents, platinum compounds, and topoisomerase II agents [3], [4]. Clinically, leukemia is classified into: Chronic and Acute. Cytomorphology, immunophenotyping and immunogenetics are used to sub-classify leukemia for diagnostic, therapeutic and prognostic purposes [5]. Chronic leukemia is characterized by the malignancy of mature and differentiated hematopoietic cells, whereas, in acute leukemia there is replacement of bone marrow with abnormal immature and undifferentiated hematopoietic cells which progresses rapidly compared to the chronic form. The FAB system uses cytomorphology to distinguish Chronic and acute Leukemias: Chronic Lymphoblastic (leukemia cells are of lymphoid origin) and Chronic Myeloid (leukemia cells are of myeloid origin) and Acute Leukemia is Acute Lymphoblastic (immunophenotypic marker: terminal deoxynucleotidyl transferase TdT positive) and Acute Myeloid (immunophenotypic marker: CD13 and/ or CD33 positive) sub-types [6].

Table 1.1.1. A summary of the immunophenotypic markers expressed as a result of ALL-causing gene fusion/ aberrations [7].

Gene fusion/ aberrations	**Immunophenotypic markers**
MLL gene translocation	$CD19^{+}/CD20^{-}/CD34^{+}/TdT^{+}/ CD10^{-}/CD24^{-}$
t (1;19) E2A-PBX fusion genes	$CD19^{+}/CD20^{+}/CD34^{-}/ CD10^{+}$
t (12;21) TEL-AML I fusion genes	$CD10^{+}/CD20^{-}/CD24^{+}/CD34^{+}/TdT^{-}/CD19^{+}$
t (9;22) BCR-ABL fusion genes	$CD10^{+}/CD19^{+}/CD24^{+}/CD34^{+}/TdT^{+} /CD20^{-}$

ALL is a sub-type of acute leukemia, caused by the malignancy of the immature and abnormal lymphoid (B-cell and T-cell) cells called “blasts” [1]. Proliferation and accumulation of blasts into the bone marrow, blood and extramedullary body sites cause different symptoms in patients like anemia, thrombocytopenia, and neutropenia. Other symptoms like fatigue, easy or spontaneous bruising and/or bleeding, and infections are common in ALL patients. Patients with B-ALL show additional symptoms such as fever, night sweats, and unintentional weight loss, along with, lymphadenopathy, bone joint pain, and hepatosplenomegaly. Malignancies of leukemia blast cells into CNS are accompanied with symptoms like cranial (meningeal) neuropathies related to increased intracranial pressure [11].

Table 1.1.2. A summary of the different types of B-ALL based on distinguishing immunophenotypic marker expression [5], [8].

Pro-B (EGIL BI)	**Common-B (EGIL BII)**	**Pre-B-ALL (EGIL BIII)**
$CD10^-$	$CD10^+$	$CD10^+$
$CyIg\mu^-$	$CyIg\mu^+$	$CyIg\mu^+$
-	-	$SmIg\mu^+$

Table 1.1.3. A summary of the different types of T-ALL based on distinguishing immunophenotypic marker expression[5], [8].

Immature T-ALL		**Common Thymocytic T-ALL (EGIL T-III)**	**Mature T-ALL (EGIL T-IV)**
Pro-thymocytic (pro-T-ALL) (EGIL T-I)	**Immature thymocytic T-ALL (pre-T-ALL) (EGIL T-II)**		
$CyCD3^+$ $CD7^+$	$CyCD3^+$ $CD2^+$ $CD5^+$	$CD1^+$ $CD4^+/CD8^+$ $SmCD3^+/SmCD3^-$	$CD1^-$ $SmCD3^+$

Table 1.1.4. Immunophenotypic markers expressed in B and T-ALL

Pro-B ALL	$CD19^+$, $CD22^+$, TdT^+
Common-B ALL	$CD19^+$, $CD20^+$, $CD22^+$, $CD10^+$, TdT^+
Pre- B ALL	$CD19^+$, $CD20^+$, $CD22^+$, $CD10^+$ TdT^+
Pro-thymocytic T-ALL	$CD34^+$, HLA-DR^+, TdT^+
Immature thymocytic T-ALL	$CD7^+$, cy$CD3^+$, $CD5^+$
Common thymocytic T-ALL	$CD7^+$, cy$CD3^+$, $CD5^+$
Mature T-ALL	$CD7^+$, cy$CD3^+$, $CD5^+$

1.2. Acute Lymphoblastic Leukemia (ALL): A Global Challenge

GLOBOCAN 2020 estimates leukemia to be the 11th leading cause of worldwide mortality due to cancer. The cases of incidence and death due to leukemia, globally per year are expected to rise until 2030 [9].

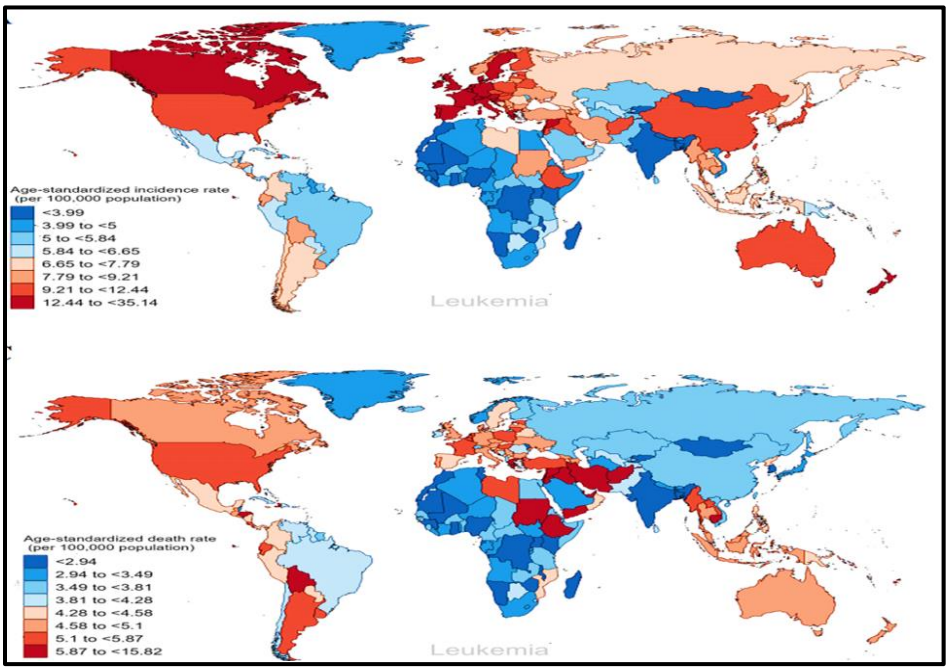


Fig.1.2.1. Global demographics of Incidence (ASIR) and Mortality (ASDR) of Leukemia [10].

The sub-type ALL accounts for about 70-80% of the known leukemia cases. 90-98% of the known ALL cases worldwide are of primary origin. Globally the annual incidence of ALL was found to be 1.08 to 2.12 per 100,000 population according to a 2015 study [11].

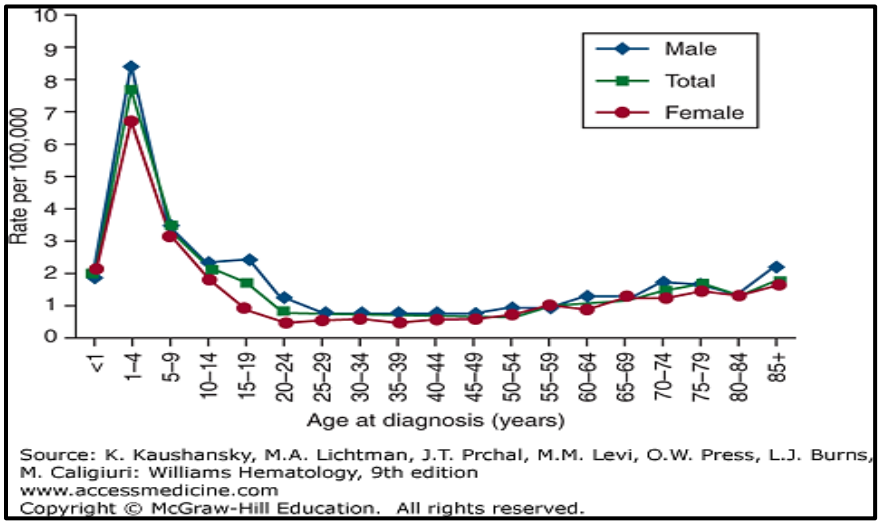


Fig.1.2.2. ALL shows bimodal distribution. It is reported to be significantly higher in males than females.

ALL shows a bimodal distribution in age groups affected, with 80% of the ALL patients being children below 10 and 20% being adults above 50. 23% of the global pediatric cancers are known to be due to ALL [12]. B-ALL is the most predominant type in pediatric and adult patients, accounting for 85% of ALL cases in children and 75% in adults [13].

1.3. Current therapies of ALL

The therapeutic regimens for ALL patients are tailored according to age stratification and risk groups (standard, high and low-risk) based on cytogenetic outcomes [7]. Despite similar regimens being administered to treat T-ALL and B-ALL, distinctions in response to different components of therapy have been observed in the two types of ALL [14].

Current therapies of pediatric B-ALL result in long-term survival of 80%–90%. In adults, while complete remission rates are similar to those of children, long-term survival is only 40%. Patients aged 15–39 years, show outcomes for B-ALL substantially inferior to those of children with 5-year overall survival (OS) rates of 50%–60% [15].

Table 1.3.1. Summary of the cytogenetic changes and the risk stratification of ALL patients [7], [15].

Risk Group	**Cytogenetic changes of the selected gene(s)**	**Adult**	**Children**
High risk	Hypodiploidy of RAS, IKZF2, TP53	5%–10%	5%–10%
	Translocations t(9;22)(q34;q11) of BCR-ABL, IKZF, CRLF2 genes	15%–25%	2%–6%
	t(4;11)/ t(9;11)/ t(19;1)/ t(3;11) of MLL gene	5%–10%	<5%
	t(8;14)/ t(8;22)/ t(2;8) of c-MYC gene	5%	2%–5%
Standard risk	t(1;14); t(10;14); t(5;14) of TCR gene	~35%	~35%
Low risk	t (12;21) (p12; q22) of ETV6-RUNX1 gene	<1%	20%–25%
	Hyper diploidy/ tetra ploidy of TP53, CREBB genes	2%–15%	10%–26%

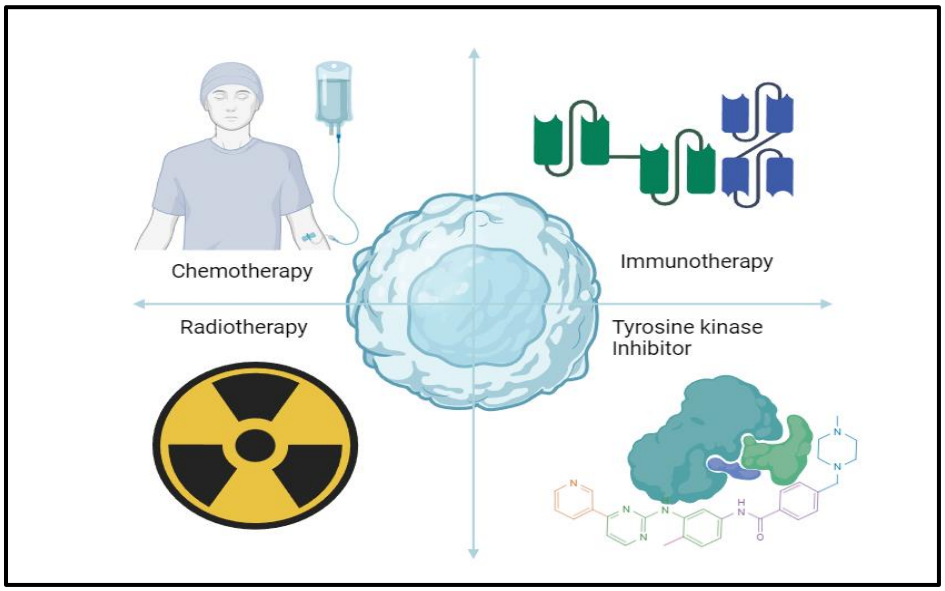


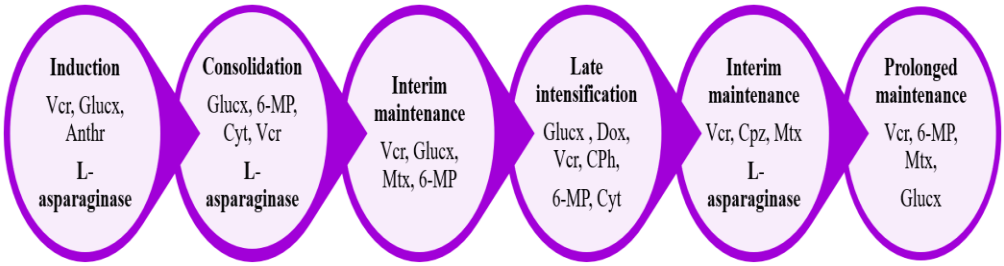
Fig. 1.3.1. Overview of the current therapeutic measures used to treat ALL

Immunotherapy has proven efficacy in (relapse after remission) and Ph^- B-ALL children and ALL adult patients. Monoclonal antibodies are specific for immunophenotypic markers. Cytotoxic drugs conjugated to monoclonal antibodies are proving useful in ALL therapy. Most of these MAbs are specific for CD19 (Loncastuximab tesirine), CD20 (Inotuzumab ozogamicin) and CD22 (Camidanlumab tesirine) receptors. Commonly used cytotoxic drugs attached are mainly DNA crosslinking/ damaging substances like pyrrolobenzodiazepine (PBD) dimers and anti-tumor antibiotics like calicheamicin. T-cell redirecting antibodies include bisubstrate antibodies [16], [17].

TKIs are used in combinatorial therapies of ALL along with chemotherapy mainly for patients with genetic aberrations like Ph+ /BRC-ABL fusions. TKIs like imatinib, dasatinib have been proved useful for therapy of such high-risk patients in both pediatric and geriatric regimens [18].

Chemotherapy of ALL consists of Induction, Consolidation, Intensification and Maintenance phases with or without CNS prophylaxis. Multiple anti-neoplastic drugs are appropriately formulated for each phase, based on risk and age stratifications. Overall, the treatment outcome results in CR of over 90% in the SR pediatric ALL following chemotherapy but comparatively modest in other pediatric risk groups, with 5-year survival rates of only 30%-40% in adult ALL.

a.



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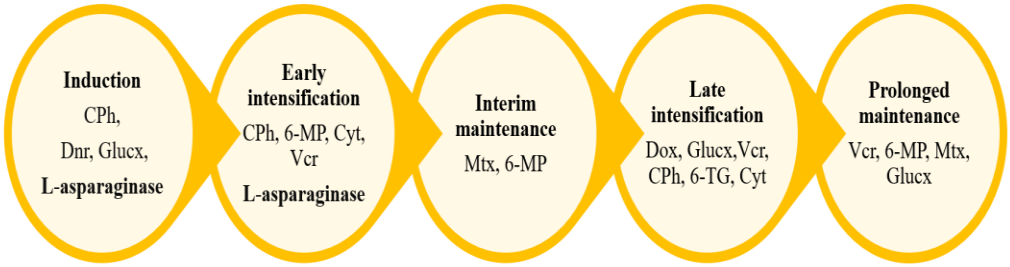


Fig. 1.3.2 a. Chemotherapeutic regimen administered to pediatric ALL patients; **b.** Chemotherapeutic regimen administered to geriatric ALL patients. L-asparaginase is an important component of both of them [19].

1.4. L-asparaginase: An overview as a chemotherapeutic drug used clinically

L-asparaginase obtained from *E. coli* (EcAII) is FDA-approved for treating ALL and has significantly improved relapse-free survival (RFS) and overall survival (OS) in pediatric and geriatric ALL globally. It is one of the first-line drugs used in ALL treatments despite the evolution

of immunotherapy. Chemotherapeutic regimen consisting of L-asparaginase is known to increase the survival rate of ALL patients to over 80% [20].

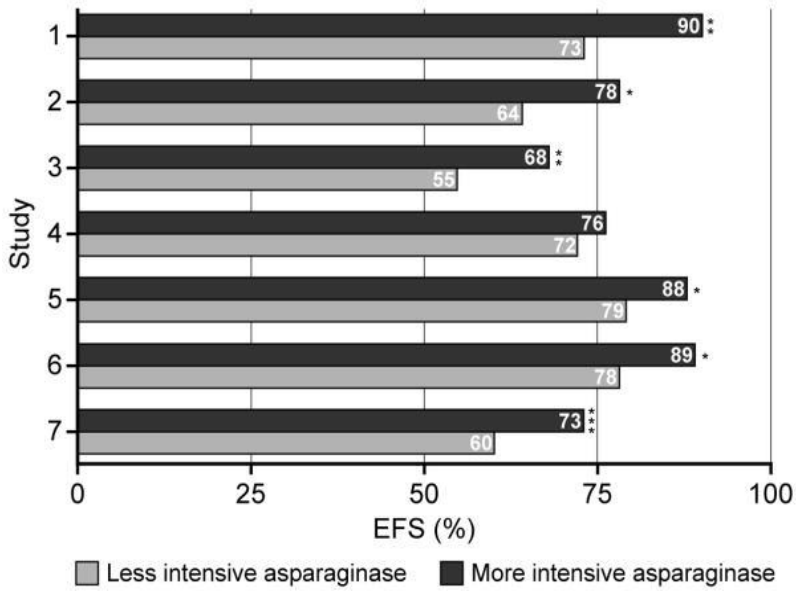


Fig.1.4.1. Study shows the importance of L-asparaginase as an indispensable component in chemotherapy in ALL patients. Intensive therapy with L-asparaginase has shown significant difference to that of a less intensive one.

Table. 1.4.1. The commercially available L-asparaginases from *E. coli* [21].

Name	**Chemical form**
Elspar®	Recombinant EcA (Type-II) Native
Kidrolase®	Recombinant EcA (Type-II) Native
Spectrila®	Recombinant EcA (Type-II) Native
Oncaspar®	EcA (Type-II) Pegylated with SS-PEG
Asparlas®	EcA (Type-II) Pegylated with SC-PEG

Although other microbial and plant sources of the type II enzyme are being investigated for their properties, to suit their use in cancer therapy, the L-Asparaginase (Type-II) from *Erwinia chrysanthemi* (under brand

names like Erwinaze® and Rylaze®) is clinically approved by FDA to be administered to patients that develop immunogenic resistance or hypersensitive reactions to therapy with *E. coli* L-asparaginase [22], [23], [24].

There are three different modes of asparagine metabolism in normal lymphoid cells: (1) extracellular uptake, (2) de-novo synthesis and (3) protein recycling [25].

L-asparaginase depletes the extracellular levels of asparagine by deaminating it to aspartic acid, releasing ammonia. DNA hypermethylation of promoter sequences of (ASNS) asparagine synthetase (the rate-limiting enzyme in the de-novo asparagine synthesis pathway) in blast cells results in their dependency on uptake from the extracellular amino acid pool or protein recycling [26].

It has been found that lymphoblast cells cannot survive relying solely on protein recycling. The lack of amino acid asparagine in them inhibits protein synthesis, arrest of cells at G_0/G_1 phase and triggers apoptosis of blast cells. Thus, the depletion of extracellular asparagine kills the lymphoblasts but not the healthy cells with normal ASNS [25], [27].

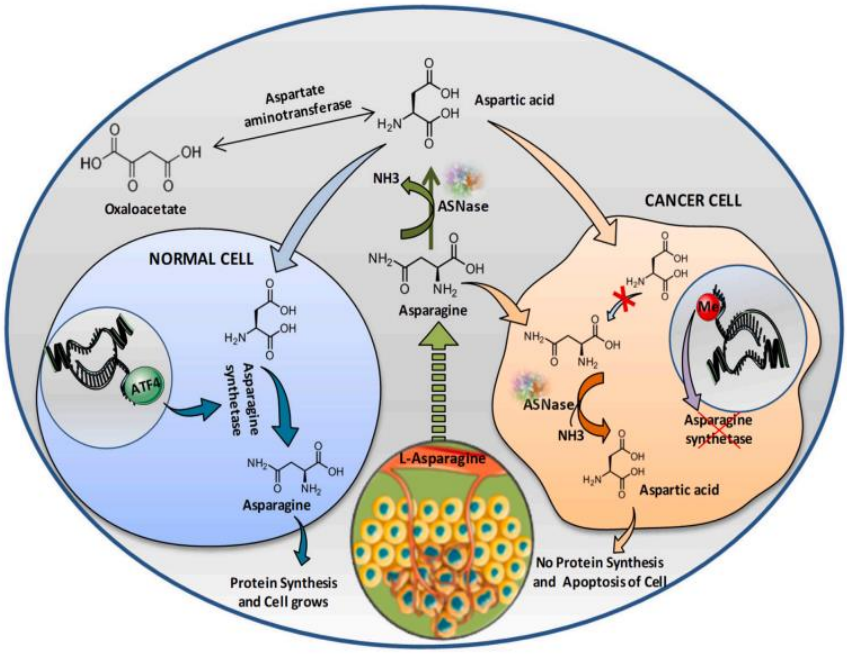


Fig.1.4.2. L-asparaginase is an important component of chemotherapy in ALL patients and acts by amino acid deprivation of the leukemia blasts. Compared to (ASNS negative) blast cells, the normal cells are capable of L- Asparagine synthesis from L-Asp due to normal ASNS production and, hence, remain unaffected by enzymatic action [28].

1.5. Challenges and limitations of using L-Asparaginase in chemotherapy

1.5.1. Toxicity

Symptoms of pancreatic and hepatotoxicity in the form of hyperglycemia, hyperbilirubinemia, neurotoxicity and venous thromboembolic events are commonly present in patients administered with the enzyme. Studies have shown that the glutaminase activity of the wild-type EcA is responsible for toxic effects. However, other studies argue that the deamination of L-glutamine is essential for the drug's effectiveness against ASNS-upregulated leukemia cells. Excessive ammonia is also known to be a cause of toxic effects due to the administration of L-asparaginase [29].

1.5.2. Immunogenicity

As the enzyme administered to patients is bacterial in origin, the enzymatic epitopes are known to cause hypersensitivity. Three types of hypersensitivity reactions are known to occur: Clinical hypersensitivity is due to antibody-mediated immune reactions and occurs only after repeated exposure to the asparaginase epitopes. Sub-clinical hypersensitivity is due to anti-asparaginase antibodies and can develop without clinical symptoms. Non-antibody-mediated reactions are due to the direct release of histamines, activation of the complement system, or rapid rise in ammonia levels associated with asparaginase administration [6].

1.5.3. Resistance

Resistance of blast cells to L-asparaginase is another drawback. Multiple factors have been associated with causing resistance which includes mutations in blasts that increase their ASNS synthesis ability, cathepsin and asparaginase endopeptidase mediated enzymatic degradation, immune cell-mediated clearance of enzyme, inactivation by neutralizing antibodies, or due to the accumulation of less cytotoxic NK cells [26].

1.5.4. Low-half life

The low half-life of the enzyme decreases its therapeutic efficacy. The lower pH of the cancer microenvironment favors the secretion of lysosomal cathepsins and human asparagine endopeptidase in circulation. These enzymes cleave L-asparaginase effectively, thereby lowering its half-life.

1.5.5. Low enzymatic activity

A study comparing in-vivo asparaginase activity of Indian generic L-asparaginases obtained from *E. coli* found them inferior to foreign biologics and innovators. The in-vivo enzymatic activity in ALL patients is lower than the label claim and demonstrated poor adherence to Critical

Quality Attributes (CQAs). High HCPs (cytoplasmic and membrane-bound) in the Indian generics indicate the lack of quality purification and extraction procedures [30].

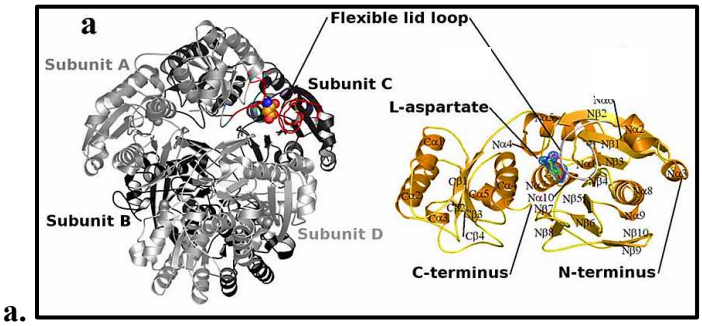
Chapter 2

Motivation

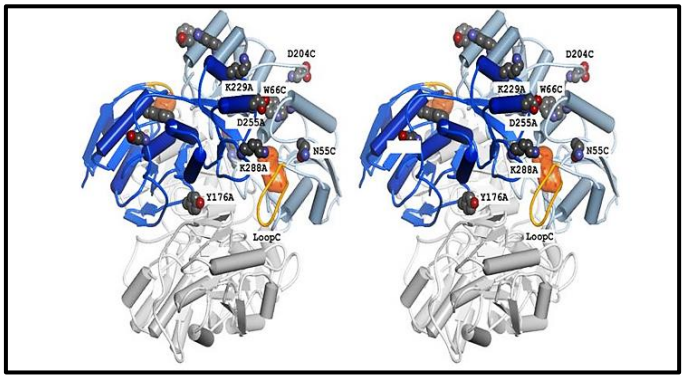
2.1. Generation of recombinant engineered mutants of L-Asparaginase

2.1.1. Selection of target amino acid for site-directed mutagenesis

The amino acid residues at the dimer-dimer interfaces (Tyr-176) and within the immunogenic epitope regions (Lys-288 and Trp-66) have been selectively targeted to develop variants with desirable properties. The EcAII encoding *ansB* gene was cloned in plasmid p-TWE1 and was used as the template to generate the single mutants, double and triple mutants were generated using a single and double mutant as template. Mutagenic primers consisted of single base substitution for the selected amino acids. Site-directed mutagenesis has been done by the PCR method [20], [31], [32].



a.



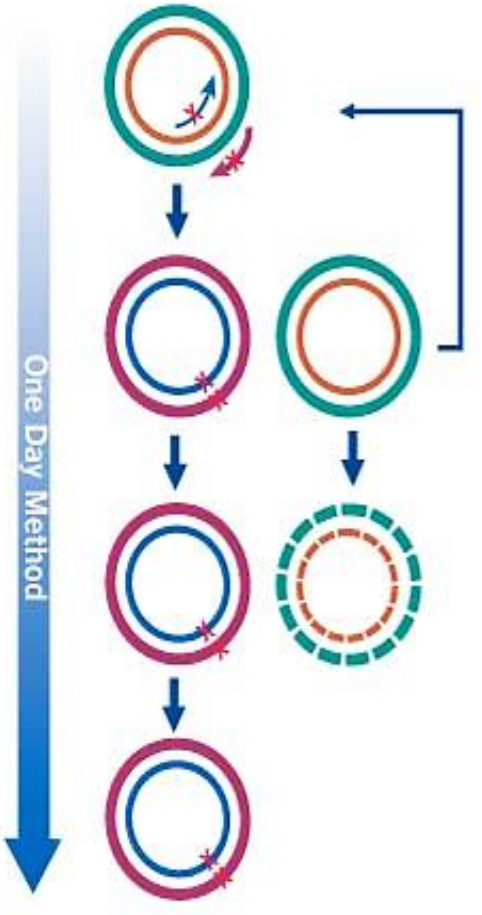
b.

Fig.2.1.1. a. Tetrameric structure of EcAII showing intimate dimers AC and BD (grey); Individual protomer of EcAII (yellow).

b. Structure of native EcAII showing the locations of the amino acids selected for substitution mutation. Subunits A (dark blue) and C (light blue), the BD dimer (grey) and the mobile loop of monomer C (orange). An aspartate residue is bound to the active site of subunit C. Individual residues are labelled according to position and subunit; e.g. Y176A stands for tyrosine at 176 position of protein sequence that is a constituent of subunit A [31].

2.1.2. Site-directed mutagenesis and generation of the engineered variants

Site-directed mutagenesis had been done by the PCR method using mutagenic primers for peptide sequences given in **Fig.2.2.1(b.** and **c.).** Double and triple mutants were prepared using mutagenic forward and reverse primers on single and double variant constructs, respectively.



1.Mutant Strand synthesis

Perform thermal cycling to:

- Denaturation of DNA template
- Annealing of mutagenic primers
- Extension and incorporation of primers with high fidelity DNA polymerase

2. Dpn I digestion of Template

Digestion of parental methylated and hemi-methylated DNA with Dpn I

3. Transformation

Transformation of mutated molecule into competent cells for nick repair

a.

b.

-----KGEQVVNIGSQDMNDDVW[66]LTLAKKINTDCDKTDGFVITHG-------

TGTAVVRSSRVPTGTTQDAEVDDAK[288]YGFVASGTLNPQKARV----------

----------VATFK SVNY[176]GPL GYIHNGKIDYQRTPARKHT----------

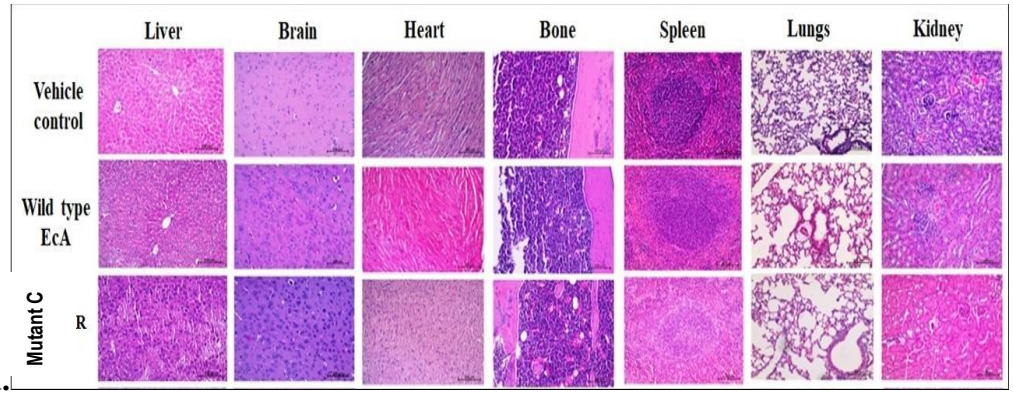
Fig. 2.1.2. a. Site directed by PCR method. Source: Quik Change XL II (Agilent);

b. Primers used target the peptide sequences (blue) and the target amino acids are numbered. Rational protein engineering was used for substitution mutations at the designated numbered amino acids. Combinations of substitution mutations at K288 and Y176 were used to generate double and triple point mutations which were further subject to pre-clinical studies.

2.1.3. Preclinical evaluation of selected mutants and their effectiveness in ALL therapy

Pre-clinical pharmacokinetic and pharmacodynamic studies of the selected recombinant mutant enzymes have shown that the variants demonstrated increased serum half-lives, reduced immunogenicity and glutaminase activity, however, retaining the asparaginase activity intact. The engineered variants have demonstrated enhanced anti-leukemic properties. Pre-clinical evaluation of four selected mutants, denoted in the study as Mutants A, B, C, and D evaluated the potency of the developed enzyme in terms of therapeutic effectiveness, toxicity as discussed in table given in **APPENDIX-A** [33]**.**

a.



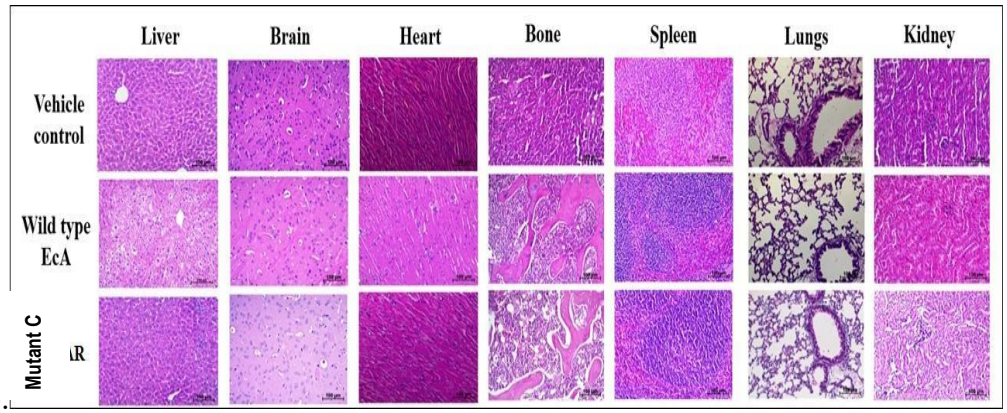


Fig.2.1.3. a. Hematoxylin and Eosin staining for histopathological examination of liver, brain, heart, bone, spleen, lungs and kidney collected from mice treated with EcAII wild-type and variants for single dose or acute toxicity study. Wild type EcAII and Mutant C were administered intravenously at a concentration of 500 IU/Kg, 2000 IU/Kg and 5000IU/Kg and were observed for 14 days.**; b.** Hematoxylin and Eosin staining for histopathological examination of liver, brain, heart, bone, spleen, lungs and kidneys collected from mice treated with EcAII wild-type and variants for multiple dose or sub-toxicity study 400 IU/Kg, 800 IU/Kg and 1600 IU/Kg of Wild type EcAII and Mutant C were administered through intra-peritoneal route five days a week for 4 weeks. The organs were collected on day 28 [20], [33].

2.2. Biosimilars in economic therapy: A note on L-asparaginase

Biosimilars are biologics that are highly similar to an existing licensed reference biological product in terms of safety, efficacy and purity but differ in production processes. A recent study projected that the clinical use of biosimilars between 2021 to 2025 could save at least $38 billion in worldwide health budgets [34].

The global annual case number for newly diagnosed ALL has increased to about 30% between 1990 to 2017, thereby causing a growing demand world-wide for clinical-grade L-asparaginase. Biosimilars of L-asparaginase have significantly lowered the economic cost of treatment of ALL. Global shortages of L-asparaginase are reported not only in all income groups, and non-availability are common even in some high-income countries [35]. Cost burden of treatment has been found to be

one of the major reasons for global disparity existing for leukemia control and prevention between countries [9]. A study comparing the quality attributes (efficacy, activity and purity) of L-asparaginase biosimilars from high, high-middle and middle-income countries found that L-asparaginase biosimilars from high-income countries (Germany) were better compared to high-middle (China), or middle-income (India) countries, thereby concluding the lack of internationalized parity in quality attributes of the different L-asparaginase biosimilars a threat to treatment safety. [23], [35]. A study has shown that existing Indian biosimilars of L-asparaginase show poor in-vivo enzymatic activity in clinical set-ups in comparison to foreign innovators [30].

2.3. Objectives of Thesis

The prime objective of the thesis is to establish the bio similarity and clinical capability of our recombinant, engineered variant of L-asparaginase (Mutant C) so that it can be clinically administered to ALL patients in India for clinical trial phase-I. This has been attempted in three ways:

- Scaling up the enzyme production from lab-scale to pilot-scale of the selected variant by suitable process development study
- Characterization of the enzyme to establish its Critical Quality Attributes for bio similarity
- Development of drug-excipient formulation suitable for parenteral (I.V/I.M.) administration similar to existing Indian L-asparaginase biosimilars.

Chapter 3

Bioprocess Scale-up

3.1. Introduction

E. coli derived L-asparaginases are oligomers consisting of 2-4 copies of a single chain composed of ~ 330 amino acids. There are two isoforms of L-asparaginase in *E. coli* (EC 3.5.1.1) Type I (encoded by *ansA* gene), a constitutively produced cytosolic enzyme with substrate affinity of about 80μM, k_{cat} ~ 6 s^{-1} compared to Type II (encoded *ansB* gene), a periplasmic isoenzyme associated with metabolism in anaerobic conditions and has K_m ~3.5 μM and k_{cat} ~ 12-60 s^{-1}. Both EcAI and II show tetrameric arrangement of oligomers, but the arrangement of individual protomers is different in them [24], [36], [37].

The native EcAII is found to be produced under anaerobic conditions without any conclusive results that adding the natural substrate L-asparagine to the media stimulates the overproduction of EcAII from the *ansB* gene [38]. For reproducibility in fermentation, the recombinant strains of *E. coli* are used to produce the enzyme, where the *ansB* gene is cloned into plasmid and the enzyme is produced in the host *E. coli* cells, usually by inducible mechanisms.

At the lab scale, the specific growth rate that needs to be maintained in the fed batches was calculated along with the optimal feed rate. Fed-batch mode was studied using an optimal feed rate control from the characterization at the lab scale and then volumetrically scaled up to pilot under constant volumetric oxygen transfer coefficients. The k_{La} criterion was studied for our recombinant engineered variant of L-asparaginase [39].

3.2. Materials and Methods

3.2.1. Chemicals and Media Components

The chemicals, solvents, and media components used in the present study were of reagent grade and were purchased either from Merck (Darmstadt, Germany), Sigma Aldrich (New Jersey, USA), Qualigens (Mumbai, India), SRL (Mumbai, India), and Hi-Media (Mumbai, India). The chemicals were used as it is without any purifications or modifications. The antibiotics (Kanamycin mono sulphate / Tetracycline chloride) used for growing cultures were purchased from Sigma Aldrich (New Jersey, USA).

The media for starting inoculum cultivation was LB broth, Miller (Sigma Aldrich) contained (g/L) tryptone (10.00), yeast extract (5.00) and sodium chloride (10.00). The pH was adjusted to 7.00

The complex media for fed-batch contained (in g/L) Dextrose monohydrate (21.80), $MgSO_4.7H_2O$ (1.02), Thiamine. HCl (0.05) and Kanamycin sulphate (0.05). Trace elements were added at 45.60 ml/L. 24% Ammonia solution was used to maintain the pH of the media during fermentation to ~7.00

3.2.2. Strain and Cell Growth Conditions

E. coli DH5α (Kindly provided by Dr. Klaus Roehm, University of Marburg, Germany) and *E. coli* BL21 (DE3) (ATCC) are used throughout the study. The strains were grown at 37ºC, 220 rpm for 14 hours in LB media (pH~7.20). The transformed BL21(DE3) was grown at 37ºC, 220 rpm for 14 hours in LB media (pH~7.20) in an orbital shaker.

3.2.3. Working Cell Bank Constitution

3.2.3.1. Glycerol stock revival

20% glycerol stocks of bacterial cultures (previously sequenced) were revived by inoculating 30 µl of the glycerol solution into 3ml LB media containing antibiotics. It was incubated at 37ºC, at 220 rpm for 14 hours. A loopful of the culture was grown on antibiotic-containing plates by streaking to isolate a colony.

3.2.3.2. Isolation of Plasmid

The plasmid of Mutant C was isolated using the Qiagen Mini Prep Isolation Kit (QIA Spin Mini Prep Kit). The protocol of the extraction kit was used. 5ml of the *E. coli* DH5α was pelleted and the pellet was dissolved in resuspension buffer, then lysis buffer and neutralization buffer. Then the solution was centrifuged at 13000 rpm for 10 minutes. The supernatant was added to the QIA Prep 2.0 Spin column. Wash buffer was added and the plasmid was extracted in the elution buffer. The plasmid was quantified spectrophotometrically at A_{260} and the ratio of $A_{260/280}$ was used to check the purity of the extracted plasmid.

3.2.3.3. Agarose Gel Electrophoresis

DNA is separated according to its length in an agarose gel matrix by applying electric fields with constant voltage. The smaller fragment of DNA travels further in the matrix, in comparison to longer DNA fragments. The wells were prepared using agarose solution in 1X TAE buffer with a fluorescent dye, Ethidium bromide (EtBr), for staining the DNA. The samples were mixed into the loading dye (containing glycerol and bromophenol blue for tracking the DNA in gel) and then loaded into the wells. 1X TAE was used as the electrophoretic buffer for the experiment. EtBr intercalates into the rings of DNA, as it passes through the gel, which can then be seen under UV. The gel was then analyzed under a Gel Documentation System.

3.2.3.4. Preparation of Competent BL21(DE3) Cells (Calcium Chloride Method)

The cells of BL21 (DE3) were grown in LB media containing Tetracycline and incubated at 37ºC for 220 rpm for 14 hours. 1% of the grown cells was used to inoculate 10 ml of LB media containing Tetracycline and grown at 37ºC, 220 rpm until the O.D. at 600 nm reached 0.6. The cells were pelleted and washed with autoclaved Milli Q water at 4ºC then treated with 0.1 M Calcium chloride solution. The mixture was incubated in ice for 2 hours. The cells were pelleted by

centrifugation at 8000 rpm for 5 min at 4ºC. The pellets were redissolved in 500 µl of 0.1 M $CaCl_2$ to constitute the final competent cell preparation.

3.2.3.5. Transformation of Competent BL21(DE3) Cells (Heat Shock Method)

10µl of the plasmid extracted from Mutant C DH5α was added to 100µl of Competent Cells in 2 ml MCT. The mixture was kept in ice for 30 minutes. The MCT was kept at 37ºC for 90 minutes. Then, it was immediately kept on ice for 10 minutes. 1 ml of LB media was added to the cells and incubated at 220 rpm at 37ºC for 1 hour. The cells were pelleted by centrifugation at 3000 rpm for 5 minutes and 1ml of the supernatant was discarded. 1 ml of fresh LB media was added to resuspend the cells. Serial dilution of the above culture was done and plated by Spread Plate Method on Kanamycin and tetracycline-containing LB Agar plates.

3.2.3.6. Cell Growth Measurement

Cell growth was calculated using the O.D. method by measuring absorbance at 600 nm. Consecutive $O.D._{600nm}$ measurements were used to calculate the specific growth rate using the formula: $\mu=\Delta \ln O.D._{600nm}/\Delta t$ [40].

3.2.3.7. Recombinant engineered variant of EcAII production in bioreactors

The recombinantly engineered variant of EcAII previously developed was studied in a 5L (working volume = 3L) glass bioreactor (Biostat C DC Bio-AFDW fermenter with PRS Bio and Automation Solution for automatic parameter generation and online screening) and a 50 L (working volume = 30L) stainless steel bioreactor (in SCIGENICS Bioreactor with online monitoring system for parameter checking). Both the bioreactors operated had temperature-control feature, Rushton impellers (3-bladed bench top fermenter and 6-bladed pilot scale fermenter) and were stirred tank-type fermenters.

Sterilization of the bioreactors was done with the media for ~40 minutes at 121ºC, using an autoclave for the 3L bioreactor (glass vessel) and sterilization in place (SIP) for the 30 L stainless bioreactor. The complex media was sterilized in an autoclave and added after bioreactor sterilization. 0.05 g/L of Kanamycin was

added after filter sterilization with 0.2µm pore size to the media. IPTG induction was done 2hrs after the start of the feed.

The WCB of mutant C was used to inoculate 10 ml of LB media containing Kanamycin (0.05 g/L) and Tetracycline (0.02 g/L) to form the pre-cultures. The cells were incubated for 12-16 hours at 160 rpm and 37ºC. This was used to inoculate seed cultures of 2 L of LB broth consisting of 1% (v/v) inoculum supplemented with dextrose monohydrate (21.800 g/L), Kanamycin (0.05 g/L) and Tetracycline (0.02 g/L). It was incubated at 220 rpm on a rotary shaker at 37ºC until the $O.D._{600\ nm}$ reached 0.6. The above inoculum was added at 5% (v/v) to the bioreactors.

3.2.5. Fed-Batch Operation

Fed-batch experiments were performed in 3 and 30 L bioreactors. The batch run was started with 2.5 and ~25 L of complex media. Dextrose-based feed comprised 766 g/L of Dextrose monohydrate, 40 g/L of Yeast extract, 2.40 g/L of $MgSO_4.7H_2O$, and 0.20 g/L of Thiamine. HCl, 0.05 g/L of Kanamycin and 5ml/L of Trace elements (pH~7). It was started after the initial glucose concentration dipped below 0.01 g/L (glucose feedback control-based). The same was performed in a 30 L bioreactor. The fed batch was operated at temperature between 30.6-29.9ºC. The airflow rate was maintained at 1 lpm during cultivation. Antifoam 204 at 20 ml/L concentration in the bioreactor medium. IPTG induction at 1mM concentration was done when the dissolved oxygen suddenly dipped <40%.

3.2.6. Scale-up criteria

The effect of the gas-liquid mass transport during fermentation in aerobic culture is an important factor in scale-up. Therefore, the volumetric mass transfer coefficient (k_{La}) was maintained constant throughout scales. The process was scaled up from 3 L to a fermenter of 30 L working volume, applying a scale-up ratio of about 1:10. The success of the scale-up process has been studied by experimental results, showing limited differences between small- and large-scale fermentation, done under the same oxygen transfer rate.

3.2.7. Volumetric mass transfer coefficient (k_{La}) calculation

The volumetric oxygen transfer rate (OTR) is one of the most notable factors that governs aerobic fermentation. The volumetric mass transfer coefficient (k_{La}) is a measure of the rate of oxygen diffusion across the area of the gas-liquid interface per liquid volume. It is one of the key process parameters that correlates the gaseous exchange, fluid dynamics in the fermenter and the properties of the vessel during scaling up of fermentation. It is given by the relation:

$$OTR = k_{La}(C_G^* - C_L) \qquad (1)$$

The volumetric mass transfer coefficient was calculated based on the physical dynamic method [39]. The k_{La} value was measured according to the equation:

$$\frac{C_L - C_{L0}}{C_G^* - C_{L0}} = 1 - E = \frac{e^{-k_{La}t}}{1 - t_E k_{La}} \qquad (2)$$ Where $t >> t_E$

3.2.8. Feed rate calculation in the exponential phase and optimization of feed rate

Growth of the *E. coli* strain was characterized at a lab scale and optical density measurements were done to establish the optimal feed rate of the substrate in a fed-batch mode in the exponential phase. The following equations of feed-rate were used for calculation [41]:

$$F_0 = \frac{X_0 . V_0 . \mu}{Y_{X/S} . \mathrm{Win}} \qquad (3)$$

$$F_t = F_0 e^{\mu t} \qquad (4)$$

$$Y_{X/S,abs} = Y_{X/Smax} . \frac{\mu_0}{\mu_0 - Y_{X/S,max} . m_s} \qquad (5)$$

$$F_O' = \frac{X_0 . V_0}{W_{in}} . \left(\frac{\mu_0}{Y_{X/S,abs}} + m_s \right) \qquad (6)$$

$$F_t' = F_0'.e^{\mu_0 t} \quad (7)$$

3.2.9. Downstream process development study

3.2.9.1. Protein extraction in periplasmic lysate at Lab Scale (3L fermentation) and pilot scale (30L fermentation)

IPTG induced cells were grown, and then harvested by centrifugation at 10000 rpm at 4 ºC until a clear supernatant was obtained. The pellets were resuspended in TES buffer and vortexed, kept in ice for 10 minutes. The mixture was centrifuged at 13,000 rpm at 4 ºC until a clear supernatant was formed. Autoclaved Mili-Q water (4 ºC) was added to the centrifuge bottles and vortexed until a solution formed and kept in ice. Centrifugation of this mixture was done at 13,000 rpm at 4 °C, till a clear supernatant was formed. Supernatant was collected and stored at 4 ºC.

3.2.9.2. Ammonium Sulphate Precipitation

Ammonium Sulphate Precipitation was done at 50% and 90% ammonium sulphate salt saturation, respectively. First, 50% ammonium sulphate precipitation was performed. To each 100 ml of the supernatant 29.5 gm of $(NH_4)_2SO_4$ was added. It was stirred for 90 minutes on a magnetic stirrer at 4 ºC. Then centrifuged at 10500 rpm for 2 hours under identical conditions. The supernatant was collected and was used for 90% ammonium sulphate precipitation. To each 100 ml of the supernatant, 27.2 gm of $(NH_4)_2SO_4$ was added. It was stirred for 60 minutes on a magnetic stirrer at 4 ºC, then centrifuged at 10500 rpm for 2 hours at the same temperature. The pellet was collected and dissolved in 20 mM Tris HCl buffer (pH of ~8.5).

3.2.9.3. Diafiltration

Diafiltration was used for de-saltation of ammonium sulphate from the protein. It is based on the principle of ultrafiltration, which selectively utilizes permeable (porous) membrane filters to separate the components of solutions based on their molecular size. Diafiltration of the crude protein obtained after steps in **3.2.9.2.**, was done using a 5kDa, and pore size of 0.5µm diafiltration membrane at a pressure

of 1kg cm^{-2} and flow rate 40 ml/min across a 0.1m^2 spectrum TFF unit. The buffer used was 20 mM Tris HCl (pH~8.21).

3.3. Results

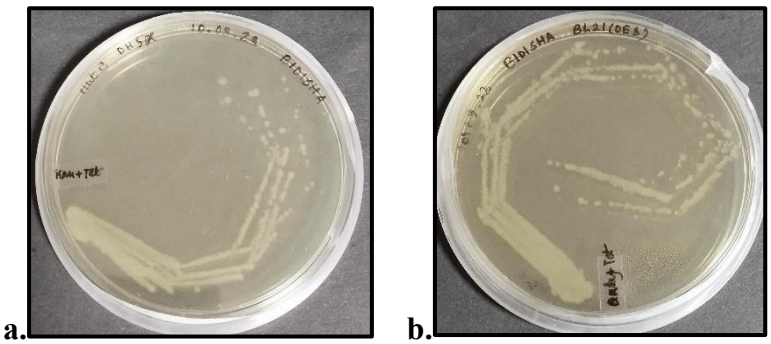


Fig.3.3.1. Glycerol stocks revived and single isolated colonies were obtained by Streak Plate Method **a.** E. coli DH5α strain grown on Tetracycline and Kanamycin containing plates**; b.** *E. coli* BL21(DE3) strain grown on Tetracycline containing plates.

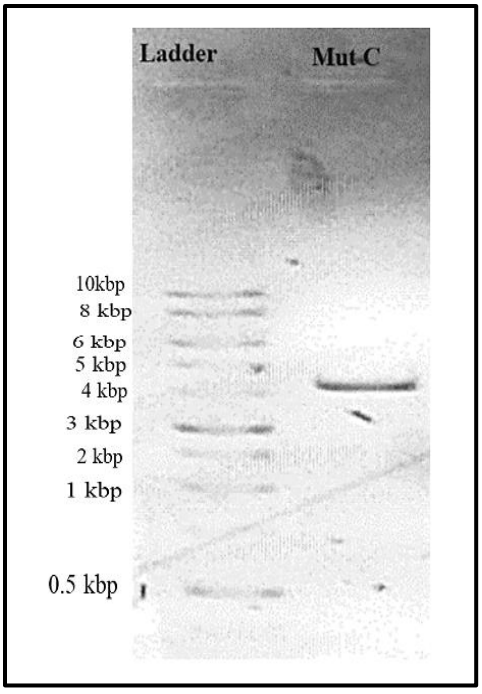
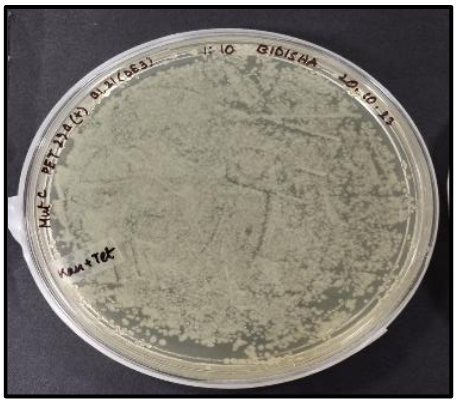
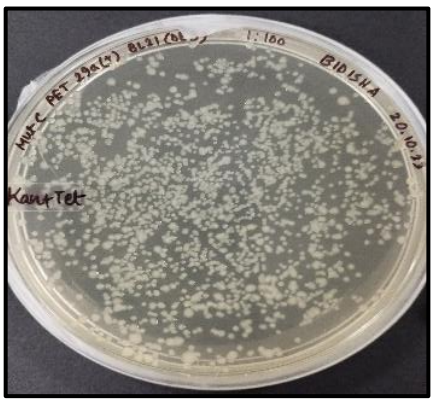


Fig.3.3.2. 0.8% Agarose gel electrophoresis was done after plasmid quantification using spectrophotometric method.

a.  **b.** 

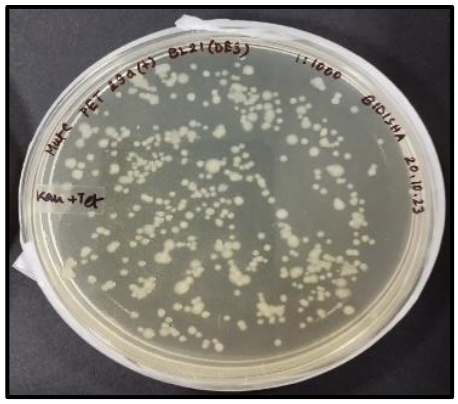
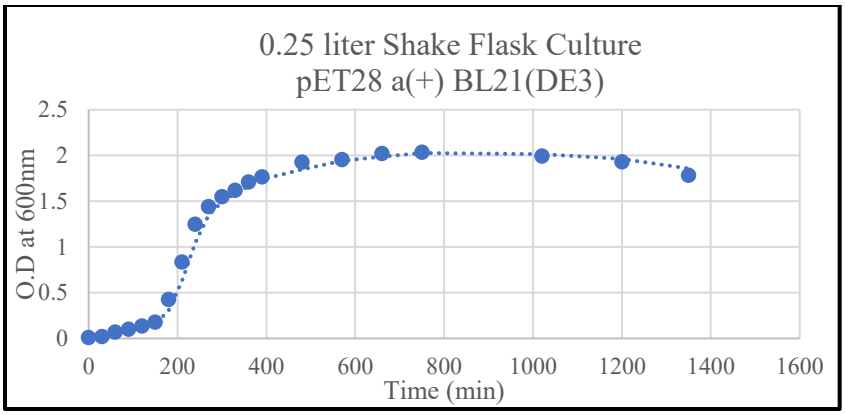
c. 

Fig.3.3.3. Competent cells of BL21(DE3) were prepared and the plasmid extracted from DH5α starin was used to transform them. Colony isolation was done was spread plate and serial dilution **a.**TNTC plate dilution at 1:10 ; **b.** Plates at 1:100 were having few confluenting colonies ; **c.** Plates with 1:1000 dilution had distinct and single isolated colonies were selected.

a.



b.

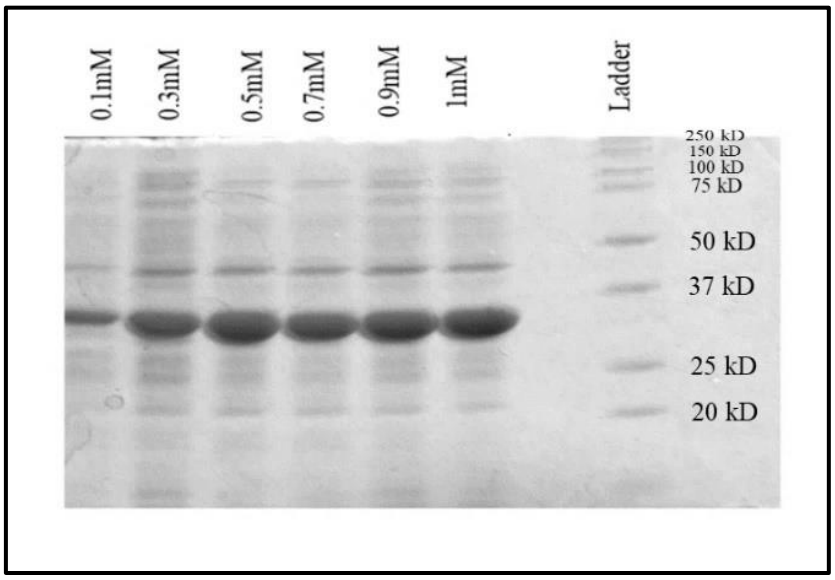
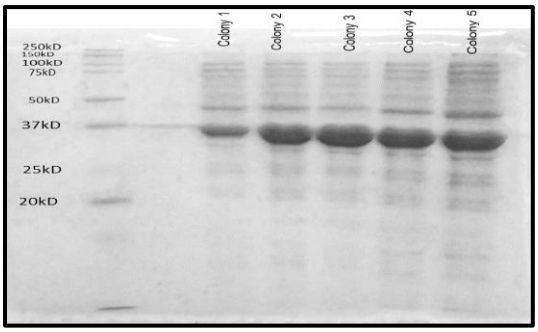


Fig.3.3.3. a. The batch kinetics of transformed and previously sequenced for Mutant C; **b**. The IPTG induction was studied at different concentrations for the 0.25L Shake Flask Culture. IPTG concentration at 0.5 mM was found to be optimal for Mutant C production for the 0.25L culture**.**

a.

b.



c.

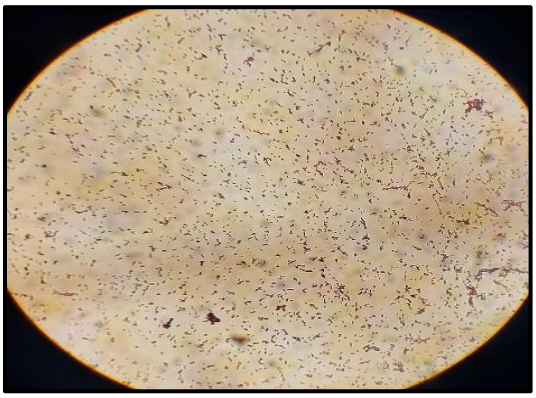
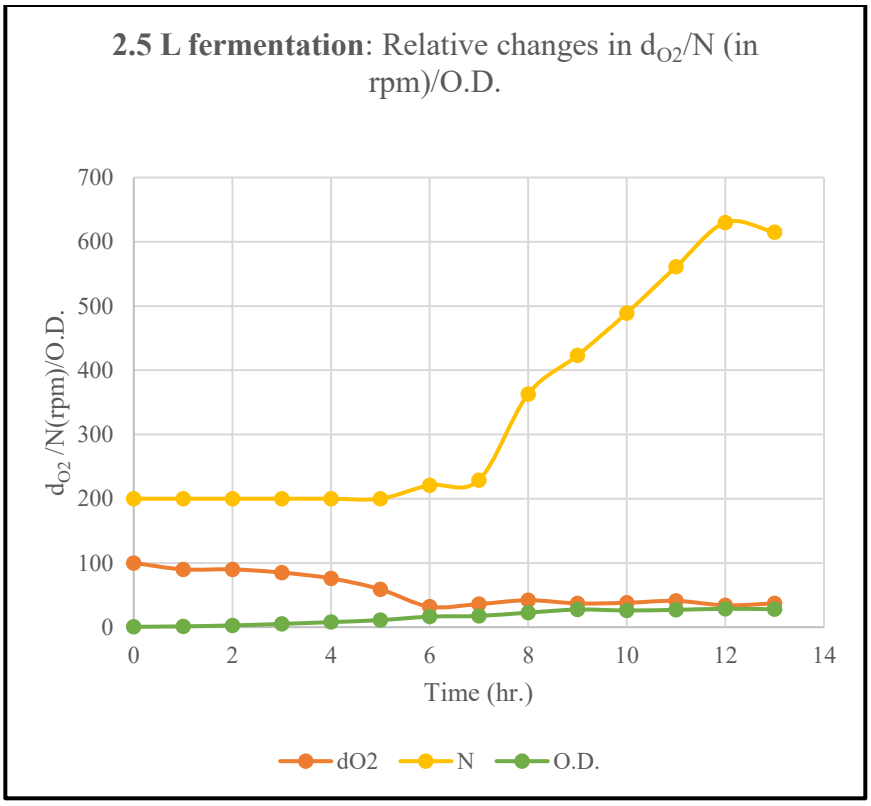


Fig.3.3.4. a. Single isolated colonies from 1:1000 dilution plates were grown using the batch conditions optimized from the results in **Fig.3.3.3**; **b.** The production of Mutant C was observed in all the 5 selected colonies. Colony 3 was found to be most specifically producing Mutant C under identical conditions and induction procedures**; c.** Colony 3 cells were grown and were used for constituting the WCB. The cells were Gram-stained prior to inoculating the fermenters to check for possible contamination.

a.



b.

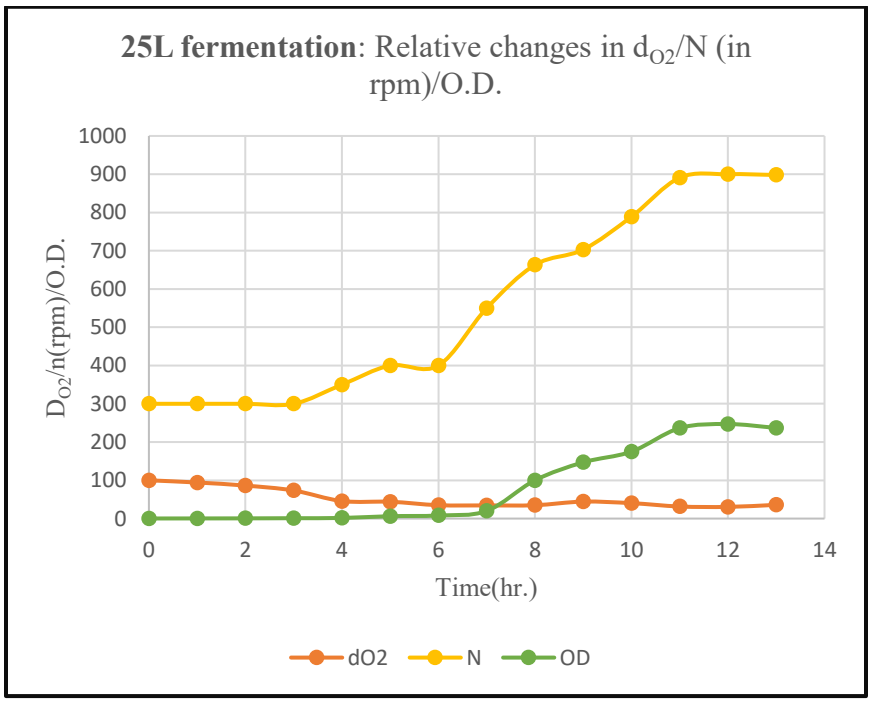


Fig. 3.3.5. a.; b. Fed-batch operations for the 2.5 L and 25 L fermenters in terms of the changes in dissolved oxygen (d_{O2})/ N (agitation speed in rpm)

and O.D. at 600nm**. a.** IPTG inductions were done at 6hr and feed was started at the 5.5 hr. time point**; b.** IPTG induction was done at 7.5hr and feed was started at 7 hr. time point.

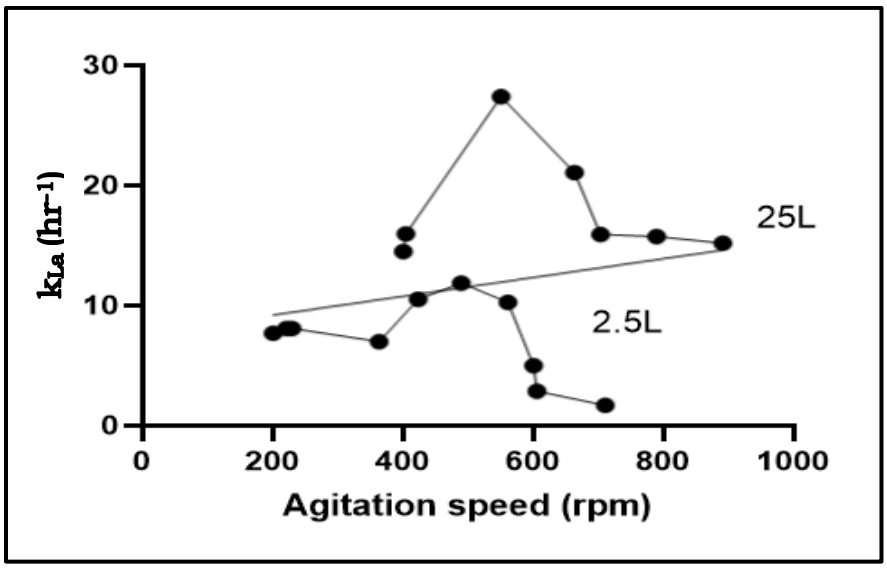
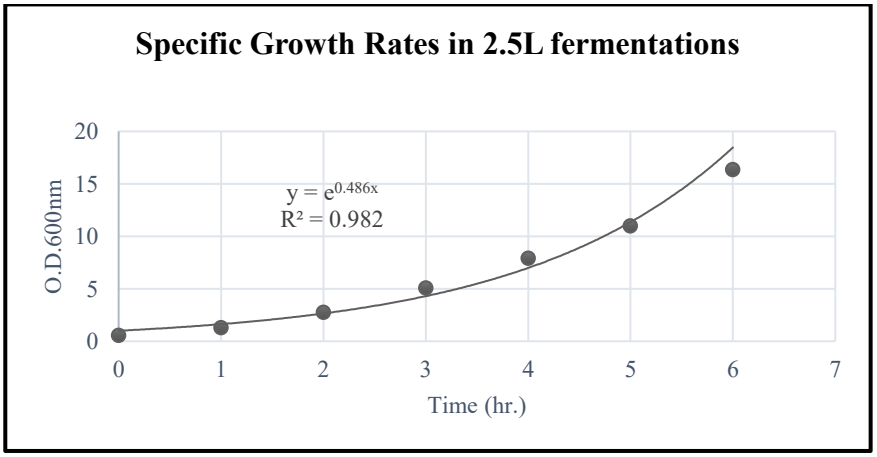


Fig. 3.3.6. Volumetric mass transfer coefficient (k_{La}) as a function of stirrer agitation speed at 30ºC and same airflow rates performed in the 2.5L fermenter and 25L fermenter**.**

a.



b.

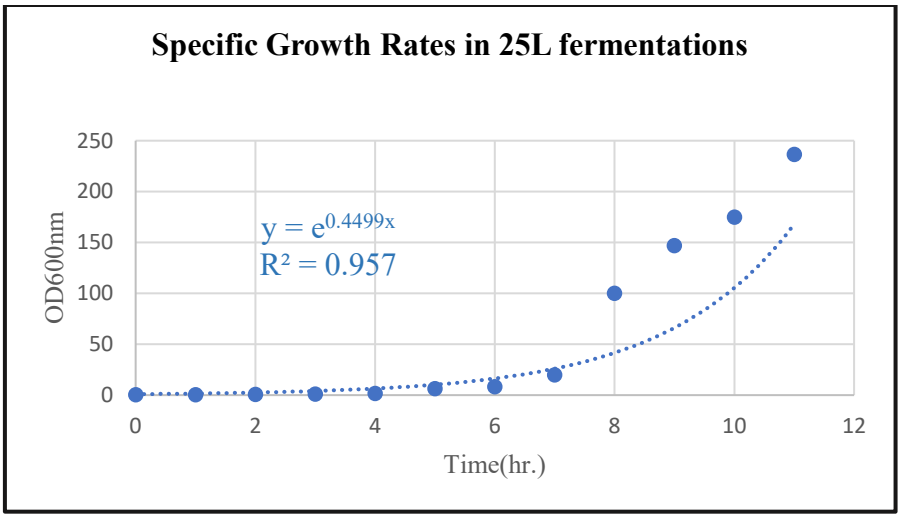


Fig.3.3.7. Specific growth rate at exponential phase constant feed rate for **a.** 2.5L was (0.486hr^{-1}) and **b.** 25L (0.4499 hr^{-1})

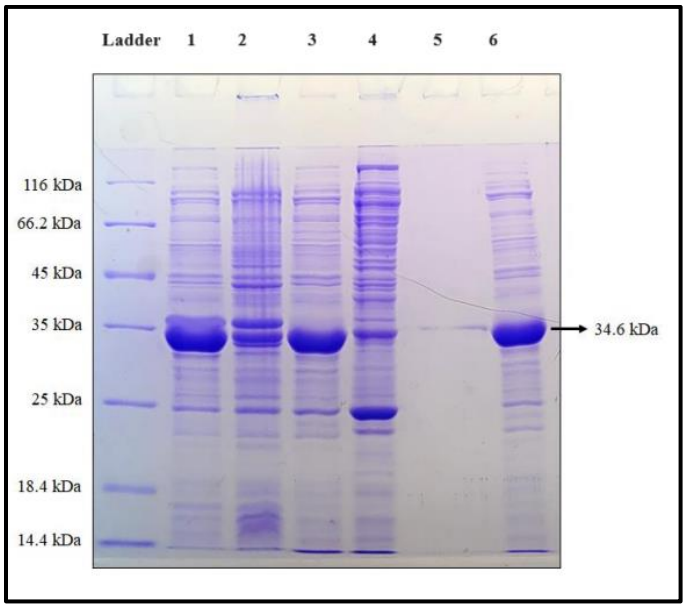


Fig.3.3.8. a. SDS-PAGE of enzyme during downstream process development.

Lane 1-TES buffer analyzed for enzyme after dissolving the cell pellets

Lane 2-Lyzed cell pellets dissolved in water analyzed for enzyme

Lane 3- The Milli Q water in which the lysed cell pellets were dissolved were analyzed for the enzyme

Lane 4-After 50% Ammonium sulphate precipitation, a pellet was formed. It was analyzed for the enzyme.

Lane 5-Supernatant was analyzed for enzyme after 90% ammonium sulphate precipitation.

Lane 6- Pellet obtained after 90% ammonium sulphate precipitation was analyzed for enzyme.

3.4. Discussion

Spectrophotometric measurements for DNA purity of the isolated plasmid from DH5α showed A_{260} at 2.898, $A_{260/280}$ at 2.06. The isolated plasmid solution had a concentration of 144.9 ng/μL. This was used for transformation of BL21(DE3) and selection of the transformed colonies on double antibiotic containing plates of Tetracycline and Kanamycin.

The fermentations validated the scale-up criterion of maintaining the volumetric oxygen transfer coefficient (k_{La}) constant during scale-ups as given in **Fig.3.3.6.** Wet cell mass concentration obtained from the 2.5 L fermentation was 0.7 kg in comparison to the 25L fermentation which was 2.8kg. The difference in concentration of biomass production was attributed to a 1.08 times lower specific growth rate during process scale-up.

An alternative model has been proposed to calculate the feed rate which uses the data obtained from at least three scaled-up fermentation to generate the value of the coefficient of microbial growth maintenance (m_s). This constant would be used to correct the feed rate equation as given in the equations (5), (6) and (7) for optimization of the biomass concentration production during the pilot scale fermentation.

Chapter 4
Biologic Drug Characterization

4.1. Introduction

4.1.1. Critical Quality Attributes of biosimilars

Characterization, also known as “critical quality attributes (CQA)”, establishes the analytical similarity of the engineered enzymes to that of an existing innovator already administered to ALL patients. For approval by regulatory authorities, the clinical bio similarity needs to be established and the CQA variations must be within a range established by innovators. Analytical bio similarity is established for a biologic using validated methods that help to map the molecule for comparative structure- function associated characterization using appropriate high throughput methods. CQA analysis of each batch of the biologic reduces the cost burden towards the regulatory requirement of extensive preclinical/ clinical similarity data of each batch, thus making the drugs more affordable [42], [43].

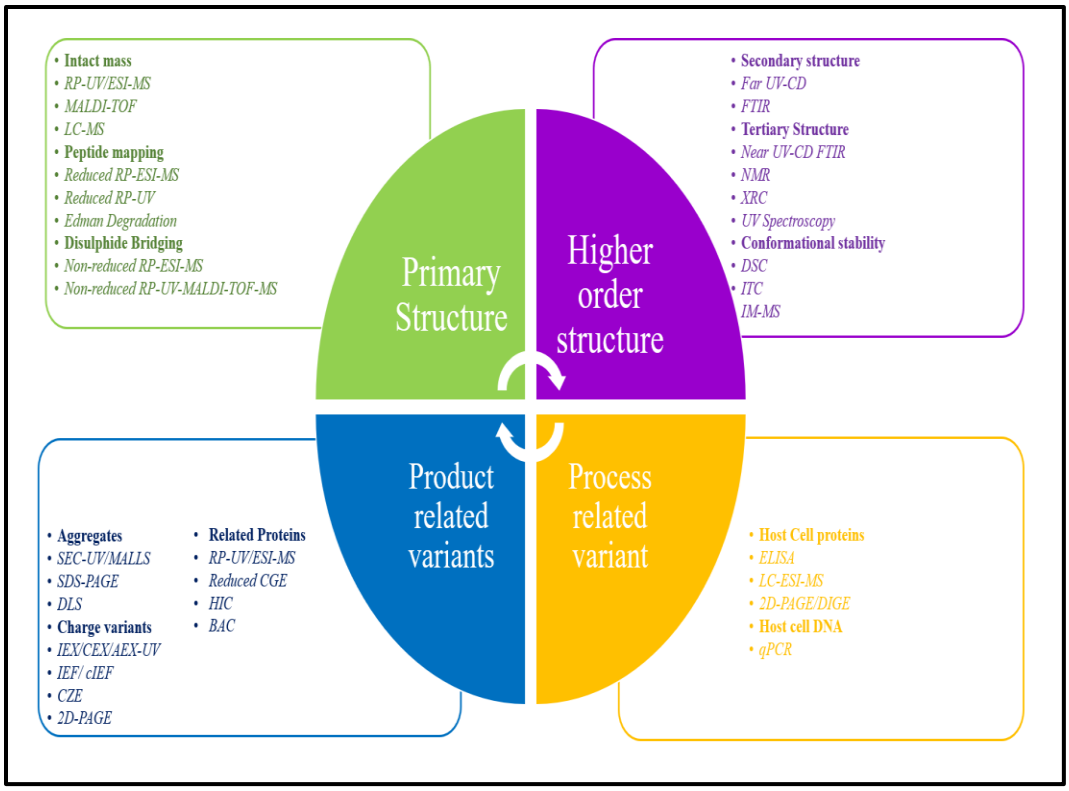


Fig. 4.1.1. Summary of the different CQAs for biologics obtained using the *E. coli* expression system and the corresponding analytical technique used to study them.

4.1.2. Structural and functional aspects of EcAII

The higher-order structure of *E. coli* L-asparaginase II (EcA II) consists of a homo-tetrameric form. A protomer of EcAII is constituted by a larger N-domain consisting of 4 α-helices and 10 β-strands formed of 195 amino acid residues and a smaller C-domain of 4 α-helices and 4 β-strands (~120 amino acid residues). An interdomain linker of 21-26 amino acid connects the smaller and larger domains. A disulfide bridge between Cys77 and Cys105, located on the surface at the N-terminal domain, is unique to EcAII. Individual protomeric units are called A, B, C, and D, wherein A and C form an extended contact called “intimate dimers”, and B and D form a less extended contact called “distant dimers” [24].

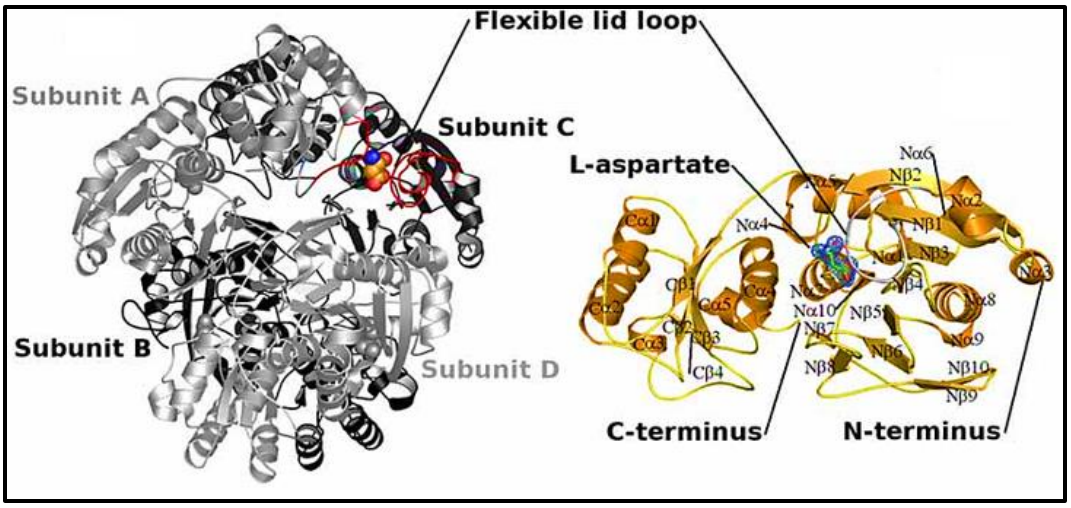


Fig. 4.1.2. Dimers forming tetramer (grey); a single protomer unit with the product in an active site pocket (yellow) [22].

Each intimate dimer forms two complete active sites, constituted by residues located in each protomeric N-terminal so that a tetramer of EcAII consists of four active sites. Only a single residue of the C-terminal domain contributes to the catalytic domain. The topological switch point formed by strands β1 and β3, with α-helices (Nα1 and Nα2), forms the active-site pocket. An induced-fit mechanism of enzyme action proposes that the active site consists of a rigid part involved in substrate binding and a flexible part that closes on the binding pocket upon substrate binding so that all the catalytically relevant residues fall into close proximity [24], [37], [44].

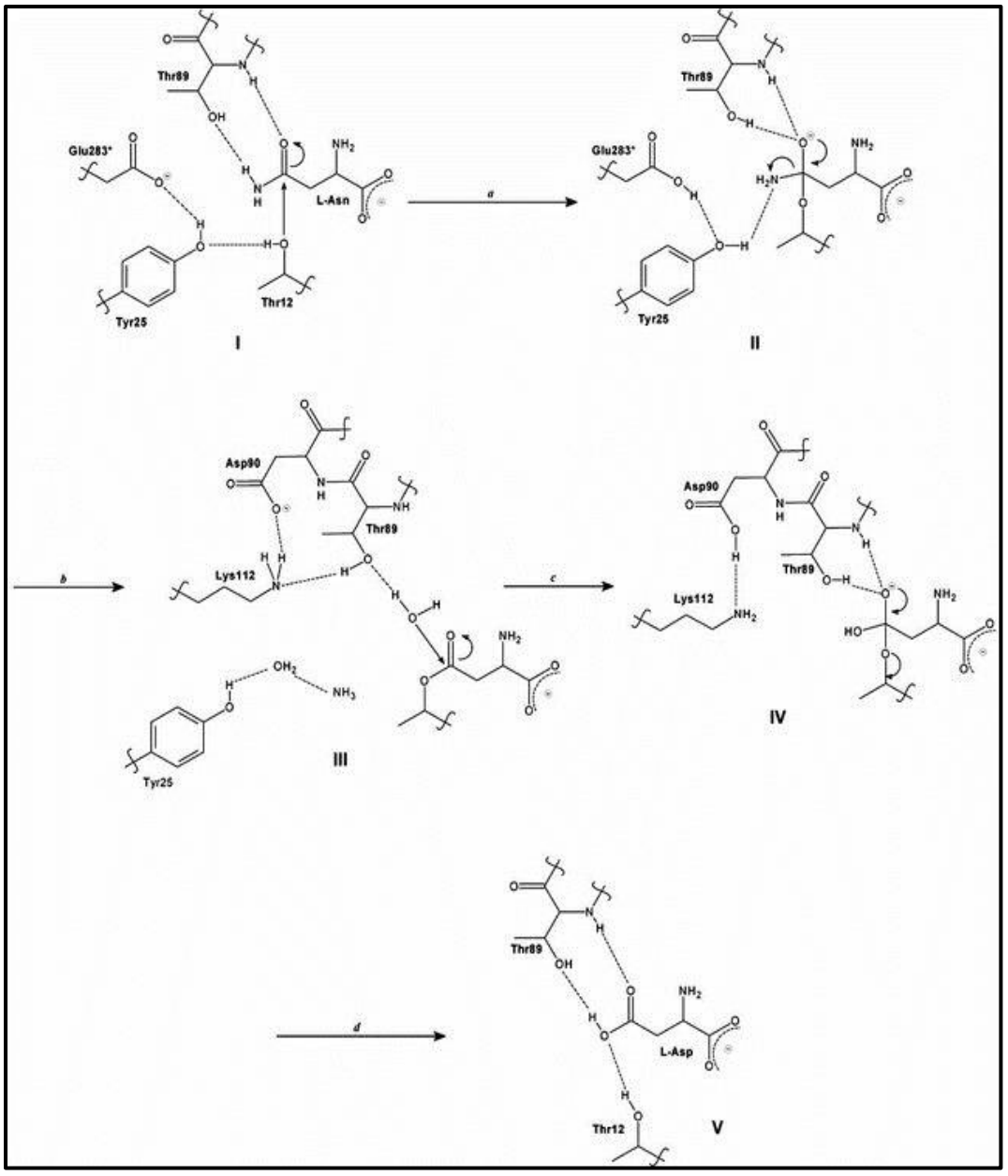


Fig. 4.1.2.3. The residues at the active site involved in enzymatic catalysis for conversion of L-asparagine to L-aspartate [45].

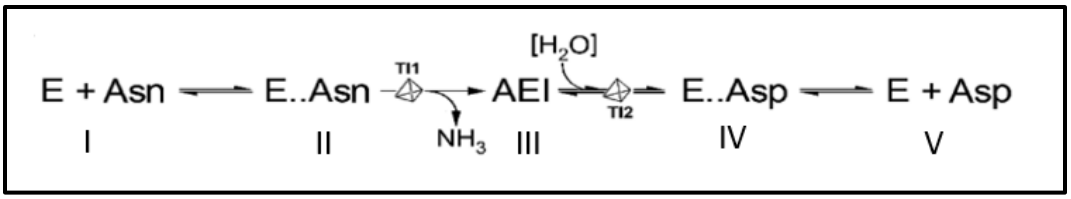


Fig. 4.1.2.3. I-V A ping-pong mechanism of enzyme action of EcAII has been proposed. The positioning of the catalytic residues in steps a-d are shown in **Fig. 4.1.2.2.** [46]

4.2. Materials and Methods

4.2.1. Anion Exchange Chromatography

Anion exchange chromatography was done using Akta Explorer. The column used for Anion exchange chromatography is the Hi-trap Q-HP Sepharose + column (Cytiva (Marlborough, USA)). It has positively charged quaternary ammonium resin that binds to charged l-asparaginase and negatively elutes the protein. The buffers were equilibration buffer (20mM Tris) and elution buffer (20mM Tris, 0.5M NaCl). The column volume was 5 cv. The Unicorn software was set at method run, and the method was designed by putting 15 column volume (CV) and a flow rate of 2 ml/minute. The sample injected comprised of the crude obtained after diafiltration.

4.2.2. Hydrophobic Interaction Chromatography

HIC was done using Akta Start. The column used for HIC was a Capto-phenyl column. It has hydrophobic groups that facilitates the binding of more hydrophobic components while speeding up the elution of hydrophilic proteins from the column. The bound hydrophobic components are eluted out using a gradient of ammonium sulphate solution. The equilibration buffer was 50 mM PBS buffer and the elution buffer was 1.2 M $(NH_4)_2SO_4$ in 50 mM PBS buffer. The purification was run in gradient mode during elution. The flow rate used was 2 ml/min.

4.2.3. Size Exclusion (Gel filtration) Chromatography

Size exclusion chromatography was done using Akta Start. The column used for Size exclusion chromatography has porous resin with a molecular weight cut-off of 200 kDa that retards the elution of the proteins (MW< 200 kDa) over the larger proteins. The buffers were equilibration buffer and elution buffer (20 mM Tris HCl, conductivity 1.4 mS/cm and pH~8). The column volume was 30 ml. Sample injection volume was between 3-5ml and a flow rate of 3 ml/minute was used for the purpose of purification.

4.2.4. SDS-PAGE

A discontinuous SDS PAGE (Laemmle, 1970) consisting of an upper layer of 4% stacking gel having a pH of 6.8 owing to the tris-HCl and a lower layer of 12% resolving gel having a pH of 8.3 owing to tris-glycine was performed. Sodium Dodecyl Sulphate, an anionic detergent, binds to the protein backbone of L-asparaginase at a constant molar ratio (mass per charge), thereby removing the effect of the intrinsic charge of the protein and conformation. β-mercapto ethanol further breaks covalent bonds removing the dependency of electrophoretic mobility due to conformation. Thus, the electrophoretic mobility of the enzyme is only due to the length of its linearized monomers. These identical units are visualized as a single band after staining with CBB-G250 solutions. Purity determination of our enzyme by 12% SDS-PAGE was done by densitometric method using Image J software [47].

4.2.5. SEC-HPLC

SEC-HPLC mainly involves the size-based separation of components in a mixture in the porous beads of the column, using a pressurized mobile phase. The retention time (RT) of the different components is dependent mainly on these interactions and conditions like minor variations in temperature, pH and composition of the mobile phase. However, non-specific binding may be responsible for inaccuracy in the RTs of the analytes. The UV detectors help to detect each component in the form of a chromatogram.

SEC-HPLC was Shimadzu UFLC LC-20 AD Prominence Liquid Chromatography, DGU 20 Degassing Unit, SPD-M20A Prominence diode array, CTO-10AS VP Shimadzu Column Oven was used for analysis. The column used was Shodex LW-803 GEL 212210. Buffer used was 1X PBS buffer at pH~7. The oven temperature was 35- 39 ºC. A calibration curve was prepared from a list of known molecular weights (MW) of proteins and their RTs achieved in the column in the same buffer composition. This was used to predict the MW of the analytes from the chromatogram. The purity determination of our protein by SEC-HPLC was done by LabSolutions CS Software, Shimadzu.

4.2.6. RP-HPLC

RP-HPLC was done using a C18 Promosil column as the stationary phase. Mobile phase constituted of acetonitrile and water in the ratio of 20:80 initially and it was run at gradient with the acetonitrile concentration being increased. The RP-HPLC run was based on the principle that as the concentration of acetonitrile increased in the mobile phase, the constituents of higher hydrophobicity would gradually develop reduced affinity for the stationary phase and consequently elute out in the mobile phase and be detected by the UV detectors.

4.3. Results

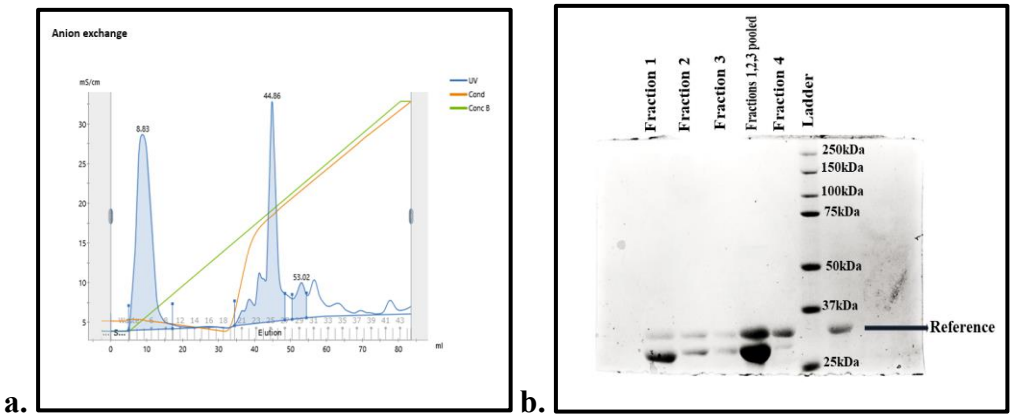
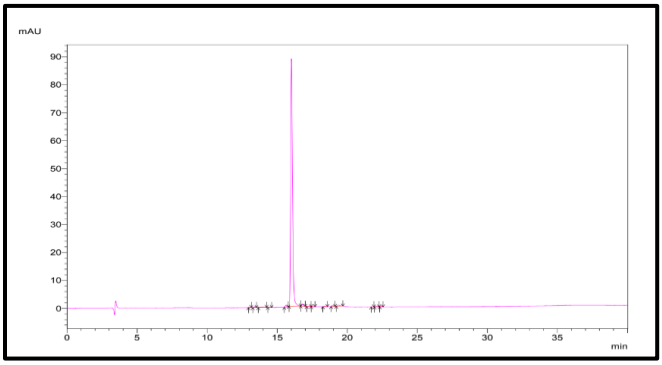


Fig. 4.3.1. a. Chromatogram of protein purified by AEC following diafiltration. The four principal fractions collected by this method were used for further experiments; **b.** 12% SDS-PAGE analysis of fractions obtained from AEC.

a.

Peak	RT (in min)	Area	Area%
1	12.649	14487	0.699
2	12.901	38060	1.838
3	14.259	35237	1.701
4	15.067	6495	0.314
5	16.000	1950921	94.197
6	17.550	1111	0.054
7	17.992	1400	0.068
8	19.798	13759	0.664
9	20.726	9641207110	0.465
Total			100.000

b.



c.

PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%	Relative Retention Time
1	13.042	338	59	0.039	0.814
2	13.392	1740	307	0.199	0.836
3	13.920	1801	114	0.206	0.869
4	14.437	402	48	0.046	0.902
5	15.608	4496	677	0.515	0.975
6	16.013	842585	88490	96.543	1.000
7	16.804	4851	616	0.556	1.049
8	17.261	1294	166	0.148	1.078
9	17.506	608	84	0.070	1.093
10	18.382	1141	131	0.131	1.148
11	18.953	2893	380	0.331	1.184
12	19.329	5297	459	0.607	1.207
13	21.789	388	61	0.044	1.361
14	22.121	3176	315	0.364	1.381
15	22.415	1749	243	0.200	1.400
Total		872758	92150	100.000	

Fig.4.3.2. a. Percent composition of constituents from chromatogram obtained for reference Indian L-asparaginase biosimilar; **b.** Chromatogram of RP-HPLC analysis of Mutant C; **c.** Percent composition of constituents from chromatogram. Percent purity of 96.54% after one-step purification of Mutant C

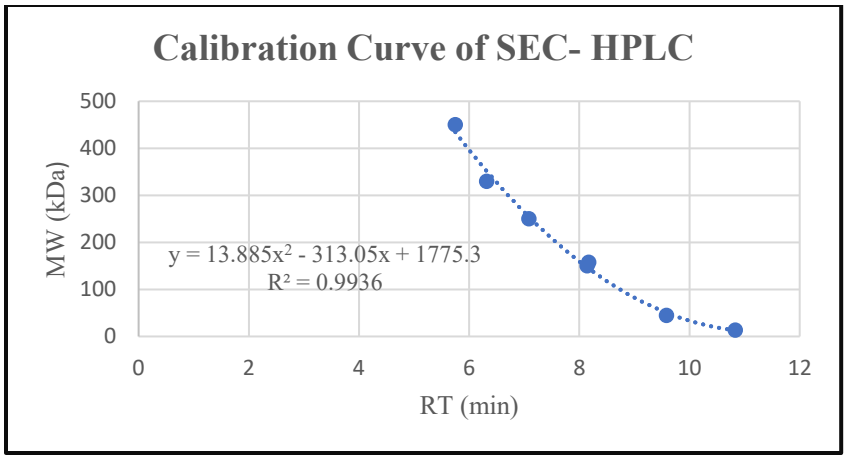
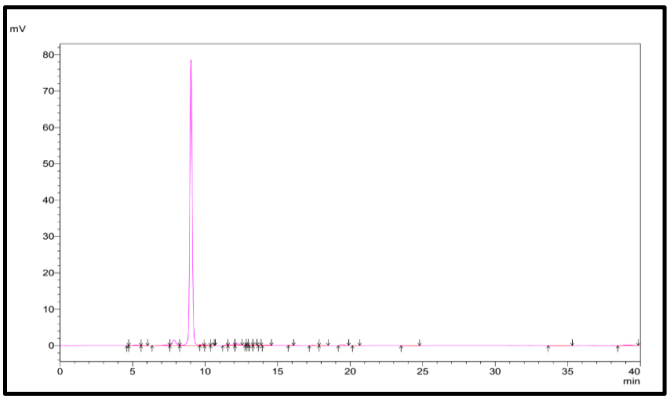


Fig.4.3.3. Calibration curve of SEC-HPLC for molecular weight determination

a.



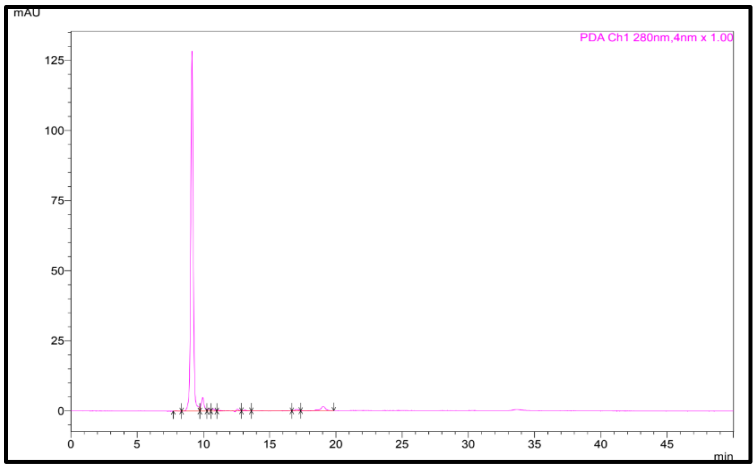
b.

Detector A 280nm

Peak#	Ret. Time	Area	Height	Area%	Relative Retention Time
1	4.608	115	37	0.012	0.511
2	5.217	3864	150	0.398	0.578
3	5.667	786	68	0.081	0.628
4	7.275	4396	154	0.452	0.807
5	7.858	24536	1523	2.525	0.871
6	9.025	908952	78584	93.529	1.001
7	9.767	1444	163	0.149	1.083
8	10.117	1251	113	0.129	1.122
9	10.483	219	27	0.023	1.162
10	11.450	1458	139	0.150	1.269
11	11.775	2152	93	0.221	1.305
12	12.267	5187	460	0.534	1.360
13	12.775	405	200	0.042	1.416
14	12.925	31	13	0.003	1.433
15	13.167	483	60	0.050	1.460
16	13.433	295	35	0.030	1.489
17	13.742	106	14	0.011	1.523
18	14.333	1005	65	0.103	1.589
19	15.883	302	28	0.031	1.761
20	17.475	1245	68	0.128	1.937
21	18.167	810	48	0.083	2.014
22	19.458	4182	246	0.430	2.157
23	20.383	564	38	0.058	2.260
24	23.967	714	20	0.073	2.657
25	34.408	750	14	0.077	3.815
26	39.108	6586	153	0.678	4.336
Total		971836	82515	100.000	

Fig.4.3.4. a. SEC-HPLC chromatogram of reference Indian L-asparaginase biosimilar; **b.** Percentage composition profile of constituents from SEC-HPLC for reference indian L-asparaginase biosimilar.

a.



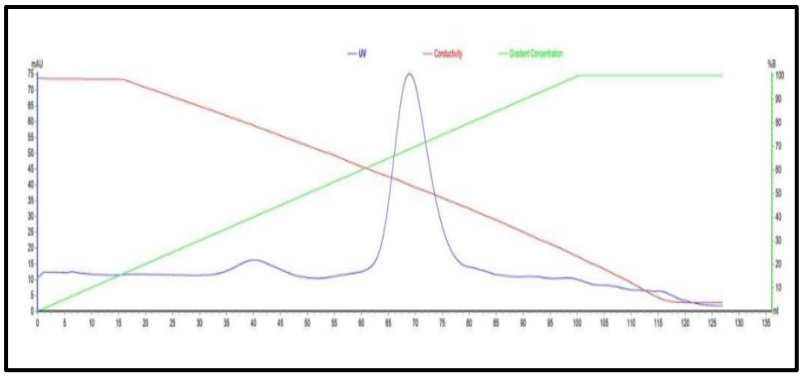
b.

PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%	Relative Retention Time
1	7.911	1638	152	0.088	0.866
2	9.136	1690726	128223	90.749	1.000
3	9.942	73422	4822	3.941	1.088
4	10.343	11963	728	0.642	1.132
5	10.707	12982	735	0.697	1.172
6	12.562	15234	691	0.818	1.375
7	13.128	5691	301	0.305	1.437
8	16.249	-437	55	-0.023	1.779
9	17.055	9517	496	0.511	1.867
10	19.042	42341	1486	2.273	2.084
Total		1863077	137688	100.000	

Fig.4.3.5. a. Chromatogram of SEC-HPLC of Mutant C; **b**. Percentage composition profile of constituents from SEC-HPLC for Mutant C L-asparaginase.

a.



b.

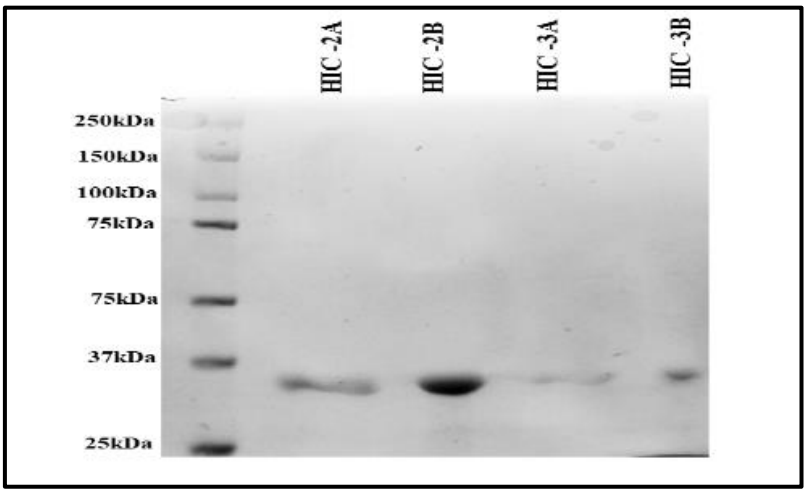
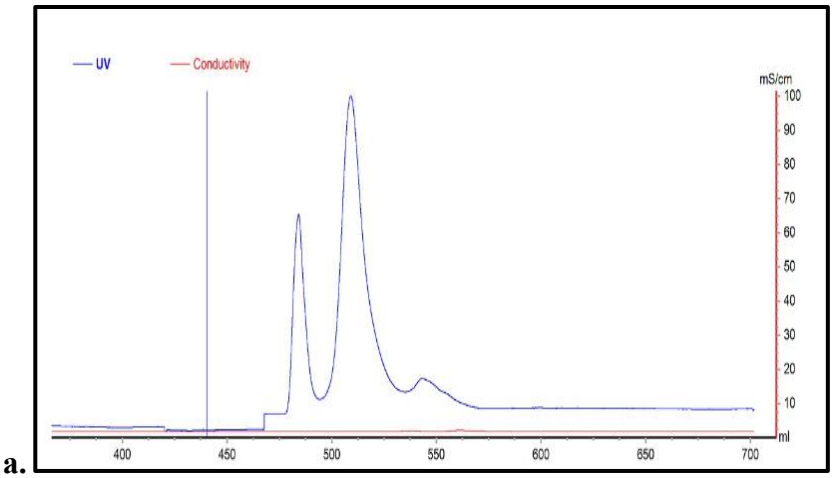
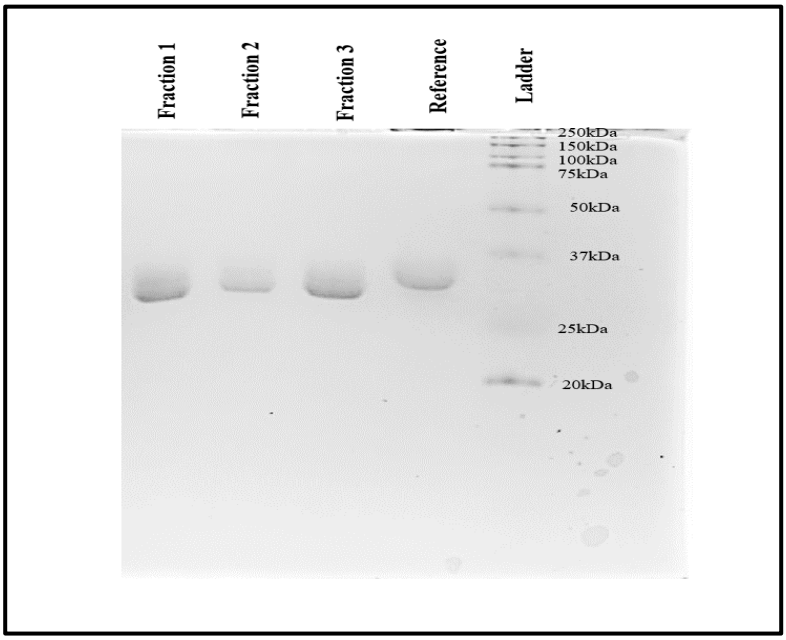


Fig.4.3.6. a. HIC chromatogram; **b.**12% SDS-PAGE of protein purified by HIC following diafiltration



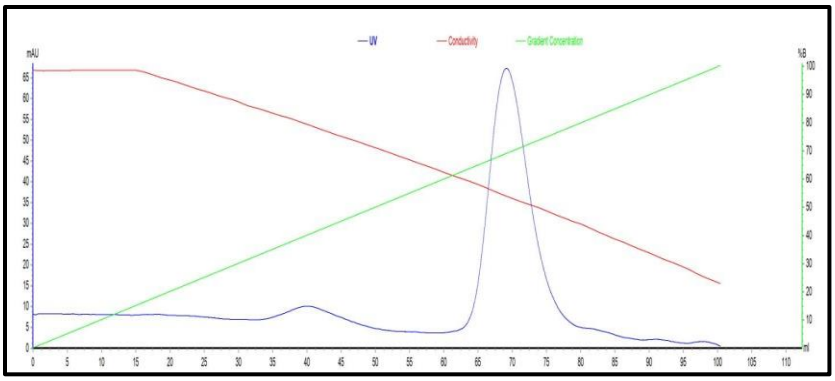
a.



b.

Fig.4.3.7. a. SEC Chromatogram of L-asparaginase after one step purification using HIC; **b.** 12% SDS-PAGE analysis of the purified fraction from SEC. The fraction with the largest peak from 4 different SEC runs performed in different solvents was analyzed. Fractions 1, 2, 3 and 4 were isolated in different solvent compositions used for SEC. Fraction 1 in 50 mM tris HCl buffer, Fraction 2 in Milli Q water and Fraction 3 in 20 mM tris HCl. The reference was isolated using water as the buffer. The band of fraction 2 was found to be at same level with that of the reference using the same solvent for dissolving the protein.

a.



b.

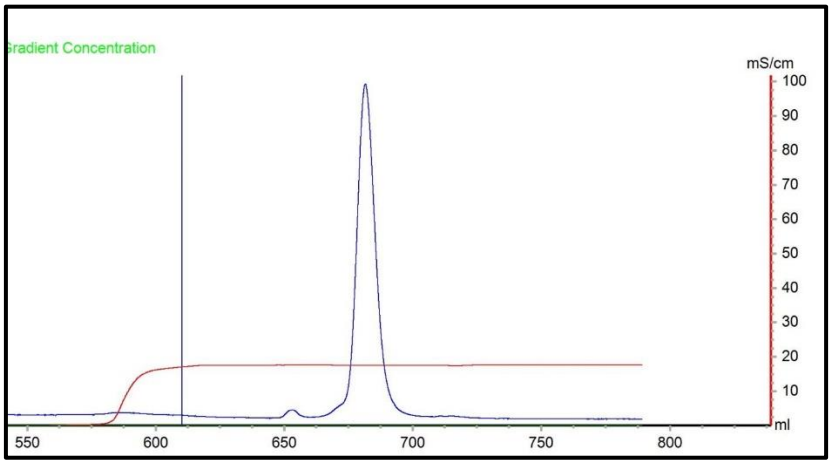


Fig.4.3.8. a. Chromatogram of Mutant C L-asparaginase obtained after third step of HIC. First step was HIC purification of crude obtained after diafiltration followed by SEC fractionation and the major fractionated peak was subjected to HIC again**; b.** SEC chromatogram of the reference Indian L-asparaginase biosimilar.

4.4. Discussion

The protein purified by single step Anion Exchange Chromatography was used to study the characteristics. RP-HPLC analysis was done for a reference Indian biosimilar and the components and their respective percentages was used to compare to that of the RP-HPLC done for Mutant C. This showed that the reference Indian biosimilar had 9 different components whereas, the Mutant C purified by AEC was having 15 components. The component at RT at 16 min was having 94.197% for the reference and RT at 16.013 min was having 96.543% composition for Mut C.

SEC-HPLC analysis was done for the same fractions of Mutant C and reference L-asparaginase biosimilar. The results showed that the reference had 26 components and at RT of 9.025 min, the component eluted had 93.529% and had molecular weight of 87.8243 kDa whereas, Mutant C had 10 components and at RT of 9.136 min (90.749%), the component had molecular weight of 81.1387 kDa. Thus, the major component in both the steps had molecular weight close to the L-asparaginase dimers (~70kDa).

Additionally, a study on the effect of different buffer compositions used in SEC was analyzed for the resolution of separation of components. Tris buffer in the mobile phase was able to provide greater separation resolution of the enzyme in comparison to water (**Fig.4.3.7. b.**).

However, for the need of higher purification (~99.9%), HIC chromatography in combination with SEC chromatography was studied. HIC chromatography was performed on the retentate of diafiltration. SDS-PAGE analysis showed single band in a collected elute 2B. This was used for SE Chromatography and was used for a third step purification using HIC method. The final chromatogram of the three-step purified Mutant C was compared to a single step SEC chromatogram of a reference Indian biosimilar and were found to be similar.

Chapter 5
Formulation Development

5.1. Introduction

Lyophilization is a well-known method that helps to increase the shelf-life and facilitate the transport of biologically active substances [48]. Other advantages of this technique involve preserving the enzyme in a dry state to reduce microbial/ degradation by denaturing agents. It reduces the oxidation of protein during long term storage and transport. The enzyme can be rapidly reconstituted and is generally dispersed homogenously in the excipient-protein formulation [49], [50].

All the L-asparaginase biosimilars are available in the form of lyophilized formulations which are reconstituted to be suitably administered intravenously/ intramuscularly to ALL patients. The objective of this study was to develop, optimize the freeze-drying cycle for our recombinant engineered variant of L-asparaginase (Mutant C) and establish a formulation similar to that of existing L-asparaginase biosimilars.

Table 5.1.1. The principle of stages of lyophilization cycles [51].

Freezing	**Primary drying**	**Secondary drying**
Cooling below triple point	Pressure is lowered and the preparation is heated to sublimate the ice (~95%)	Used to completely remove the water (dehydration) to ensure long-term stability of the enzyme at higher temperatures that primary drying
Freezing at ordinary temperature	Temperature of the enzyme remains is kept below its critical temperature	Pressure is lowered for desorption

5.2. Materials and Methods

5.2.1. Anion Exchange Chromatography

Anion exchange chromatography was done using Akta Explorer. The column used for Anion exchange chromatography is the Hi-trap Q-HP Sepharose + column (Cytiva (Marlborough, USA)). It has positively charged quaternary ammonium resin that binds to charged l-asparaginase and negatively elutes the protein. The buffers were equilibration buffer (20mM Tris) and elution buffer (20mM Tris, 0.5M NaCl). The column volume was 5 cv. The Unicorn software was set at method run, and the method was designed by putting 15 column volume (CV) and a flow rate of 2 ml/minute. The sample injected comprised of the crude obtained after diafiltration.

5.2.2. Lyo-cycle design for excipient-drug formulation

The Lyo cycle for the formulations was designed based on the principle of freezing, primary drying and secondary drying in a vacuum (pressure of about 0.01 bar of constant pressure). Lyophilization cycles were done using the LABCONCO lyophilizer. The freeze-drying cycle was optimized with excipient- water and protein- water. After successful trials excipient- protein in water was used to obtain the final lyophilized formulation.

5.2.3. Enzyme concentration determination

The estimation of protein concentration was done by using the UV spectroscopy method [52]. The coefficient of absorption Ɛ for the mutant L-asparaginase in Milli-Q water as solvent was estimated by this method. 1mg of lyophilized powder (without excipient) was weighed and dissolved in 1 ml of Milli-Q autoclaved water. Then the solution was serially diluted in the ratio of 1:10 with autoclaved Milli-Q. The O.D. of the known diluted solutions were taken at 280 nm using UV-1800 Shimadzu UV-Spectrophotometer in 2ml quartz cuvettes. A linear graph was plotted, and using Beer Lambert's law: $A=\mathcal{E}cl$, the slope of the graph was used to find Ɛ.

5.2.4. Enzyme activity determination

The enzyme solution of known concentration was diluted to 50µg/ml in tris HCl buffer at a pH of 7.3. To 10mM of the substrate solution (L-asparagine solution in the same buffer), 10µg of the enzyme was added and then incubated at 37ºC for 30 minutes. Then 1.5 M TCA solution was added to stop the reaction. Ammonia released by the enzyme in the presence of an asparagine solution of known concentration was determined by Nessler's Reagent, using colorimetric analysis at an O.D. of 480nm. A standard curve was prepared using a gradient of known ammonium sulphate solutions of concentrations in tris buffer at pH= 8, from 0,5,10,15,20 and 25 mM, and their O.D was taken at 480 nm. This was used to estimate the concentration of the product, that is, ammonia. Enzymatic activity (IU) is the amount of enzyme which produces 1µmol min^{-1} of ammonia at 37ºC at pH~7.3 (in accordance with pharmacopoeia of existing L-asparaginase biosimilars)

5.2.5. Excipient- drug composition for formulation preparation

The label claim of at least three Indian biosimilar L-asparaginases was studied for developing the drug-excipient formulation. For bio similarity, the drug: excipient ratio was kept in the order of 1:2 (w/w). All formulations were studied using autoclaved Milli Q as solvent to maintain similarity with existing biosimilars that are prepared using water for injection as the medium. The enzyme used for formulation development was 91% pure (HPLC data), 94.56% purity by 12% SDS PAGE.

5.2.6. Storage and reconstitution of formulations

All the formulated, non-formulated lyophilized and non-lyophilized enzymes were stored between 2-8 ºC under identical factors for a period of 90 days. They were all reconstituted with autoclaved Milli Q filter sterilized with 0.22 µm membrane.

5.2.7. Statistical Analysis

ANOVA (one-way) was used to analyze the enzyme activities of the formulated and non-formulated enzymes. Null hypothesis is tested by this method with the assumption that samples in all groups are drawn from populations with the same mean, whereas the alternate assumes them not to be drawn from the same populations. It compares the ratio of variances between several samples.

Mann-Whitney method was used to analyze the percent of activity recovered for the enzyme. The Mann–Whitney *U* test tests a null hypothesis of a non-parametric data set such that the probability distribution of a randomly drawn observation from one group is the same as that of a randomly drawn observation from the other group (equally likely) against an alternative that those distributions are not equal (not equally likely). U is calculated as:

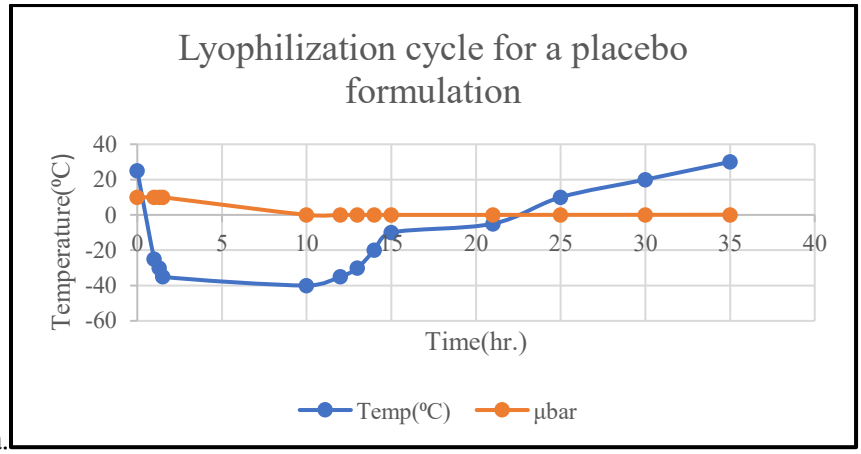
$$U_1 = n_1.n_2 + \frac{n_1(n_1+1)}{2} - R_1; U_2 = n_1.n_2 + \frac{n_2(n_2+1)}{2} - R_2 \qquad (9)$$

The p value (probability under the assumption of no effect or no difference of obtaining a result). A p-value of 0.05% in this method indicates that the null hypothesis has a 0.05% chance of being true and forms the basis of the rejection criteria for null hypothesis. A p-value <0.05% is indicated by *, p<0.01% is indicated by ** and p<0.001% by ***.

Graph pad Prism 5 was used for all statistical analysis in these experiments.

5.3. Results

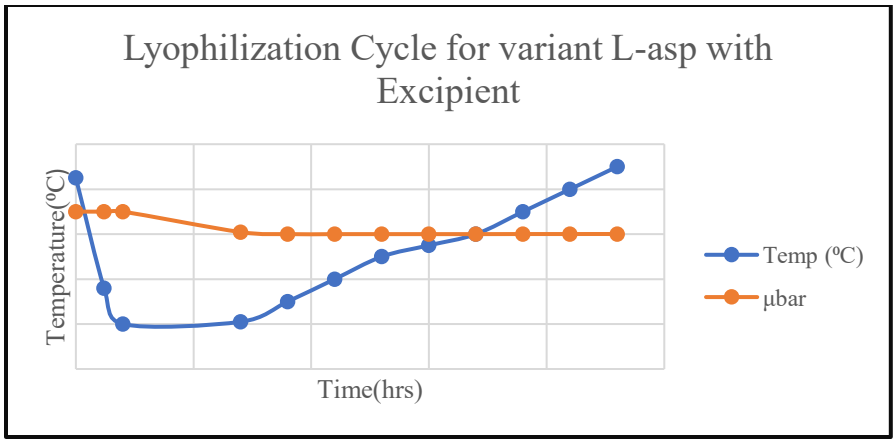
a.



b.

Fig.5.3.1. a. Lyophilization cycle optimized for a placebo formulation; **b**. Vials were tested for only excipient solutions in their requisite concentrations which were later used in drug formulation

a.



b.

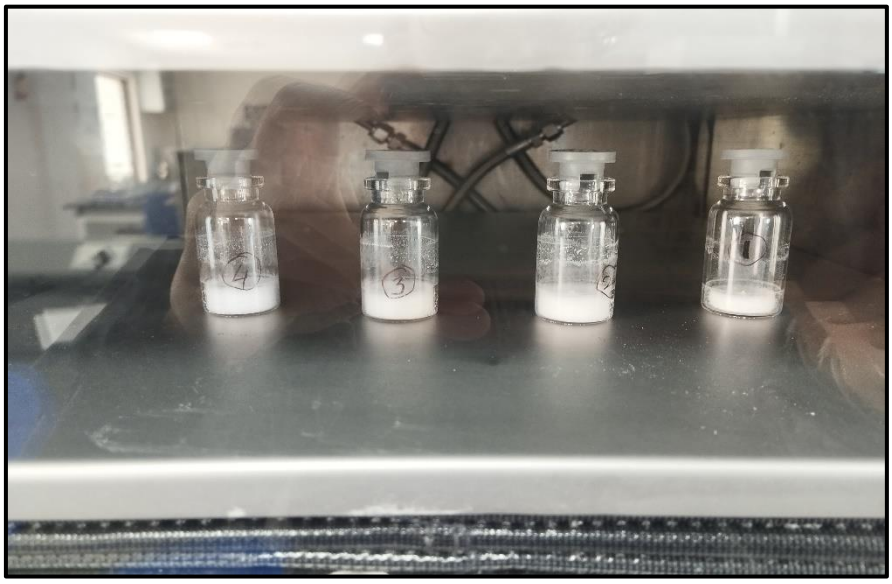
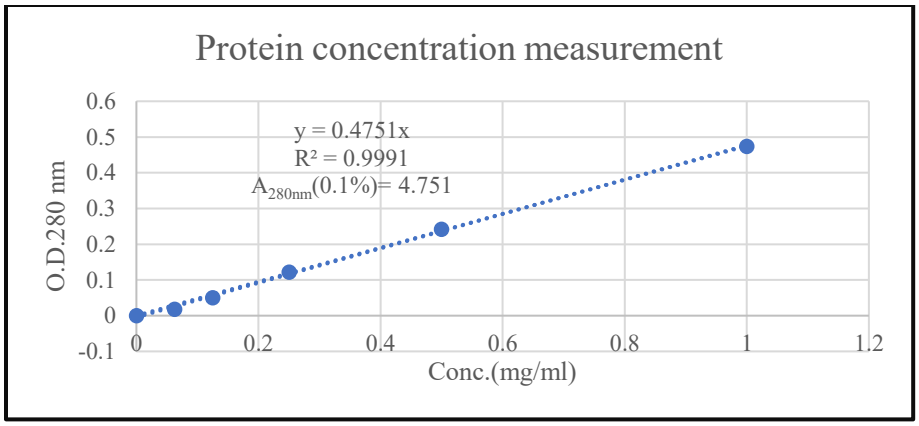


Fig. 5.3.2. a. Lyophilization cycle optimized for developing the injectable formulation that is shelved in powdered form (2-8ºC); **b.** Protein-excipient lyophilized vial in the lyophilizer.

a.



b.

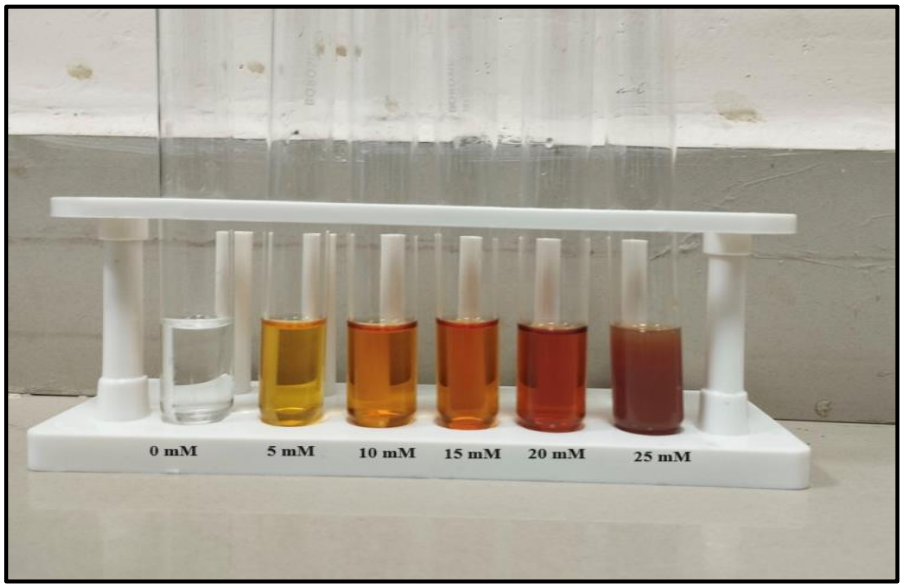
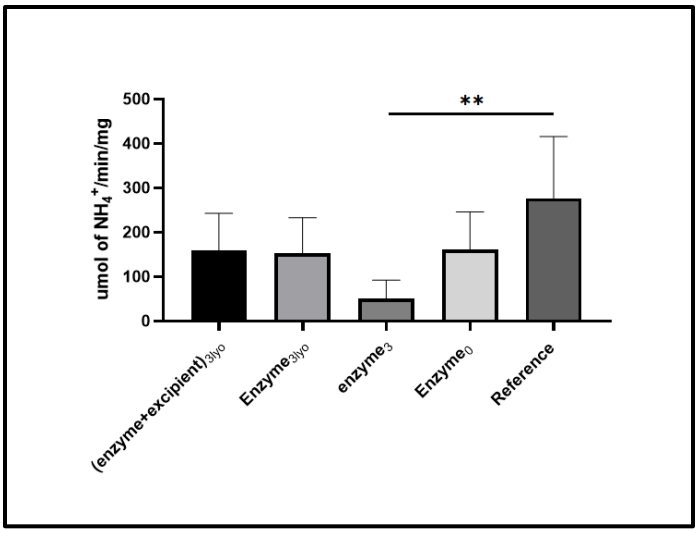


Fig.5.3.3. a. Protein concentration determination was done using spectrophotometric method at O.D.280nm; **b**. Activity determination by Nesslerization assay.

a.



b.

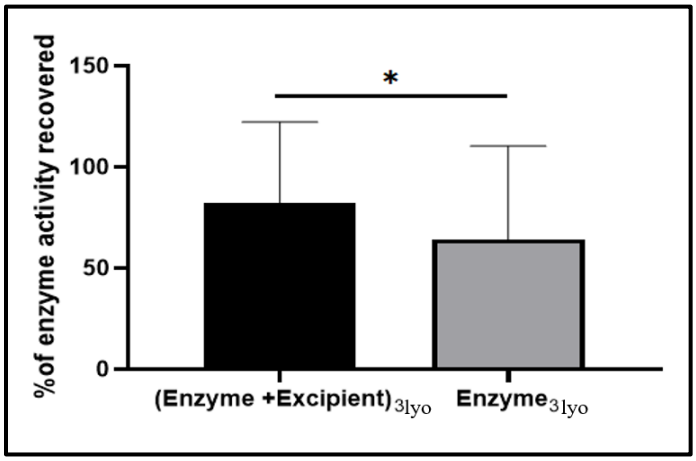


Fig.5.3.4. a. The activity of the enzyme without lyophilization and after 90 days in lyophilized state (all stored at 2-8 ºC)

Enzyme$_0$: Enzyme activity 90 days prior of purified protein

(Enzyme + excipient)$_{3lyo}$: Enzyme lyophilized with excipient

Enzyme $_{3lyo}$: Enzyme lyophilized without excipient

Enzyme$_3$: No excipient in enzyme, not lyophilized

Reference: Activity of reference biosimilar***;***

b. Percent of enzyme activity recovered in formulation in comparison to non-formulated enzyme both stored under identical conditions for 90 days in 2-8 ºC

(Enzyme + excipient)$_{3lyo}$: Enzyme lyophilized with excipient

Enzyme$_{3lyo}$: No excipient in enzyme and lyophilized

5.4. Discussion

The recombinant variant of L-asparaginase was purified using a single step anion exchange chromatography yielded protein of purity 91% purity (HPLC analysis) and 94.56% purity (12% SDS PAGE). A_{280nm} (0.1%) = 4.571 relation estimated using UV spectrophotometric method was used to quantify protein concentration. Enzymatic activity (IU) is defined as the amount of enzyme which produces 1µmol min^{-1} of ammonia at 37ºC at pH~7.3 (in accordance with pharmacopoeia of existing L-asparaginase biosimilars).

The activity of our enzyme in formulation was found to show no significant difference (ns) when compared to the reference l-asparaginase biosimilar.

However, the enzyme which was lyophilized without the excipient showed $p<0.05$ difference with the percent recovery of activity in comparison to the one which was lyophilized with the excipient. The enzyme which was not lyophilized (with/ without any excipient) showed significant reduction in activity ($p<0.01$) and there was fouling which indicated degradation after 3 months.

Chapter 6

Conclusions

6.1. Synopsis

L-asparaginase biosimilars, according to several reports are known to significantly improve the outcome of ALL treatment. However, global disparity exists in the availability and the lack of international parity in the quality attributes of the drug. Therapeutic limitations like toxicity, immunogenicity, short half-life and poor in-vivo enzymatic activity also cause a lot of side-effects associated with administration of the drug. With these problem statements, the thesis delves into the drug development of a novel, engineered, recombinant variant of L-asparaginase which has shown to be lesser immunogenic and toxic, however maintaining the enzymatic activity and half-life similar to that of the wild-type enzyme, based on in-vivo mice model studies and pre-clinical studies using blood samples from ALL patients.

Most of the lab-grown technologies in medicinal biotechnology fail to reach the patient due to factors like non-scalability, non-transferability and/ or most during clinical trial stages. Drug developmental stages before the biologic can be taken to the clinical trial stage includes, bioprocess scalability, bio similarity attribute assessment to qualify regulations and feasible formulation for administration of the drug.

Both the upstream and downstream processes have been evaluated along with the positive results in scalability of fermentation batches. The growth rate during the 2.5 L fed-batch fermentation was 7.42% higher than that in the 25 L fermentation. However, the scale up criterion as a function of agitation speed was studied and the graphs indicated acceptable and comparable changes in both during growth in the exponential feeding mode. This opens a scope for feed rate determination optimization during scale up and a model of how it can be done has been outlined in the thesis.

The pre-requisite of characterization involved attaining a 99.9% purity of the enzyme straight from the crude processed following downstream using suitable purification methods and/or combinations of several such methods. The efficiency of a SEC-HIC based chromatographic purification has been given.

The final work of this thesis has been to show the development of a suitable injectable formulation at least at par with existing L-asparaginase biosimilars used clinically. The performance of the enzyme in the excipient and without excipient has been studied, the enzymatic activities have been found to be comparable / not significantly different from the activity of a reference biosimilar that uses the same excipient in their lyophilized formulations.

6.2. Scopes

There are several scopes in this work:

- The feed rate calculation model needs validation after optimizing three 25L fermentations and the corrected feed rate needs to be determined. Further, experimentation should be done to determine the accurate growth rate by the dry cell weight method.

- The CQA analysis profile needs to be created using several biophysical techniques like LC-MS, peptide sequencing for primary structure, secondary and tertiary structure determinations using UV Spectroscopy, CD, the isoelectric point of Mutant C using isoelectric focusing, and percentage of prevalent variants in the 99.9% pure enzyme solution.

- Further experimentation using additives in the formulation solvent that can increase the enzymatic activity upon reconstitution of lyophilized formulations.

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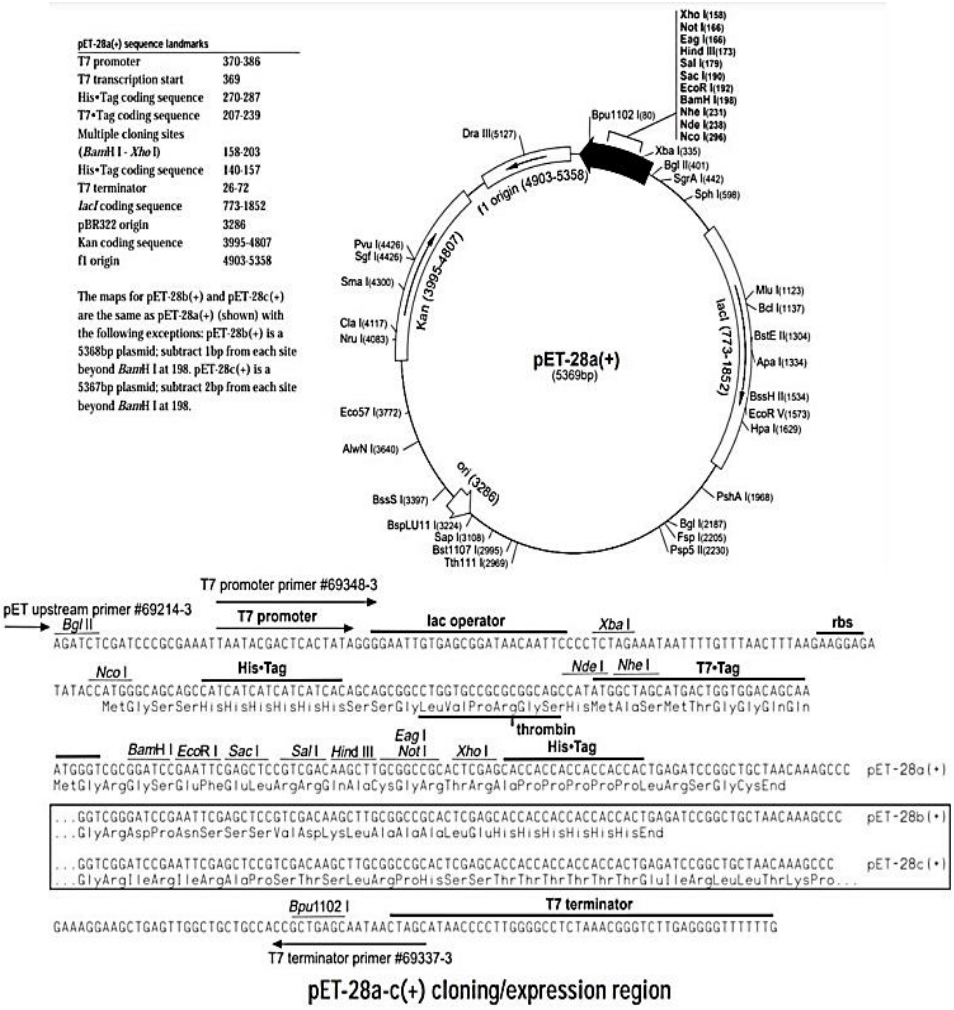
APPENDIX-A



pET-28a-c(+) cloning/expression region

Table: Summary of the selected engineered variants that were developed after screening and their properties.

Mutants	Pharmacokinetic property in mice models	Immunogenicity in mice models for antibodies in primary ALL	Immunogenicity in mice models for antibodies in relapsed ALL	Comments
Mutant A	Half-life ***(267.28 ± 9.74)	IgG ns	IgG ns	-
	Mean Residence Time ***(290.44±5.85)	IgM ns	IgM ns	-
Mutant B	Half-life **(213.29 ± 6.53)	IgG **	IgG***	32.63% less than wild type EcAII in binding to antibodies
	Mean Residence Time **(263.98±23)	IgM**	IgM**	2-fold less binding to antibodies in relapse free ALL patient samples
Mutant C	Half-life ns	IgG **	IgG***	27.43% less than wild type EcAII 2-fold less binding to antibodies in relapse free ALL patient samples
	Mean Residence Time ns (171.79± 7.58)	IgM***	IgM***	4-fold lower titers of IgM in comparison to wild type
Mutant D	Half-life ***(273.83 ± 35.45)	IgG**	IgG**	49.09% less than wild type EcAII in binding antibodies, 2-fold less binding to antibodies in relapse free ALL patient samples
	Mean Residence Time *(273.90± 11.48)	IgM***	IgM***	5-fold lower titers of IgG and lower titers of IgM in comparison to wild type