A DISSERTATION ON

"Downstream Process Development for Bio-therapeutic Monoclonal Antibody"

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF B. TECH-M. TECH DUAL DEGREE
IN BIOTECHNOLOGY

BY
MD.AASIF
B. TECH-M. TECH BIOTECHNOLOGY (X SEMESTER)
ROLL NO: 1801010023
UNDER THE SUPERVISION OF
Dr. ABYSON JOSEPH
RESEARCH SCIENTIST AND LEAD DOWNSTREAM PROCESSING R&D GROUP
PREMAS BIOTECH PVT. LTD.,Manesar

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD

LUCKNOW- 226026

DECLARATION FORM

I'm Md. Aasif, a student of **B.Tech - M.Tech Dual Degree Biotechnology (5th year/10th semester),** Integral University has completed my six months' dissertation work entitled **"Downstream Process Development for Bio-therapeutic Monoclonal Antibody"** successfully from **Premas Biotech Pvt. Ltd.** under the able guidance of **Dr. Abyson Joseph (Research scientist).**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Md. Aasif

Date

Dr. Reena Vishwakarma

Date

Premas Biotech

CERTIFICATE

This is to certify that Mr. **Md. Aasif,** Enrollment Number **1800101943** has carried out the research work presented in this thesis entitled **“Downstream Process Development for Bio-therapeutic Monoclonal Antibody”** for the award of (**B.Tech-M.Tech Dual Degree Biotechnology** from Premas Biotech Pvt.Ltd. under my supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University. The dissertation was a compulsory part of his (**B.Tech-M.Tech Dual Degree Biotechnology**).

I wish him good luck and bright future.

Dr. Abyson Joseph

(Supervisor)

Research Scientist

Premas Biotech Pvt. Lim.
Department of R&D Downstream

INTEGRAL UNIVERSITY

LUCKNOW - INDIA

Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809

Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Md. Aasif** a student of Integral University has completed his six month's dissertation work entitled **"Downstream Process Development for Bio-therapeutic Monoclonal Antibody"** successfully. He has completed this work from **Premas Biotech Pvt. Ltd.** under the able guidance of **Dr. Abyson Joseph (Research scientist).** The dissertation was a compulsory part of his **B. Tech-M.Tech. Dual degree Biotechnology**.

I wish him good luck and bright future.

Dr. Roohi
Professor
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA

Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Md. Aasif** a student of **B. Tech - M. Tech Dual Degree Biotechnology (5th year/ 10th semester),** Integral University has completed his six months' dissertation work entitled **"Downstream Process Development for Bio-therapeutic Monoclonal Antibody"** successfully. He has completed this work from **Premas Biotech Pvt. Ltd.** under the able guidance of **Dr. Abyson Joseph (Research scientist).** The dissertation was a compulsory part of **B. Tech-M.Tech. Dual degree Biotechnology**.

I wish him good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

ACKNOWLEDGEMENT

I am grateful to The Almighty for the good health and blessings necessary to complete this project. Exceptional gratitude goes to my friends and family, and well- wishers for their love, support, encouragement, and blessings throughout my thesis.

Prof. S.W. Akhtar, Hon’ble Chancellor and founder, his visionary leadership and commitment to academic excellence have paved the way for the creation of this esteemed institution. His encouragement has inspired me to strive for excellence in my research

Dr. Syed Nadeem Akhtar, Hon’ble Pro Chancellor his unwavering dedication to the university's growth and development has been instrumental in creating an environment conducive to learning and research. His support has motivated me to push my boundaries and contribute to the scholarly community.

Prof. Javed Musarrat, Hon’ble Vice Chancellor, his guidance and mentorship have been instrumental in shaping my research journey. His commitment to fostering academic curiosity and nurturing young scholars has been a constant source of inspiration for me.

Prof. (Dr.) Mohd. Haris Siddiqui, Registrar I am grateful for your administrative support and assistance throughout the thesis submission process. His guidance has helped me navigate various procedures smoothly.

Prof. T. Usmani, Dean his expertise and insightful feedback have been invaluable in refining the direction of my research. His dedication to academic excellence has motivated me to strive for the highest standards in my work.

I would also like to thank **Prof. (Dr.) Alvina Farooqui**, Head, Department of Bioengineering, Integral University, Lucknow, her expertise and encouragement have been instrumental in steering me through the challenges of my research. Her passion for the subject matter has been contagious, motivating me to explore new horizons. Dr. Khawaja Osama, U.G. Coordinator, **Dr. Khawaja Osama** and **Dr. Reena Vishwakarma**, Course Coordinator, Assistant Professor of bioengineering department, for providing us with all necessary support throughout my journey.

I am also grateful to **Dr. Roohi**, Professor, **Dr. Ashish kalicharan**, Assistant professor, **Dr. Zeeshan Rafi,** Department of Bioengineering, Integral University, Lucknow. Their support, guidance, and advice have helped me through the entire process of my dissertation. I thank all of them sincerely for all the efforts

I would like to express my deepest gratitude and special thanks to the **Dr. P.K. Kundu** and **Dr. Nupur Malhotra** who gave me this golden opportunity to work here. The Dissertation opportunity I have with Premas Biotech is a great chance for learning and professional development. Therefore, I consider myself as a very lucky individual as I was provided with an opportunity to be a part of it.

I am thanking of my Supervisor **Dr. Abyson Joseph** Research Scientist Department of DSP in Premas Biotech Pvt.lim**,** for their help and guidance about all the techniques, analytical and theoretical aspect of the overall subject matter whenever needed. I am thankful my DSP team member **Dr. Nupur Pandey** Senior manufacturing head, **Mr. Prince Mathur**, **Ms. Sakshi** and **Mr. Mantosh Singh** for helping to this project successfully.

I am thankful to Analytical team member **Dr. Saurab Singha, Dr. Eswar Reddy and Mr. Abheek Sen** Gupta for their careful and precious guidance which were extremely valuable for my study both theoretically and practically.

I also want to thank **Ms. Ankita, Ms. Ananya, Ms. Dixit** and **Ms. Sarita Saini** for their efforts that made this work successful.

I am thankful to Human Resource team **Ms. Barkha** Singh and **Mr. Arun Raturi** for permitting me to complete my whole Dissertation work and providing me necessary facilities for carrying out the present work.

Date: 30/07/2023 **MD. AASIF**

ABBREVIATIONS

TFF	Tangential Flow Filtration
UFDF	Ultrafiltration Dia-filtration
DV	Dia-filtration Volume
TMP	Total Membrane Pressure
RT	Retention time
FT	Flow Through
CV	Column Volume
AC	Affinity Chromatography
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid.
EQB	Equilibration Buffer
FT	Flow through
gm	Gram
mg/mL	Milligram per milliliter
mL	Milliliter
mM	Millimolar
NaCl	Sodium Chloride
ng/µl	Nano gram per microliter
SDS	Sodium dodecyl sulphate
TFF	Tangential Flow Filtration
UF/DF	Ultrafiltration & dia-filtration

LIST OF CONTENTS

S.NO.	PARTICULATES	PAGE NO.
1	Introduction and Objectives	13-16
2	Review of Literature	17-30
3	Materials and Methods	31-43
4	Results and Discussion	44-51
5	Conclusion	52
6	References	53-55

List of Figure

Fig.No.		Page. No.
1.	Market Figure value of Bio-therapeutics.	19
2.	Structural compression between the full-length antibody and Single chain fragment variable (scFv).	23
3.	Production of Recombinant Antibody.	26
4.	Recombinant his tag protein formation.	28
5.	Interaction of His tagged protein with Ni-NTA ligand.	29
6.	Periplasmic Incubation for 16 hours at 28°C.	33
7.	After centrifugation Supernatant was collected in bottle	34
8.	Filtration of Sample with the help of vacuum pump.	34
9.	Sample loading on column chromatography.	35
10.	Sample was placed on Rocker.	37
11.	Sample filled-in Dialysis bag.	38

12.	illustrates the utilization of a 5ml prepacked Ni-NTA column for purifying a His-tagged protein.	38
13.	Representative diagram of different step of western blotting.	41
14.	Difference between Normal and Tangential Flow Filtration.	43
15.	Process chromatogram profile of Capto L run is given.	44
16.	12% Reducing SDS-PAGE analysis of Capto L chromatography fractions.	45
17.	Batch binding SDS Result of scFv with Ni-NTA resin.	46
18.	Process chromatogram profile of 200mL Sample Ni-NTA run is given.	47
19.	12% Reducing SDS-PAGE analysis of Ni-NTA chromatography fractions.	47
20.	Western result of scFv antibody.	48
21.	12% Reducing SDS-PAGE checking the fractions.	49
22.	The figure is showing the purity level of purified protein.	49
23.	Process chromatogram profile of MabSelect Prism A run.	50
24.	Result of full length Ig G Non-Reducing and Reducing SDS- PAGE Gel.	51

List of Table

Table. No.		Page. No.
1.	BUFFERS USED IN 1st METHOD	31
2.	BUFFERS USED IN 2nd METHOD	32
3.	Steps in Capto L Procedure	36
4.	Steps in chromatography Ni-NTA Procedure	39
5.	Loading Parameter in SDS-PAGE 12% Capto L Gel	45
6.	Loading Parameter in 12% Ni-NTA Gel	47
7.	Loading Parameter in western blotting	48
8.	The concentration of the purified protein varies slightly when assessed through these three analytical methods.	48
9.	Data of Densitometry	49

INTRODUCTION

Monoclonal antibodies (mAbs) have solidified their position as effective and established pharmacological treatments. They operate by targeting and addressing crucial factors implicated in conditions like inflammation, cancer, and autoimmune diseases. As per report of world health organization nearly 10 million deaths in 2022. The global monoclonal antibodies market is witnessing significant growth due to the escalating global cancer cases. Monoclonal antibodies play a vital role in cancer treatment, driving their widespread utilization in this field. (Grilo, A. L., *et* al 2019). That why we are producing mAbs which are specific to cancerous cell and have ability to reduce the migration rate of cancerous cells. The demand of mAbs day by day increases because mAbs are specific to the target as compared to the chemotherapy less toxic in chemotherapy use of high dose of chemicals which are also affect the non-cancerous cell in the body lead that is lead to hair loss and extreme weakness. mAbs are made up of protein which specific bind to the surface receptor present on surface of cancer cell. Monoclonal antibody (mAbs) lesser toxic as compared to chemotherapy. (Adams, G. P., *et* al 2005). Heterogeneity of purified antibodies (immunoglobulin, Ig) based on the sample chemical modification of selected amino acid site is of considerable importance in the biotechnology field.In general, mAbs like many proteins, have charge heterogeneity that optimizes the balance of gaining favorable electrostatic interactions and determines their structure, stability, binding affinity, chemical properties and hence their biological reactivity. During manufacture, various forms of micro heterogeneity in size, charge and other parameters occur due to enzymatic processes or spontaneous degradation modifications. mAbs undergo chemical degradation via several different mechanisms including oxidation, deamination, isomerization and fragmentation which result in formation of various charge variants and heterogeneity, thus modifying their isoelectric points (pI). Monoclonal antibody technologies are providing exciting opportunities for new biopharmaceutical developments and new approaches to the diagnosis, treatment and prevention of diseases. monoclonal antibodies are also that are produced by using recombinant DNA technology. Conventional monoclonal antibodies are generated by immunizing animals with immunogens to provoke immune responses. B cells are extracted from immunized animals and merged with a myeloma cell line, resulting in immortalized hybridomas Recombinant monoclonal antibodies, on the other hand, are produced using in

vitro cloning. Genes for an antibody's light and heavy chains are inserted into expression vectors, which are then transfected into host cells for expression. This approach very fruitful for the production of therapeutic molecule because monoclonal antibodies are produce in high amount, fast production, cost effective and very efficient.

In 1972 and 1973, Peter Lobban pioneered the concept of recombinant proteins and the replication of recombinant DNA, marking a significant advancement in the field (P, Surat.2019). Recombinant protein produces by fusion of sequence which are generally not present in host organism. (Huang, C. J., *et* al 2012). The inserting the gene into the host cell by help of various physical and biological methods. Host cell replicate and express the gene of interest yield protein. Recombinant antibodies (rAbs) are derived from recombinant antibody coding genes and display technology. They offer notable advantages such as enhanced reproducibility, specificity, and scalability. Unlike traditional monoclonal antibodies (mAbs) produced through hybridoma methods, rAbs eliminate the need for hybridoma cells and animal use in production when synthetic genes are employed. This innovation streamlines the production process and underscores the potential of synthetic gene-based approaches (Ahmad, Z. et al 2012). The biotechnological production of protein-based therapeutic is one the fastest growing sectors of bio-pharmaceutical industry. In present era, recombinant protein plays a key role for making therapeutic drugs in biotechnological field. One such recombinant protein is monoclonal antibodies which gained significant attention as potential therapeutics due to the high degree of specificity in binding to target antigens, ability to initiate immune response to the target antigens and long serum persistence, thereby reducing the need for frequent dosing

The topic of my Dissertation is optimizing protein purification which is totally a part of live project going on in Premas Biotech pt. limited The purification process for biomolecules can be broken down into three main stages: Recovery, separation, purification, and final polishing. Separation involves mechanical methods like microfiltration or centrifugation to remove cells. Chromatography is the primary technique for purification due to its selectivity and gentle approach. The key chromatographic methods include Affinity chromatography, Ion exchange, Size exclusion (SEC), and Hydrophobic interaction (HIC). Although Affinity chromatography offers high selectivity, it is limited by the complexity of developing suitable ligands and the cost of resins. However, it remains the go-to method for purifying mAbs (monoclonal antibodies), Fabs (antigen-binding fragments), and scFv (single-chain fragment

variables). These fragments are often purified using two types of ligands: antigens that leverage antigen-binding capabilities, or less specific ligands that bind to the constant domain (Fc fragment). Notably, Protein G and Protein A are widely used Fc-binding natural immunoglobulin-binding ligands, despite their drawback of being costly.

The Single Chain Fragment Variable (scFv) stands as a pivotal protein fragment within the realm of bio therapeutics, setting itself apart from full-length antibodies like Immunoglobulin G (IgG) in both structure and function. Crafted from the fusion of the variable regions of the antibody's heavy (VH) and light (VL) chains, the scFv comprises a solitary polypeptide chain. This linkage is bridged by a peptide linker, ensuring fluid collaboration between VH and VL. scFv fragments are harnessed through recombinant DNA technology, with VH and VL genes either combined in vitro or integrated into host cells for expression. These fragments commonly find their expression in the Periplasmic space of Escherichia coli (E. coli) bacteria. (Griffiths, A. D.*et* al 1998).

The scFv's more compact size, weighing approximately 25-33kDa, contributes to its diminished immunogenicity compared to larger antibody counterparts. Impressively, scFv fragments can be fine-tuned to specifically target a diverse array of molecules, encompassing haptens, proteins, carbohydrates, receptors, tumor antigens, and viruses. (Huston, J. S., et al.,1996) ;(Jones, S. D. et al.,1998), (Batra, S. K. et al.,2002). The realm of medical therapies and diagnostic applications is where scFv fragments radiate potential. Their adaptability extends to serving as the cornerstone for targeted drug delivery systems, antibody-drug conjugates, and radioimmune conjugates in cancer treatment. Furthermore, scFv fragments assume a critical role in diagnostic assays, biosensors, and advanced imaging techniques (Ahmad, Z. A., et al., 2012). Diving into the specifics, the VH (Variable Heavy) domain constitutes the variable segment of the antibody's heavy chain, spanning around 110-130 amino acids. Positioned at the scFv's N-terminus, the VH domain boasts three complementarity-determining regions (CDRs) that orchestrate antigen recognition and binding. In parallel, the VL (Variable Light) domain, housing approximately 90-110 amino acids, resides at the ScFv’s C-terminus. This VL domain also embraces three CDRs pivotal for antigen binding and recognition Facilitating the seamless collaboration between VH and VL, a flexible peptide linker intervenes, usually comprised of 10 to 25 amino acids. This pliable connection grants the VH and VL domains the capacity to function harmoniously as a unified polypeptide chain, embodying the essence of the scFv's intricate design.

Objectives

1. The main aim was the purification of protein of interest i.e., scFv from the given sample.
2. To do the confirmation assay by SDS-PAGE and Western blot analysis followed by the formulation of the molecule.
3. Two different strategies were taken forward for the purification of scFv (Capto L followed by Ni-NTA and Ni-NTA)

Review of literature

2.1. Bio-therapeutics

Bio-therapeutic proteins are the biological molecule defined as a large complex of protein which is engineered in the laboratory for pharmaceutical use, (Griswold, K. E., *et* al 2016). unlike other medicines; they are not synthetically produced, but are usually produced through microbial fermentation or mammalian or insect cell culture. This Bio-therapeutics of large-molecule has revolutionized the treatment of a variety of diseases in areas such as oncology, inflammatory and autoimmune diseases, hemophilia, cardiovascular disease, infectious diseases, and rare genetic diseases. In comparison with small-molecule Bio-therapeutics, bio-therapeutics have a higher approval success rate and a similar development phase length. Recombinant DNA technology has made a revolutionary impact in the area of human healthcare by enabling mass production of safe, pure and effective drugs. The first such substance approved for therapeutic use was biosynthetic 'human' insulin made via recombinant DNA technology. The majority of biopharmaceuticals marketed today, are recombinant therapeutic protein molecule. Currently, several categories of rDNA products, like antibody fragments, growth factors, blood coagulation products, thrombolytic agents, anticoagulants, interferon" interleukins and therapeutic enzymes are being produced using rDNA technology for human use (Ghilardi, N., *et* al 2020). Biopharmaceuticals, including proteins, antibodies, and nucleic acids, are produced using biotechnology and serve therapeutic or in vivo diagnostic purposes. Approval for human use entails extensive clinical trials spanning several years. Currently, over 160 biopharmaceutical agents are approved in the USA and/or EU, with a significant focus on cancer treatment. Beyond cancer and diabetes, biopharmaceuticals have advanced the management of rheumatoid arthritis and various blood disorders. The biopharmaceutical market has witnessed substantial growth in recent years, driven by both an increasing product count and the utilization of these therapies for chronic conditions in large quantities (Coker, V. 2012). To cater to a broader patient base, expanding manufacturing capacity is necessary for the production of these efficient biopharmaceuticals. In comparison to traditional medicines, bio therapeutics offer numerous advantages.

Firstly, they are highly specific and targeted, meaning they can be designed to only affect certain cells or tissues in the body. This reduces the risk of side effects and makes them more effective in treating diseases

Secondly, Bio-therapeutics can be efficiently manufactured at a large scale while maintaining exceptionally high levels of purity, ensuring a reliable standard of quality and potency. This is particularly important for chronic diseases that require long-term treatment. Antibody-based

Bio-therapeutics have rapidly emerged as a swiftly expanding category of pharmaceuticals, spanning across diverse therapeutic domains (Licari, G., *et* al 2022). Considering the ongoing rate of approximately four new product approvals annually, Estimates suggest that approximately 60-80 monoclonal antibody products will have entered the market by the year 2020.Consequently, the cumulative global sales for these products are anticipated to approach a substantial figure of nearly $125-130 billion Ecker (D. M., *et* al 2015).

These products include various types of treatments, such as recombinant proteins, antibodies, vaccines, gene therapies, and stem cell therapies (Johnson, D. E. 2018). Bio therapeutics play a crucial role in treating a broad array of diseases and medical conditions. Bio-therapeutics have transformed. disease treatment, and targeted therapies have shown promising outcomes (Mauro1, 2018). The production of these Bio-therapeutics agents has witnessed remarkable progress over the past decade, and one of the key factors driving this advancement is the utilization of recombinant DNA technology. This technology allows for the expression and synthesis of therapeutic proteins in mammalian cell lines, which serve as efficient protein production factories. Among the strategies employed to increase the levels of protein expression in these cells, codon optimization has proven to be highly effective. Recombinant proteins play a vital role in this field, and scFv, a specific type of recombinant protein, offers high affinity and specificity for disease-related targets. This section introduces the concept of bio therapeutics, the significance of targeted therapies, and the emergence of scFv as a valuable tool in this domain. The purpose and scope of the review are outlined.

Bio-therapeutics, also known as biologics or biological therapeutics, refer to medical products derived from living organisms or produced using biotechnology. These therapies are used to treat various medical conditions, including genetic disorders, autoimmune diseases, cancers, and other chronic illnesses. Unlike traditional small-molecule drugs, Bio-therapeutics are large and complex molecules, often proteins or nucleic acids, that interact with specific targets in the body to produce therapeutic effects. Bio-therapeutics are a rapidly growing portion of the total pharmaceutical market accounting for almost one-half of recent

new drug approvals (Johnson, D. E. 2018).

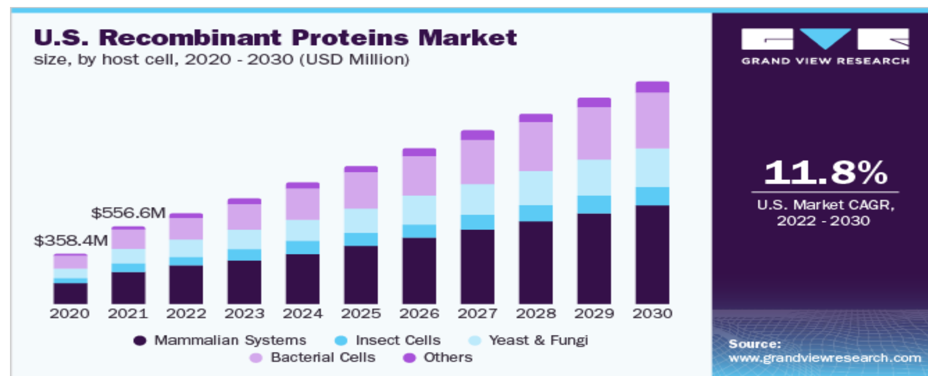

Fig 1. Market Figure value of Bio-therapeutics

As per the Pharmaceutical Research and Manufacturers of America (PhRMA), biopharmaceutical companies are actively engaged in the development of 887 cancer medications, all these products are currently either in various stages of clinical trials or are undergoing evaluation by the FDA This comprehensive count encompasses 98 treatments targeted at lung cancer, 91 for breast cancer, 80 for prostate cancer, and 55 for colorectal cancer. Notably, among the 27 new pharmaceuticals and biologics approved by the FDA in 2010, 12 were biologic-based products. A notable trend emerged in the field until 2007, where the focus shifted from vaccine development to products like monoclonal antibodies (mAbs). However, subsequent to that period, there has been a resurgence in vaccine research. For instance, in 2008, the count of candidate vaccines in the development pipeline surged from a mere 62 in 2006 to 223. Despite this rise, monoclonal antibodies, previously the leading category in the pipeline, were surpassed, even as the number of investigational drugs increased from 160 to 192 during the same timeframe (Coker, V. 2012).

2.2. Types of Bio-therapeutics:

Monoclonal Antibodies (mAbs): Monoclonal antibodies are engineered proteins that target specific molecules or cells in the body. They can be designed to block disease-causing proteins, activate the immune system, or deliver drugs to specific cells.

Recombinant Proteins: These are therapeutic proteins produced using recombinant DNA technology. They include growth factors, cytokines, hormones, and clotting factors, among others.

Vaccines: Vaccines are Bio-therapeutics products that trigger the immune system to produce protective antibodies that act on against specific pathogens, providing immunity against infectious diseases. (Metzger, 1983)

Gene Therapies: Gene therapies involve the introduction, alteration, or removal of genetic material to treat or prevent genetic diseases. They aim to correct faulty genes or replace missing or defective genes with functional ones.

Cell Therapies: Cell therapies involve the use of living cells, such as stem cells or immune cells, to repair, regenerate, or replace damaged tissues or to boost the immune response against diseases like cancer.

Nucleic Acid-Based Therapies: These therapies use nucleic acids, such as DNA or RNA, to modulate gene expression or target specific disease-causing genes.

2.3.Antibody

An antibody, or immunoglobulin (Ig), is a sizable Y-shaped protein generated by the immune system to identify and counteract foreign substances known as antigens. Antibodies have a pivotal function in safeguarding the body against infections and illnesses. They constitute a vital aspect of the adaptive immune response, signifying their distinctiveness for every distinct antigen confronted by the immune system (Murphy, et al., 2016).

2.4. Structure of Antibody

Antibodies consist of four polypeptide chains, with two identical heavy chains and two identical light chains. These chains are interconnected by disulfide bonds, creating the fundamental Y-shaped configuration. Each arm of the Y holds a distinct antigen-binding area termed the variable region, accountable for identifying and attaching to the antigen (Davies, D. R., & Metzger, H. 1983).

2.5. Types of Antibodies: There are Five primary categories of antibodies exist each with distinct functions:

IgM: The first antibody produced during an initial immune response. It is effective in agglutination and complement activation.

IgG: The most abundant antibody in the blood. It provides long-term immunity and crosses the placenta to protect the fetus.

IgA: Found in body secretions like saliva, tears, and breast milk, providing localized immunity at mucosal surfaces.

IgD: Present in minimal quantities, this antibody class contributes to B cell activation

IgE: Involved in allergic reactions and defense against parasitic infections.

2.6. Functions of Antibodies

Antigen Recognition: Antibodies are highly specific to antigens, such as bacteria, viruses, toxins, or foreign particles. When an antigen enters the body, antibodies with complementary binding sites are produced to specifically recognize and attach to the antigen (Forthal, D N. 2014).

Neutralization: Upon binding to the antigen, antibodies can neutralize its harmful effects by preventing it from infecting or damaging cells. This process can make the pathogen or toxin harmless.

Opsonization: Antibodies can enhance the process of phagocytosis, where immune cells called phagocytes engulf and destroy pathogens. Antibodies interact to the antigens on the surface of the pathogens, marking it for recognition by phagocytes.

Activation of Complement System: Antibodies can trigger the complement system, a series of proteins that help destroy invading pathogens. This process culminates in the creation of membrane attack complexes, which possess the ability to directly eliminate specific pathogens.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC): Antibodies can recruit other immune cells, such as natural killer (NK) cells, to target and destroy cells that are infected with pathogens.

2.7. Monoclonal Antibody

A Monoclonal antibody (mAb) is a laboratory-created protein designed to emulate the immune system's capacity to identify and pinpoint specific substances, such as pathogens or abnormal cells. Monoclonal antibodies are engineered to bind to a single, specific target molecule, known as an antigen (Nelson, P. N., et al 2000). They have gained immense significance in medicine and biotechnology due to their precision and effectiveness in targeting specific cells or molecules. Monoclonal antibodies provide a promising path for focused anticancer therapy, harnessing the immune system to inhibit cancer cell function and eradicate tumors. The triumph of IgG monoclonal antibodies has spurred the creation of diverse therapeutic antibody variants, encompassing antibody fragments, bispecific antibodies, and antibody derivatives such as antibody-drug conjugates and immunocytokines. These versatile antibodies provide flexible strategies for diagnosing and treating complex tumor environments (Jin, S., *et* al.,2022).

2.8. Single Chain fragment variable

scFv is composed of variable heavy and light chain domains connected by a flexible peptide linker. (Griffiths, A. D., & Duncan, A. R. (1998). This section provides an in-depth explanation of the structural components of scFv, emphasizing the importance of the linker region in maintaining the functionality and stability of scFv (Ahmad, Z. A *et* al., 2012). Single-chain variable fragments (scFv) are antibody fragments commonly produced in E. coli using recombinant DNA technology. scFv has shown remarkable potential in targeted therapy. This section highlights the ability of scFv to specifically deliver therapeutic agents to diseased cells, minimizing off-target effects and enhancing treatment efficacy. It presents case studies and examples of scFv-based targeted therapies, such as the use of scFv against tumor-specific antigens in cancer treatment (Batra, S. K, et al., 2002). scFv has gained significance in diagnostics due to its high specificity and sensitivity. This section explores the use of scFv in detecting disease-related biomarkers and molecules. It discusses the advantages of scFv in diagnostic assays, such as rapid and accurate detection, and provides examples of scFv-based diagnostic applications, including the detection of infectious agents and biomarkers in various diseases (Jones, S. D., *et* al., 1998). It highlights the versatility of scFv in molecular biology techniques, such as enzyme-linked immunosorbent assays (ELISA) and Western blotting, and its contribution to understanding protein (Winter, G *et* al., 1994).

2.9. Structure of single chain fragment variable

The scFv format consists of the variable regions of the heavy chain (VH) and light chain (VL) of an antibody connected by a flexible peptide linker, resulting in a single polypeptide chain. (Glockshuber, R., *et* al., 1990). The precise length of the flexible DNA linker connecting the two V domains is crucial for ensuring proper folding of the polypeptide chain. Previous studies suggest that the peptide linker should cover a distance of approximately 3.5 nm (35 Å), spanning from the carboxy terminus of one variable domain to the amino terminus of the other domain, while still allowing the domains to fold correctly and form a functional antigen-binding site. Huston, J. S., *et* al., (1991). VH (Variable Heavy) Domain the VH domain is the variable domain of the heavy chain of the antibody. It consists of approximately 110-130 amino acids and is located at the N-terminus of the scFv. The VH domain contains three complementarity-determining regions (CDRs) that are responsible for antigen

recognition and binding. VL (Variable Light) Domain the VL domain is the variable domain of the light chain of the antibody (Griffiths, A. D., *et* al 1998). It consists of approximately 90-110 amino acids and is located at the C-terminus of the scFv. The VL domain also contains three CDRs that contribute to antigen recognition and binding. Flexible Peptide Linker the VH and VL domains are connected by a flexible peptide linker, typically consisting of 10 to 25 amino acids. The peptide linker provides flexibility and allows the VH and VL domains to function together as a single polypeptide chain. In addition to the linker's length, the composition of amino acids also holds a crucial significance in designing a functional linker peptide. It is imperative for these linkers to possess a hydrophilic sequence, preventing any potential insertion of the peptide either within or between the variable domains during the process of protein folding. Argos, P. (1990). Presently, the prevailing and extensively employed designs involve sequences containing segments of Glycine and Serine residues. These residues are chosen for their flexibility, often complemented by charged residues like Glutamate and Lysine, which are interspersed to further bolster solubility (Whitlow, M., *et* al 1993).

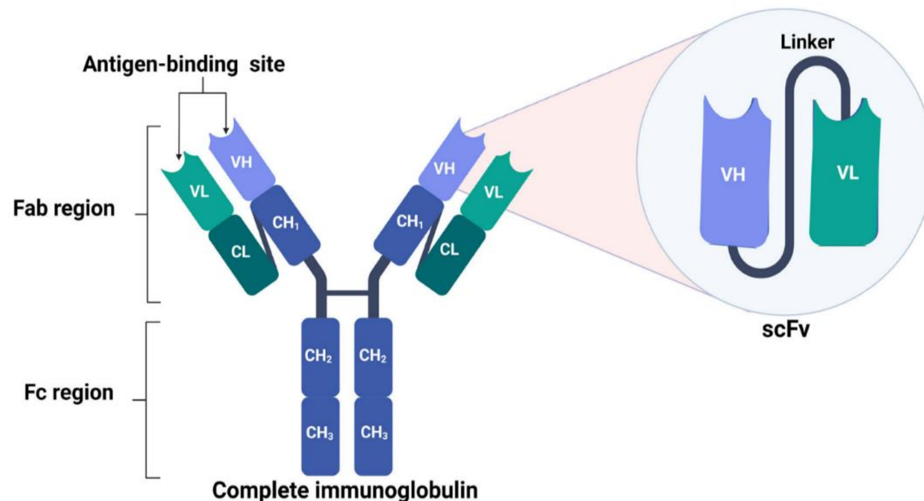

Fig 2. Structural compression between the full-length antibody and Single chain fragment variable (scFv).

2.10. Advantages of Single chain variable fragment

Single-chain variable fragments (scFv) offer several advantages in various applications due to their unique structure and properties. The small antigen-binding molecule of scFv (single chain fragment variable) antibodies could offer several advantages over a whole antibody molecule in therapeutic applications (Colcher, D., *et* al 1998), (Hudson, P. J.

1999). Some of the key advantages of scFv include:

Small Size-scFv molecules are smaller than full-length antibodies, making them ideal for use in therapeutic applications where tissue penetration and access to target sites are critical

Single Polypeptide Chain- scFv is composed of a single polypeptide chain, which simplifies the production and expression process compared to full-length antibodies, leading to higher yields and easier purification.

High Specificity-Like conventional antibodies, scFv retains the high specificity and affinity to their target antigens, enabling precise targeting of specific molecules.

Versatility- scFv can be easily engineered and modified to target different antigens by changing the variable regions, making them highly versatile for various applications.

Faster Onset of Action- Due to their smaller size, scFv can bind to target antigens more rapidly Yokota, T.*et* al (1992)., leading to a faster onset of therapeutic action in certain applications.

Lower Immunogenicity- scFv is less immunogenic compared to full-length antibodies, reducing the risk of an immune response when administered in therapeutic settings (Kipriyanov, *et* al 2002).

Stability and Solubility-scFv molecules can be designed to be stable and soluble, allowing for better formulation and storage as therapeutic agents.

Production in Microorganisms-scFv can be efficiently produced in microorganisms, such as bacteria and yeast, which reduces production costs and facilitates large-scale manufacturing.

Multivalent Constructs- scFv can be engineered to form multivalent constructs, allowing for enhanced binding avidity and improved therapeutic efficacy.

Bispecific Antibodies- scFv can be easily combined to generate bispecific antibodies, enabling simultaneous targeting of multiple antigens, which is beneficial in certain therapeutic applications.

2.11. Application of Single chain fragment variable

Single-chain variable fragments (scFv) have diverse applications in various fields, particularly in biomedical research, diagnostics, and therapeutic development Chester, K., *et* al (2004). Their small size, high specificity, and ease of production make them valuable tools for a wide range of applications. Some common applications of scFv include:

Therapeutic Antibodies

scFv can be engineered to create therapeutic antibodies for the treatment of various

diseases, including cancer, autoimmune disorders, and infectious diseases. They can be used as targeted therapies to specifically bind and neutralize disease-causing agents or cells.

Diagnostics

scFv can be utilized in diagnostic assays to detect specific antigens or pathogens. They are used in techniques like enzyme-linked immunosorbent assays (ELISA), lateral flow assays, and immunohistochemistry for disease diagnosis and monitoring (Luka, J., *et* al 2011).

Imaging and Targeting

scFv can be conjugated to imaging agents, such as fluorescent dyes or radionuclides, to facilitate non-invasive imaging of specific biomarkers in tissues or cells. They are also used in targeted drug delivery to deliver therapeutic agents specifically to disease sites.

Research Tools

In research, scFv are valuable tools for studying protein-protein interactions, characterizing cell surface receptors, and identifying specific biomolecules in complex mixtures.

Immunoassay Development

scFv-based immunoassays are widely used for quantifying analysts of interest, such as hormones, cytokines, and infectious agents, in biological samples (Kontermann, R. *et* al 1997).

Bio-separation and Purification

Immobilized scFv on chromatographic columns can be used for the purification of target proteins from complex mixtures. This technique, known as immune affinity chromatography, enables selective isolation of specific proteins.

Therapy for Venomous Animal Bites

scFv can be engineered to neutralize the toxic components of venomous animal bites, providing potential therapeutic benefits for snake bites and other venomous envenomation's.

Biosensors

scFv can be integrated into biosensor devices to detect specific targets, such as environmental contaminants, pathogens, or toxins, in real-time.

Tissue Targeting in Cancer Therapy

scFv can be used to target specific cancer cells, leading to more precise and targeted cancer therapies.

Bioimaging and Molecular Probes

scFv can be employed as molecular probes in bio-imaging studies to visualize specific cellular components or processes.

Overall, the versatility of scFv makes them valuable tools in various applications across the fields of medicine, biotechnology, diagnostics, and research. Their ability to specifically recognize and bind to target molecules or cells provides a powerful platform for developing innovative and targeted solutions in numerous areas of biomedicine and beyond.

2.12. Production of Single chain fragment variable

The production process of scFv involves several steps. This section discusses the isolation and characterization of target-specific antibodies, which serve as the starting point for scFv production. It explains the cloning of antibody variable regions and the assembly of the scFv gene. Furthermore, it delves into the expression and purification techniques used to obtain functional and high-quality scFv.

2.12. Overview of the steps involved in scFv production in E. coli:

The genes encoding the VH and VL regions of the antibody are isolated from B cells or generated through molecular cloning techniques. The VH and VL genes are then linked with a flexible peptide linker sequence to create the scFv gene (Hu, X., 2005).

The scFv gene is inserted into an expression vector suitable for E. coli, typically a plasmid. The vector contains regulatory elements, such as a promoter, ribosome binding site, and terminator, to control the expression of the scFv gene (Hust, M., et al., 2009). The recombinant expression vector carrying the scFv gene is introduced into E. coli host cells by a process called transformation.

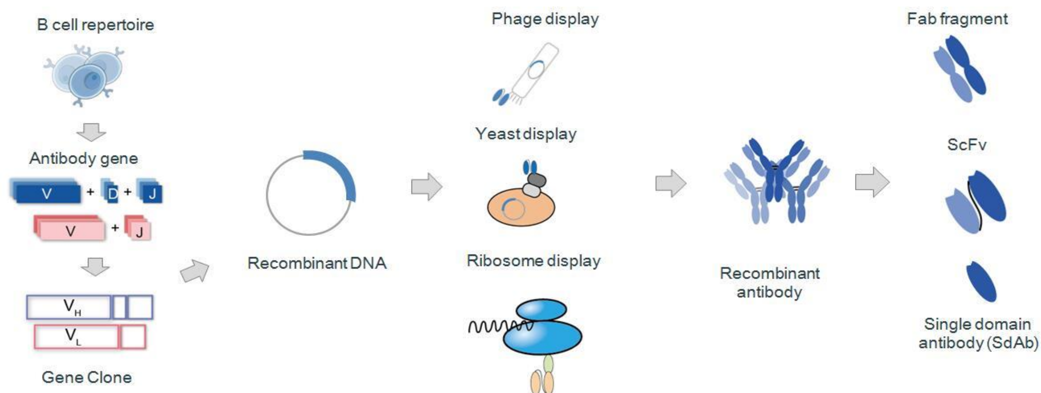

Fig 3. Production of Recombinant Antibody

E. coli cells harboring the expression vector are grown in a culture medium. At a specific growth stage, the expression of the scFv gene is induced by adding an inducer, such as isopropyl β-D-1-thiogalactopyranoside (IPTG).

The induced E. coli cells begin to synthesize the scFv protein using the genetic information from the inserted scFv gene. In some cases, the scFv protein may be expressed as insoluble aggregates called inclusion bodies. This occurs when the scFv protein tends to misfolded or aggregate in the E. coli cells.

After the desired expression time, the E. coli cells are harvested and lysed to release the scFv protein.

2.13. Protein Purification

The scFv protein needs to be purified from the E. coli cell lysate. If the scFv is expressed as inclusion bodies, an additional step of inclusion body solubilization and refolding is performed before purification (Gupta, V., *et* al 2020).

2.13. Chromatographic Purification:

Various chromatographic techniques, such as affinity chromatography or immobilized metal affinity chromatography (IMAC), are commonly used to purify the scFv protein based on its specific properties. Protein A chromatography is widely employed for monoclonal antibody capture and purification from complex cell culture harvest streams owing to its high degree of specificity (Gagnon, 1996). Protein A is a component of the cell wall of Staphylococcus aureus which interacts with Fc region of monoclonal antibodies. As an affinity ligand, protein A is coupled to Sepharose so that these regions are free to bind IgG. One molecule of protein A can bind at least two molecules of IgG. In many cases, starting with the culture supernatant, purity levels greater than 99% have been achieved. In a general protocol, the cell culture supernatant is loaded on to a Protein A column, binding occurs at neutral pH and the host cell proteins, etc. are removed by washing at an intermediate pH. The bound target protein is recovered from the column at a low pH.

2.12. Capto L resin

Capto L is a specialized affinity chromatography medium designed for the efficient capture of antibodies and antibody fragments. It utilizes a high-flow agarose matrix that offers excellent performance and high throughput capabilities. The key feature of Capto L is its immobilized immunoglobulin-binding recombinant protein L ligand, which possesses a

robust affinity for the variable region of an antibody's kappa light chain. Due to this unique ligand, Capto L is well-suited for capturing a diverse array of antibody fragments, including Fabs (antigen-binding fragments), single-chain variable fragments (scFv), and domain antibodies (Dabs). The variable region of an antibody's light chain is particularly important in determining its specificity and binding to antigens. Capto L's specific interaction with the kappa light chain allows for the selective capture and purification of a wide range of antibody fragments. Capto L's combination of a high-flow agarose matrix and the strong binding capacity of the recombinant protein L ligand makes it an efficient and effective tool for antibody and antibody fragment purification. The high-flow characteristics enable faster processing and higher productivity during chromatographic purification processes. Additionally, the resin's excellent selectivity ensures the retention and purification of the target antibody fragments, while minimizing interference from other contaminants.

The application of Capto L is particularly valuable in the production and research of therapeutic antibodies, diagnostics, and antibody-based biotechnological applications. Its versatility in capturing various antibody fragments makes it a valuable tool in the downstream processing of antibody-based products, contributing to the advancement of Bio-therapeutics and biopharmaceutical development.

2.12. Ni- NTA (nickel-nitrilotriacetic acid)

Ni-NTA Agarose is a powerful affinity resin specially designed for the purification of recombinant proteins containing a poly-histidine (6xHis) sequence, commonly referred to as the His tag. This technology facilitates the straightforward and efficient one-step purification of almost any His-tagged protein from various expression systems, whether under native or denaturing conditions.

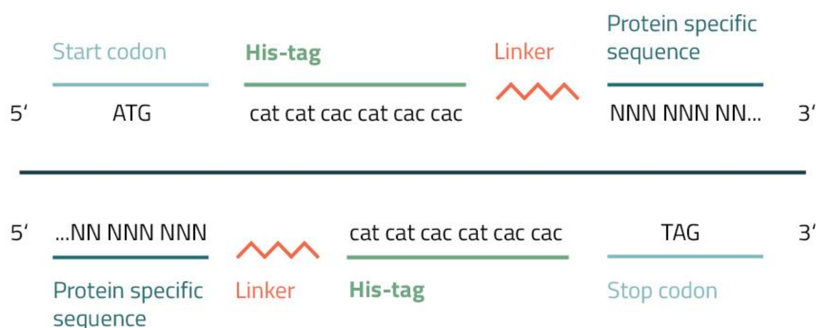

Fig 4. Recombinant his tag protein formation

The purification process of His-tagged proteins relies on the interaction between the poly-histidine sequence and divalent metal ions, with Ni2+ being the predominant ion used in Ni-NTA Agarose. The basic imidazole ring of histidine has a strong affinity for these metal ions, allowing for a selective and reversible binding interaction. The purification process is typically carried out at a neutral to slightly basic pH range (around 7.5 – 8), where the His-tag is capable of binding to the Ni2+ ions. Under these conditions, the His-tagged protein forms a stable complex with the Ni-NTA Agarose resin. Ni-NTA Agarose is constructed by coupling the chelating ligand nitrilotriacetic acid (NTA) to a cross-linked 6% agarose resin. The agarose matrix provides a stable and robust support for the NTA ligands, ensuring that the resin maintains its integrity and efficacy during purification.

This affinity resin is highly versatile and can be used in various purification formats, including batch and gravity flow applications. It offers advantages in terms of high binding capacity, rapid binding kinetics, and excellent selectivity, enabling efficient and specific purification of His-tagged proteins.

The use of Ni-NTA Agarose has revolutionized protein purification, making it a widely adopted method in both academic research and industrial applications. Its ability to purify His-tagged proteins in a single step has streamlined the protein purification process, saving time and resources while ensuring high yields of pure and functional recombinant proteins

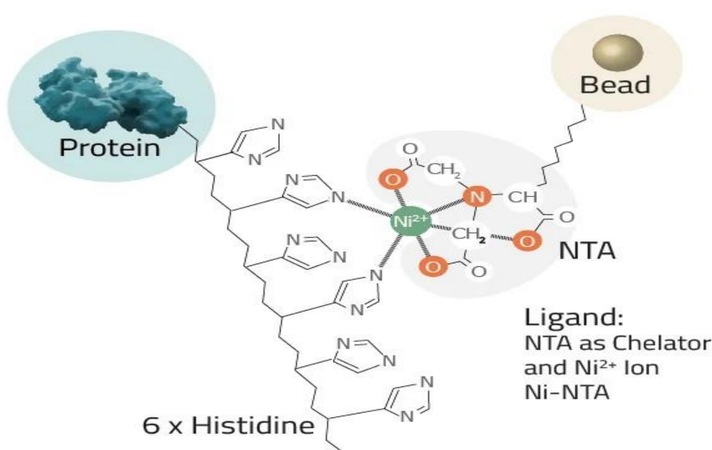

Fig 5. Interaction of His tagged protein with Ni-NTA ligand.

2.13. Analysis and Characterization

The purified scFv protein is analyzed for its quality, stability, and functionality using techniques like SDS-PAGE, Western blotting, ELISA, and binding assays.

It's important to note that the production of scFv in E. coli can be challenging due to factors such as protein mis-folding, aggregation, and low yields. Optimizing expression conditions, using appropriate host strains, and implementing efficient purification strategies are critical for obtaining high-quality scFv proteins.

This section presents notable success stories of Bio-therapeutics based on scFv. It focuses on three exemplary therapies: adalimumab, trastuzumab, and rituximab. The clinical applications, efficacy, and impact of these scFv-based therapies are discussed, emphasizing the role of scFv in their success and its contribution to personalized medicine. While scFv holds great promise, it is not without challenges and limitations. This section addresses these obstacles, including immunogenicity, stability issues, development of resistance, and manufacturing considerations. It discusses ongoing research and strategies aimed at overcoming these challenges, such as protein engineering approaches and improved manufacturing processes.

MATERIALS AND METHODS

3.1. Reagents:

- Tris base (Merck / J.T. Baker)
- Sodium Chloride (NaCl) (Merck/ J.T. Baker)
- Glycine. (Merck)
- Imidazole
- Potassium chloride
- Potassium dihydrogen phosphate
- Di-sodium hydrogen Phosphate
- Ethylene-diamine tetra acetic acid (EDTA)
- Sucrose
- Sodium Hydroxide (NaOH)

3.2. BUFFER USED

Table.No.1. BUFFERS USED IN 1st METHOD.

Buffers	Composition
Lysis buffer	0.1M Sodium phosphate + 10mM EDTA pH-7.2
EQULIBERATION BUFFER	1X PBS pH 7.2
WASH 1	1X PBS pH 7.2
WASH 2	1X PBS+1M NaCl pH 7.2
ELUTION BUFFER	0.1M Glycine pH 3.0

Table.No.2. BUFFERS USED IN 2nd METHOD.

Buffers	Composition
Lysis Buffer	Tris – 20 mM+ EDTA-0.5 mM+ Sucrose 20% pH 8.0
Dialysis Buffer	Tris – 20 mM+ NaCl – 150mM + Imidazole – 10mM pH 8.0
EQULIBERATION +Wash	Tris – 20 mM+ NaCl – 150mM + Imidazole – 10mM pH 8.0
Elution Buffer	Tris – 20 mM+ NaCl – 150mM + Imidazole – 500mM pH 8.0

3.3. Resin used

- Ni-NTA
- Capto L
- Mab select

3.4. Equipment handled:

- AKTA process chromatography systems.
- TFF assembly including cassette holder and peristaltic pump.
- Nano-Drop
- pH meter
- Conductivity meter
- Spectrophotometer
- Vertex
- Centrifuge
- Rocker
- Magnetic Stirrer

METHOD 1

3.5. Purification of scFv (Single chain Fragment variable) using Capto L followed by Ni-NTA.

3.5. Periplasmic Extraction

The Periplasmic lysis is the breaking down outer layer of bacterial cell. The scFv protein is present in Periplasmic space so to obtain the we have to only lyse of outer layer of the bacteria cell. There are different kind of Periplasmic lysis methods for Periplasmic protein release: OS (Osmotic Shock), EDTA/heat extraction, and DOC (De-oxycholate) extraction are present like; Here we use EDTA/Heat for the extraction of Periplasmic protein 10gm of cell biomass was taken and suspended in 100ml of Lysis buffer in the ratio of 1:10and Mix thoroughly and incubated in incubator for 16 hours at 200 rpm and 28°C.

Fig 6. Periplasmic Incubation for 16 hours at 28°C

3.5. Centrifugation

Centrifugation is a widely used laboratory technique for separating and isolating components of a mixture based on their density and size. It involves the application of centrifugal force to a sample placed in a container (centrifuge) to accelerate the sedimentation of particles. This process allows for the separation of substances with different densities, such as cells, organelles, proteins, and nucleic acids, from a liquid mixture. In this process we use fixed angle rotor for separation of cell debris centrifuged at 10000g for 30min at 4°C. the result of centrifugation is

pellet are suspended down and my protein of interest are soluble in supernatant.

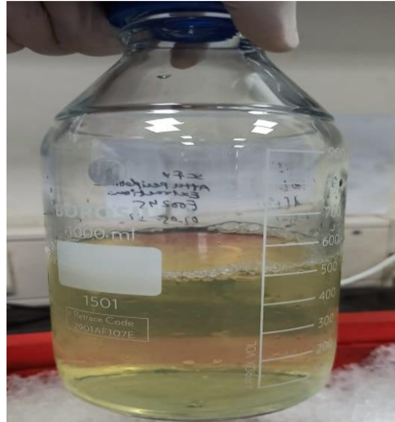

Fig 7. After centrifugation Supernatant was collected in the bottle.

3.5. Filtration

Filtration is a separation process that involves passing a mixture through a porous material or membrane to separate its components based on their size, shape, or other properties. The medium's pores allow smaller particles or substances to pass through, while larger particles or substances are trapped or retained. The medium acts as a barrier that separates the components based on their size after centrifugation supernatant was collected and filtered with the help of filter vacuumed using 0.45um PVDF membrane.

Fig 8. Filtration of Sample with the help of vacuum pump

3.5. Affinity Chromatography

Affinity Chromatography is a powerful technique that separates proteins based on specific interactions between a protein and a ligand attached to a chromatographic matrix. This method is particularly useful for capturing or isolating

proteins of interest when a suitable ligand is available. Affinity Chromatography offers high selectivity, resolution, and capacity for the target protein.

In the case of Periplasmic extraction from E. coli, the protein of interest present in the Periplasmic space is harvested using this method. After removing the rest of the cells through centrifugation, the Periplasmic sample is carefully collected. The next step involves loading the sample onto an affinity column, such as Capto L or Ni NTA, both of which are commonly used for protein purification.

To begin, the column resin is equilibrated with an equilibration buffer (EQB) to expose the ligand to which the protein will specifically bind. This allows the target protein to selectively bind while impurities are washed through in the flow-through fraction and subsequent wash steps. A secondary wash, known as a high-salt wash, helps remove weakly associated proteins and nonspecific components.

Before elution, another application of EQB ensures the removal of any remaining components from the high-salt wash and prepares the column for elution. Elution buffer is then applied to selectively release the desired protein from the column resin. Finally, the resin is sanitized and stored in a solution containing 20 percent ethanol.

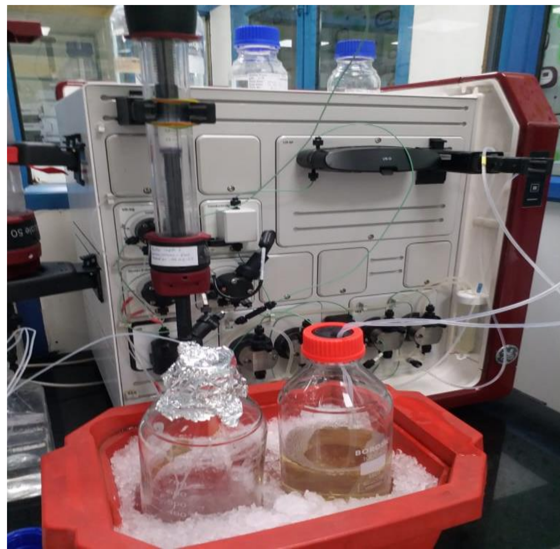

Fig 9. Sample loading on column chromatography

Table.No.3: Steps in Capto L procedure

Buffer / solution	Column Volume	Function	Watch / Action
Flow rate: 2.5mL/min		Residence time: 2 minutes	
Equilibration buffer	5 CV	Equilibration	pH and conductivity are sameas that of equilibration buffer.
Sample Loading	-	-	Air should not enter the columnwhile loading sample.
Wash 1	5 CV	Unbound impurities removal	pH and conductivity are sameas that of equilibration buffer.
Wash 2	5 CV	Loosely associated impurities removal	Collect peak from mAUascending arm to mAU descending arm if any.
Elution buffer	5 CV	Product elution	Collect peak from ascending arm to descending arm Labelit and measure the volume.

Method 2

3.6. Purification of scFv (single chain fragment variable) using Ni-NTA

3.6. Periplasmic Extraction

We used Osmotic shock for the extraction of protein because it is a fast, reliable, and relatively clean way to obtain Periplasmic protein from cells 10gm of cell biomass was taken and re-suspended in 100ml of Lysis buffer in the ratio of 1:10. Mix thoroughly and incubated on rocker for half an hour at 4C and after that incubated on room temperature This process is repeated for 5 times. This process done at low temperature provide less stress to the molecule. After that Centrifuged at 10000g for 10min at 4°C.

Fig 10. Sample was placed on Rocker.

3.6. Dialysis of sample

Dialysis for buffer exchange is a gentle method that minimizes the risk of denaturation or damage to biomolecules. Dialysis can effectively reduce the salt and buffer concentration in the sample, making it suitable for various experiments and analyses. The sample to be dialyzed is placed in a dialysis bag or a tube made from the dialysis membrane. The bag is sealed, or the tube is closed, ensuring the sample is enclosed. A larger volume of the desired dialysis buffer is prepared. This buffer should have the same pH, ionic strength, and other relevant properties as the sample

buffer, but without the contaminants or unwanted components here we use buffer B and leave the closed semipermeable membrane for 16 hours at 4C. Over time, small molecules move through the dialysis membrane down their concentration gradient, effectively exchanging the buffer around the sample. This results in the removal of salts and other contaminants from the sample.

Fig 11. Sample filled-in Dialysis bag.

3.6. Purification

After dialysis sample is filtered with 0.45 PVDF membrane with the help of vacuum filter. Sample loading on 5ml Ni-NTA prepacked column through the use of FPLC (Fast Protein Liquid Chromatography).

Fig.12. illustrates the utilization of a 5ml prepacked Ni-NTA column for purifying a His-tagged protein.

Table.No.4: Steps in chromatography Ni-NTA Procedure

Buffer / solution	Column Volume	Function	Watch / Action
Flow rate: 2mL/min Residence time: 2.5minutes			
WFI	5 Column Volume (CV)	Storage solution removal	-
Equilibration buffer	5 CV	Equilibration	pH and conductivity are sameas that of equilibration buffer.
Sample Loading	-	-	Air should not enter the columnwhile loading sample.
Post load wash by Equilibration buffer	10 CV	Unbound impurities removal	pH and conductivity are sameas that of equilibration buffer.
Elution buffer	0-100% gradient	Product elution	Collect peak from ascending arm to descending arm. Label it and measure the volume.

3.7.SDS-PAGE (SODIUM DODECYLE SULPHATE POLY ACRYLAMIDE GEL ELECTROPHORESIS)

Electrophoresis is a technique that separates molecules based on their movement under an electric field. In protein electrophoresis, a polyacrylamide gel is commonly used, formed by combining acrylamide and Bis-acrylamide. The gel polymerization process is initiated by ammonium persulfate and accelerated by TEMED (Tetra-Methyl-Ethylene-Di-amine), which generates free radicals that trigger polymerization. These growing polymer chains are cross-linked by Bis-acrylamide, creating a gel with specific porosity determined by the polymerization conditions.

In SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), SDS, an anionic detergent, is used in both the sample and running buffer. SDS binds to

proteins at a consistent ratio, denaturing them to their primary structure. As SDS is negatively charged, it imparts a uniform negative charge to proteins, masking their inherent charges. Consequently, all proteins carry a similar charge, making their mobility (Rf) solely dependent on their molecular mass. This allows for effective separation of proteins based on size during electrophoresis.

$$\mathbf{Rf = (ZE)/\ f}$$

Where,

Z represents the charge on the molecule.
E stands for the electric field strength, which is the voltage applied to the system.
f denotes the frictional force experienced by the molecule.

3.8. QUANTIFICATION

Proteins absorb UV light in proportion to their aromatic amino acid content (Out of 3 aromatic amino acids; tryptophan and tyrosine exhibit more UV absorbance especially tryptophan exhibits very strong UV absorbance but phenylalanine contributes very less to absorbance). The absorbance of a solution is influenced by the concentration of the substance and its specific molar absorptivity (ε) value, which is expressed in units of M-1 cm-1. For example, a solution containing tryptophan will exhibit an absorbance of 5500 M-1 cm-1, while a solution containing tyrosine will have an absorbance of 1490 M-1 cm-1. This means that a 1 Molar solution of tryptophan in a standard cuvette with a path length of 1 cm will yield an absorbance of 5500. The reason for the significant difference in absorbance between tryptophan and tyrosine is due to their distinct molar absorptivity values.

To quantify protein using this principle, a micro droplet approach is employed. In this method, a very small sample volume of 2μL is loaded onto a substrate. This substrate could be a pellet, which acts as a platform for holding the micro droplet. By measuring the absorbance of the sample using the specific molar absorptivity values of its constituent amino acids, the protein concentration can be determined. This micro droplet technique allows for accurate protein quantification even with very small sample volumes.

$$A280 = \varepsilon \times c \times l$$

Where:

A280: Absorbance at 280nm

ε: Molar absorptivity coefficient (extinction coefficient) of the protein in M^-1 cm^-1

c: Concentration of the protein in Molarity (mol/L)

l: Path length of the cuvette in centimeters

3.9. WESTERN BLOTTING

Western blotting or Immunoblotting is a well-established and widely used technique for the detection and analysis of proteins. The method is based on building an antibody-protein complexvia specific binding of antibodies to proteins immobilized on a membrane.

Transfer of the proteins from the gel to membrane (Nitrocellulose or PVDF) is achieved by a technique known as electro-blotting or protein blotting or western blotting.

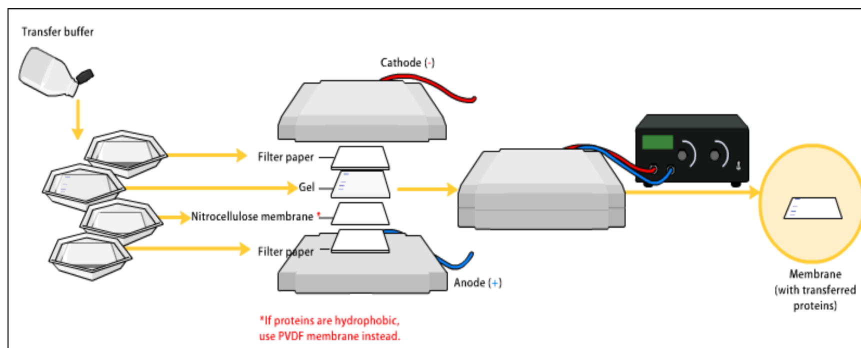

Fig 13. Representative diagram of different step of western blotting.

After the transfer of the protein to the membrane, a preliminary step involves incubating the membrane with non-fat dried milk. This milk acts to block any remaining hydrophobic binding sites on the membrane's surface. Subsequently, the membrane is exposed to a diluted solution of a primary antibody, specifically targeting the protein of interest. In this case, an anti-His antibody is used. If the antigen is detected, the primary antibody binds to the membrane, allowing for

identification of the protein of interest.

To visualize this binding interaction, the membrane is then subjected to another incubation step with an enzyme-labeled secondary antibody. Following this, a substrate for the enzyme is introduced. This substrate undergoes a chemical reaction catalyzed by the enzyme, resulting in the formation of an insoluble colored product that precipitates onto the membrane's surface. The appearance of a colored band indicates the precise location of the protein of interest.

Common enzymes used in enzyme-linked antibody techniques are alkaline phosphatase and horseradish peroxidase. Alkaline phosphatase converts a colorless substrate called 5-bromo-4-chloro-indolylphosphate (BCIP) into a blue-colored product. Alternatively, horseradish peroxidase, when combined with hydrogen peroxide (H2O2) and specific substrates like 3-amino-9-ethylcarbazole or 4-chloro-1-naphthol, generates insoluble brown or blue precipitates, respectively. This method enables clear visualization and confirmation of the presence of the protein of interest on the membrane.

3.10. Ultrafiltration and Dia-filtration (UFDF)

In general, after chromatography steps, the purified product was concentrated and Dia-filtered using a membrane module of required pore size. The sample was concentrated through membranes of $1/3^{rd}$ – $1/6^{th}$ of molecular weight cut off of the sample and then Dia-filtered (buffer exchanged) with minimum 8 dia-filtration volumes (DV) of dia-filtration buffer.

3.10. Material of construction of membrane:

- Material of membrane: - Regenerated cellulose and Polyether sulfone
- Sanitization buffer: 0.5 N NaOH
- Membrane storage solution: 20% ethanol/ 0.1N NaOH

3.10. Tangential Flow Filtration (TFF)

Tangential flow filtration (TFF) is a highly effective and essential step in the downstream processing (DSP) of biologics, playing a vital role in clarifying and purifying these substances. This technique involves the use of membrane filtration, a well-established

method for separating solid particles from liquids, widely employed in the biotechnology industry. The membranes used in this process can be categorized into two main types: microfiltration and ultrafiltration, based on their pore size

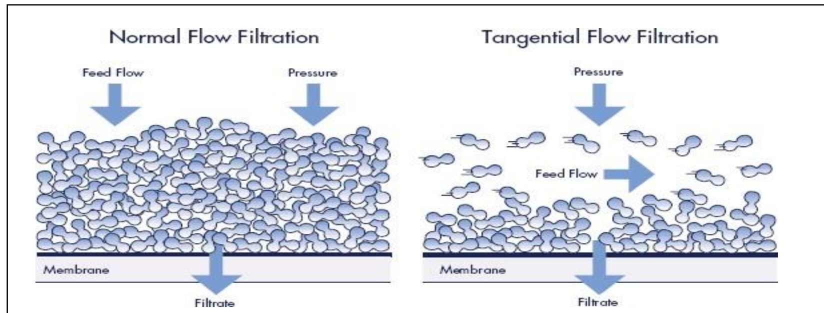

Fig 14. Difference between Normal and Tangential Flow Filtration

Transmembrane Pressure (TMP) is the average applied pressure from the feed to the filtrate side of the membrane.

$$TMP\ [bar] = [(P_F + P_R)/2] - P_f$$

Pressure Drop (ΔP) is the difference in pressure along the feed channel of the membrane from the inlet to the outlet

$$\Delta P\ [bar] = P_F - P_R$$

Results & Discussion

4.1. Purification of scFv (Single chain Fragment variable) using Capto L followed by Ni-NTA

Incubation for 16 hours at 28 $^{\circ}$C → Centrifuged 10000g for 30min at 4 $^{\circ}$C → Filtered 0.45um → Loding on Capto L Resin

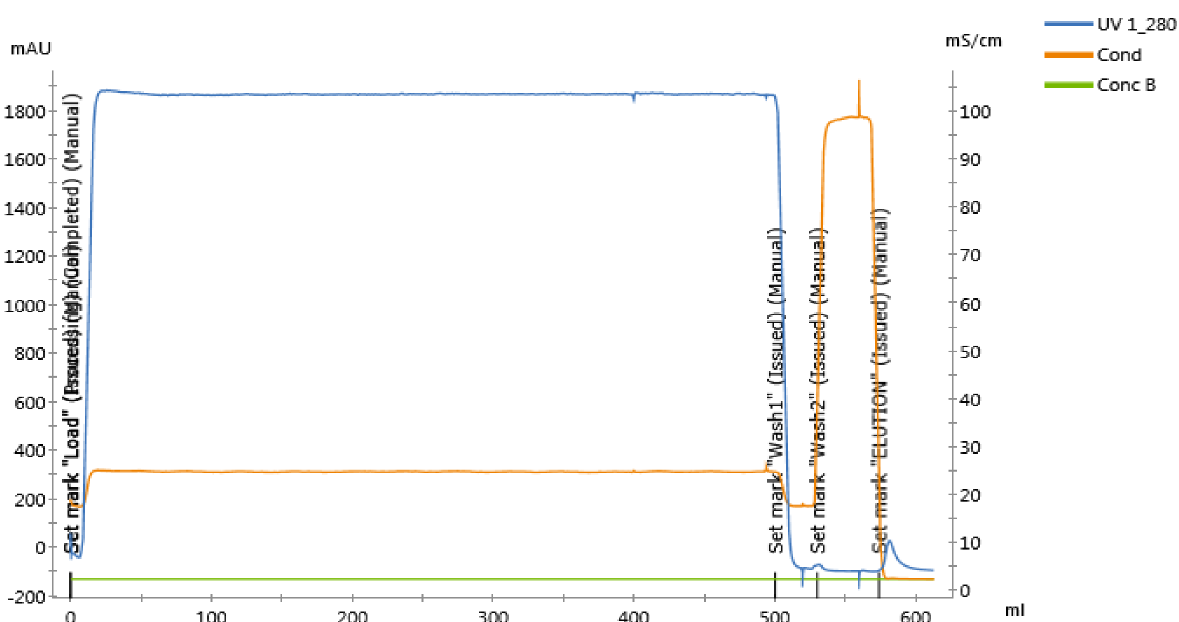

Fig 15. Process chromatogram profile of Capto L run is given.

NOTE: The chromatogram's blue traces represent the absorbance readings at 280nm. The chromatogram displays a distinct peak corresponding to the protein of interest, which becomes evident after the passage of 1 column volume (1CV) of elution buffer (0.1M Glycine pH 3.0). This peak is observed within the elution volume ranging from 550mL to 600mL. This specific fraction was subsequently collected for further analysis.

SDS-PAGE Result

The SDS-PAGE of the output samples was performed in reducing method, the samples were treated with reducing dye containing reducing agent i.e., Beta-mercapto-ethanol in boiling water for 10 minutes prior to loading further processed with Commassie brilliant blue staining. The result of SDS-PAGE is shown below:

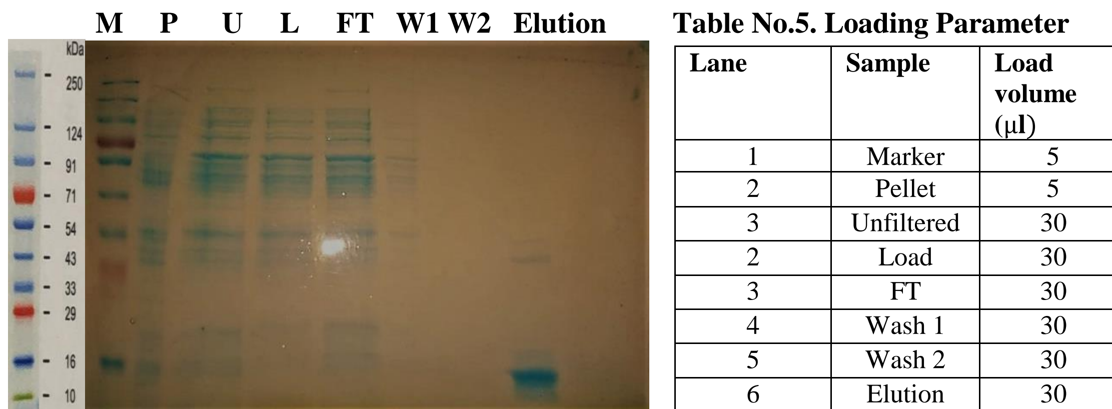

Table No.5. Loading Parameter

Lane	**Sample**	**Load volume (µl)**
1	Marker	5
2	Pellet	5
3	Unfiltered	30
2	Load	30
3	FT	30
4	Wash 1	30
5	Wash 2	30
6	Elution	30

Fig 16. 12% Reducing SDS-PAGE analysis of Capto L chromatography fractions

Observation

The SDS-PAGE gel electrophoresis results reveal that the elution peak comprises the desired protein. However, it is noticeable that the protein has undergone fragmentation into two distinct fragments, approximately 33kDa and 16kDa in size. This phenomenon is clearly evident in the SDS-PAGE analysis.

Method 2

4.2. To purify scFv by using affinity chromatography Ni-NTA resin to obtain pure form of scFv.

- We tried another method for extraction of Periplasmic scFv antibody fragment.
- Instead of EDTA heat extraction we are using Osmotic shock method.
- So first of all we started with another Periplasmic extraction method which is heat shock method.
- We changed the buffer condition as in previous experiment we use TES buffer.
- Dialysis of Sample because EDTA hinder in binding with Ni NTA resin.
- We started with batch binding method with 1 ml of Ni-NTA.

4.2. Batch Binding

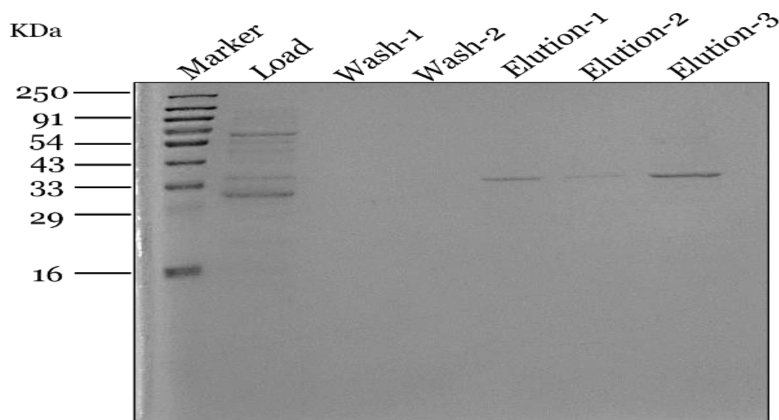

Fig 17. 12%SDS PAGE Batch binding SDS Result of scFv with Ni-NTA resin.

Observation – During the Elution steps labeled as 1, 2, and 3, distinct bands corresponding to my protein of interest, which holds a size of approximately 33kDa, are distinctly visible in the SDS-PAGE analysis. This clear visualization affirmed our decision to opt for this particular extraction method. This choice is rooted in the fact that the protein of interest remains intact and doesn't undergo fragmentation into two separate fragment.

4.2. After successfully purifying the protein in batch binding method we scale this process to column chromatography with 5ml Resin.

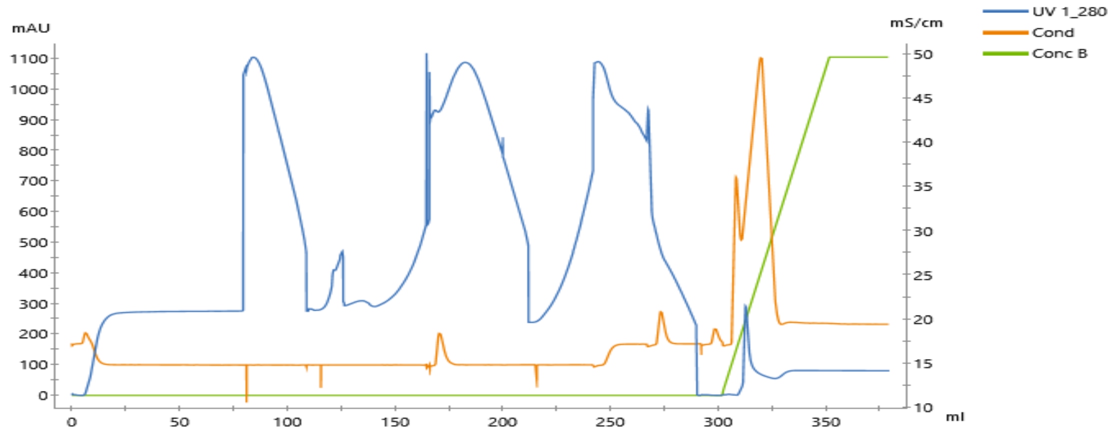

Fig 18. Process chromatogram profile of 200mL Sample Ni-NTA run is given. NOTE: The blue traces in the chromatogram represent the absorbance measured at 280nm. The peak corresponding to the protein of interest was detected after approximately 20-30% of the elution buffer had passed through the system. This peak was observed within the elution volume range of 300-330 mL, and this specific fraction was collected for further analysis.

4.2. SDS-PAGE Result

The SDS-PAGE of the output samples was performed in reducing method, the samples were treated with reducing dye containing reducing agent i.e., Beta-mercapto-ethanol in boiling water for 10 minutes prior to loading further processed with Commassie brilliant blue staining. The result of SDS-PAGE is shown below:

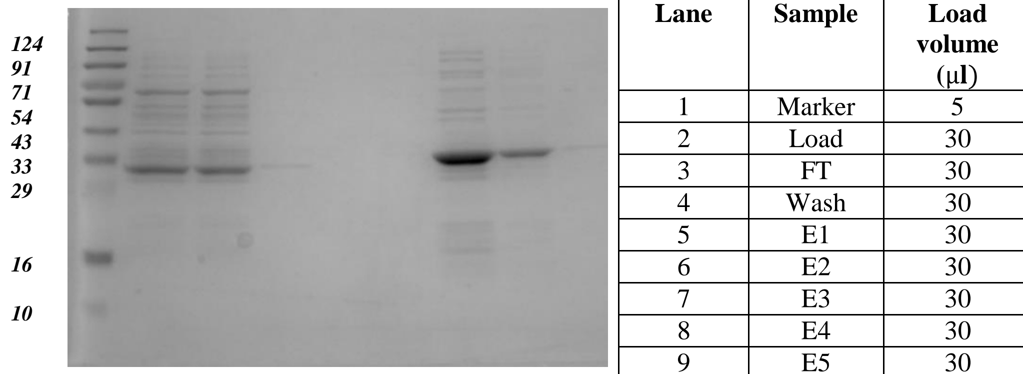

Table No.6. Loading Parameter

Lane	Sample	Load volume (µl)
1	Marker	5
2	Load	30
3	FT	30
4	Wash	30
5	E1	30
6	E2	30
7	E3	30
8	E4	30
9	E5	30

Fig 19. 12% Reducing SDS-PAGE analysis of Ni-NTA chromatography fractions

4.2. Observation-After analyzing the SDS-PAGE gel electrophoresis results, it is evident that the elution peaks 3 and 4 comprise the desired protein, which is approximately 33kDa in size. This outcome aligns with my intended goal for further analysis of the fractions. Subsequently, Western blotting will be pursued for a more comprehensive investigation of these fractions.

4.2. Western Blotting Result:

Specific bands of purified sample are obtained by performing western blot technique.

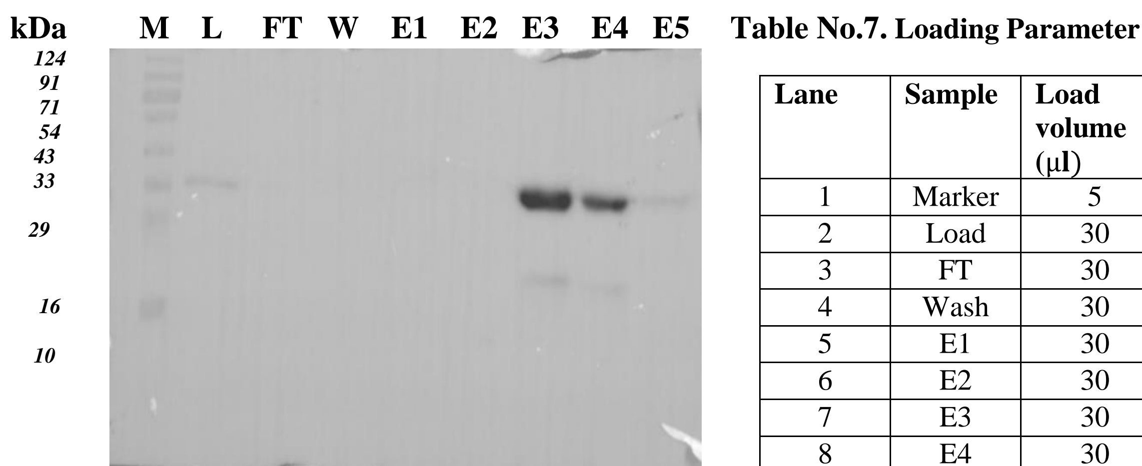

Table No.7. Loading Parameter

Lane	Sample	Load volume (μl)
1	Marker	5
2	Load	30
3	FT	30
4	Wash	30
5	E1	30
6	E2	30
7	E3	30
8	E4	30
9	E5	30

Fig 20. Western result of scFv antibody

4.2. Observation: The results obtained from SDS-PAGE gel electrophoresis indicate that elution peaks 3 and 4 contain proteins of the desired size. To further validate this finding, we performed Western blotting using an Anti-His primary antibody. The Anti-His antibody exhibited a strong binding affinity to the protein of interest in both elution 3 and elution 4. The subsequent application of a secondary antibody resulted in noticeable fluorescence signals, confirming the presence of the desired protein bands.

Table No. 8. The concentration of the purified protein varies slightly when assessed through these three analytical methods.

Sr. No.	A280(mg/ml)	BCA (mg/ml)	Bradford(mg/ml)
E3	**0.231**	**0.364**	**0.489**
E4	**0.059**	**0.046**	**0.206**

The overall concentration of the purified protein is 0.9 mg.

4.2. Densitometry result

In this context, we are assessing the purity of the purified sample using densitometry.

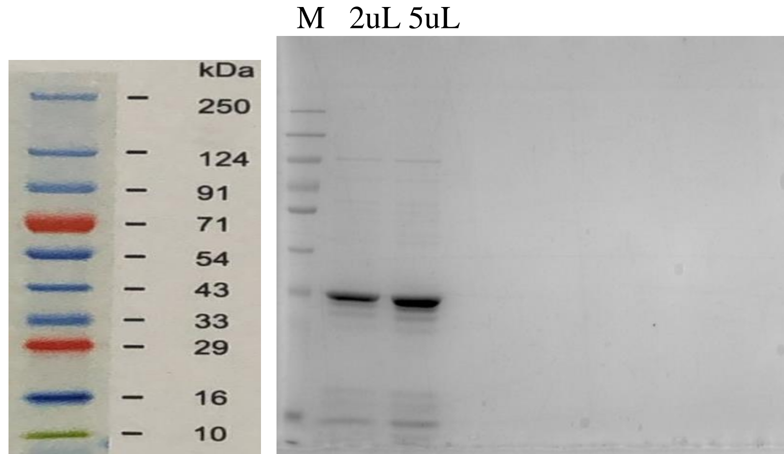

Fig 21. 12% Reducing SDS-PAGE checking the fractions

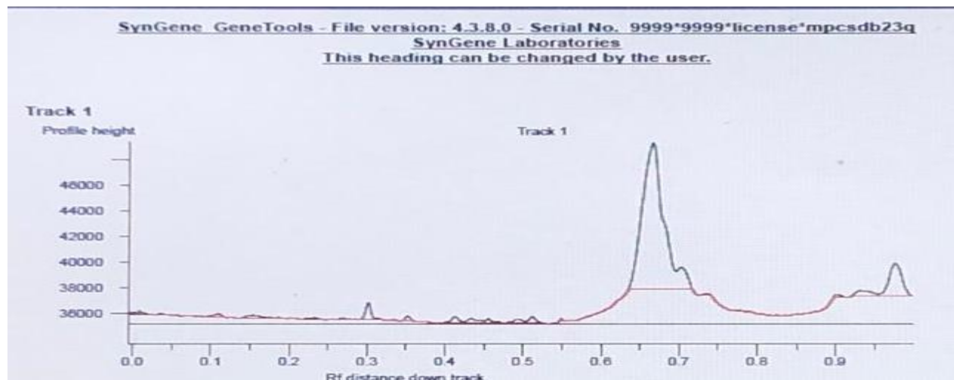

Fig 22. The figure is showing the purity level of purified protein.

Table No.9. Data of Densitometry

Number	Height	Raw Volume	% Raw vol.
1	1344.325	739480.25	2.475
2	505.363	388916.	1.302
3	512.871	352810.50	1.181
4	11507.769	23172590.00	77.570
5	1715.199	1723147.13	5.768
6	383.406	336577.63	1.127
7	2538.632	3159453.50	10.576

4.2. Observation: Following the purification of scFv, we are evaluating the purity level of elution 3 by examining the percentage of raw volume occupied by peak 4, which is 77.5%.

4.3. Purification of full length Ig G antibody using Mabselect prismA Resin

We are working on producing full-length IgG of scFv that will be fragmented over a period of time.

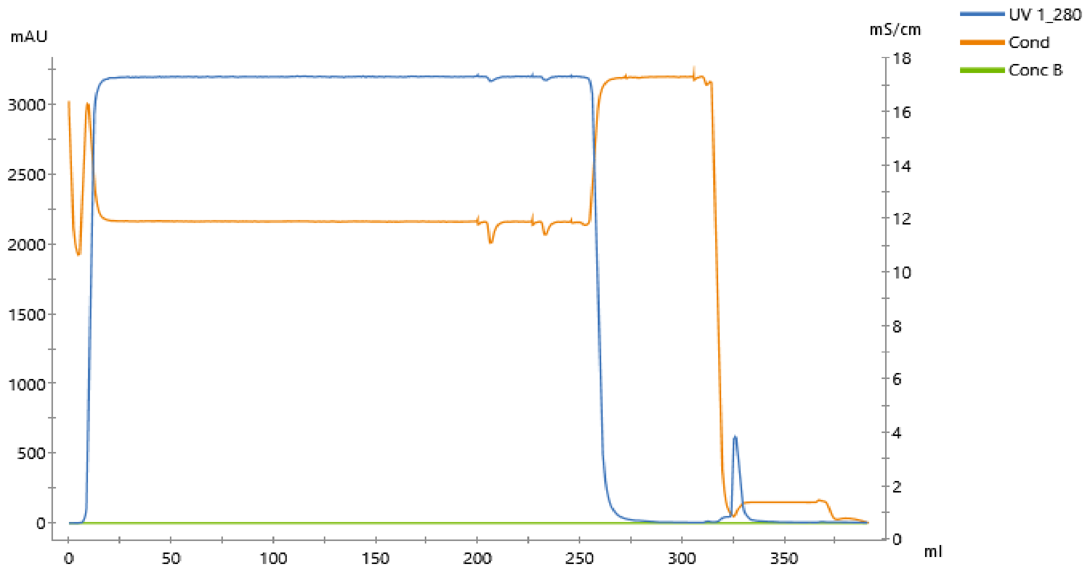

Fig 23 – Process chromatogram profile of MabSelect Prism A run

NOTE: The blue traces in the chromatogram correspond to the absorbance at 280nm. The peak representing the protein of interest is evident after the passage of 1 column volume (1CV) of elution buffer (0.1M Glycine pH 3.0). This peak is observed within the range of 310-350 mL and has been collected for further analysis.

4.3.SDS-PAGE Result

The SDS-PAGE of the samples performed in reducing and non-reducing method both in 12% SDS-PAGE gel. For reducing the samples were treated with reducing dye containing reducing agent i.e., Beta-mercapto-ethanol in boiling water for 10 minutes prior to loading. For non-reducing, samples were prepared in non-reducing dye further processed with Commassie brilliant blue staining. The result of SDS-PAGE is shown below:

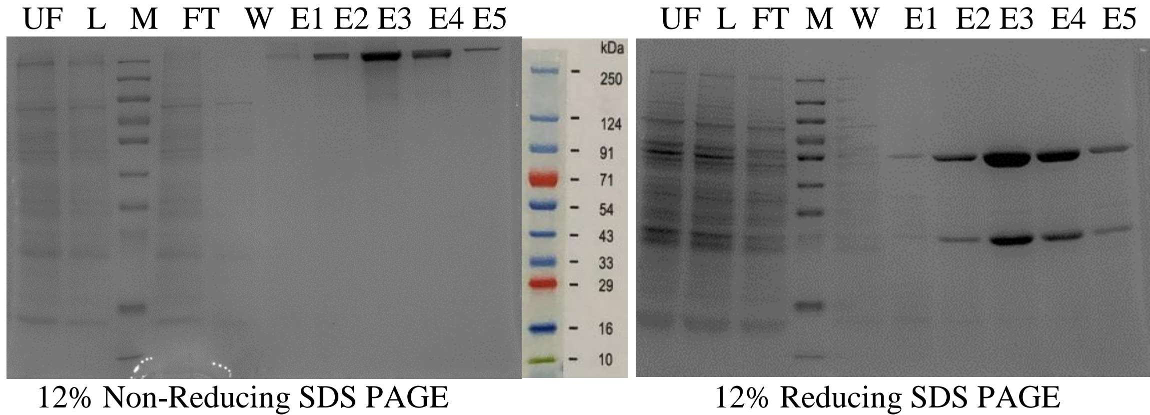

Fig 24. SDS- PAGE Result of full length Ig G Non-Reducing and Reducing

4.3. OBSERVATION: Upon elution from MabSelect Prism A, the elution fractions E2-E5 exhibited two distinct bands at approximately 33 kDa and 54 kDa when subjected to reducing conditions (as depicted in the figure). Conversely, under non-reducing conditions, a single band was observed at around 250 kDa (as shown in Figure 1). After concentrating, the E2-E5 and E3-E4 fractions were pooled together, resulting in final protein concentrations of 0.157 mg/mL and 0.450 mg/mL, respectively, as determined through UV280 analysis.

In the context of concentration, a total of 0.427 mg of protein from the E2-E5 fractions and 2.11 mg of protein from the E3-E4 fractions were obtained following dialysis in 1X phosphate-buffered saline (PBS). Dialysis is an advantageous method for buffer exchange, as it is gentle and reduces the likelihood of denaturation or damage to biomolecule.

Conclusion

We observe two bands in the reducing SDS-PAGE. This could be due to time-dependent cleavage of the molecule. The molecule of interest is expressed in the Periplasmic space of *E. coli*. Thus, the first step involves the release of molecule from the Periplasmic space. For this the method used involves treating the cells with high EDTA containing buffer and incubating at 28º C for a period of 16 hrs. We hypothesized that this treatment might be the root cause of the protein cleavage. Upon reviewing the literature, we have come about the osmotic shock method for Periplasmic extraction which is less time consuming a softer method of extraction. Based on this we used osmotic shock method using sucrose for extraction.

The escalating utilization of Recombinant Proteins in the realm of Bio-therapeutics necessitates a sturdy, dependable, and cost-efficient production process. This purification procedure, a pivotal component of the overall manufacturing process, fulfills the requisites of purity, throughput, and yield. Utilizing chromatographic purification, particularly Affinity chromatography (currently limited to the initial purification step due to the project's early stage), has yielded a definitive protein presence as evident from the protein bands observed in SDS-PAGE analysis under reducing conditions. Additionally, it effectively eliminates impurities like host cell proteins, DNA, and aggregates. This procedure exhibits resilience, reliability, and a yield of approximately 75%.

References

Ahmad, Z. A., Yeap, S. K., Ali, A. M., Ho, W. Y., Alitheen, N. B. M., & Hamid, M. (2012). scFv antibody: principles and clinical application. *Journal of Immunology Research*, *2012*.

Mauro, V. P. (2018). Codon optimization in the production of recombinant biotherapeutics: potential risks and considerations. *BioDrugs*, *32*(1), 69-81.

Huang, C. J., Lin, H., & Yang, X. (2012). Industrial production of recombinant therapeutics in Escherichia coli and its recent advancements. *Journal of Industrial Microbiology and Biotechnology*, *39*(3), 383-399.

Jones, S. D., & Marasco, W. A. (1998). Antibodies for targeted gene therapy: extracellular gene targeting and intracellular expression. *Advanced drug delivery reviews*, *31*(1-2), 153-170.

Batra, S. K., Jain, M., Wittel, U. A., Chauhan, S. C., & Colcher, D. (2002). Pharmacokinetics and biodistribution of genetically engineered antibodies. *Current opinion in biotechnology*, *13*(6), 603-608.

Griffiths, A. D., & Duncan, A. R. (1998). Strategies for selection of antibodies by phage display. *Current opinion in biotechnology*, *9*(1), 102-108.

Davies, D. R., & Metzger, H. (1983). Structural basis of antibody function. *Annual review of immunology*, *1*(1), 87-115.

Winter, G., Griffiths, A. D., Hawkins, R. E., & Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annual review of immunology*, *12*(1), 433-455.

Glockshuber, R., Malia, M., Pfitzinger, I., & Plueckthun, A. (1990). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry*, *29*(6), 1362-1367.

Hu, X., O'Dwyer, R., & Wall, J. G. (2005). Cloning, expression and characterisation of a single-chain Fv antibody fragment against domoic acid in Escherichia coli. *Journal of Biotechnology*, *120*(1), 38-45.

Hust, M., Steinwand, M., Al-Halabi, L., Helmsing, S., Schirrmann, T., & Dübel, S.

(2009). Improved microtitre plate production of single chain Fv fragments in Escherichia coli. *New Biotechnology*, *25*(6), 424-428.

Dai, K., Zhu, H., & Ruan, C. (2003). Generation and characterization of recombinant single chain Fv antibody that recognizes platelet glycoprotein Ibα. Thrombosis research, 109(2-3), 137-144.K.

Sheikholvaezin, A., Sandström, P., Eriksson, D., Norgren, N., Riklund, K., & Stigbrand, T. (2006). Optimizing the generation of recombinant single-chain antibodies against placental alkaline phosphatase. Hybridoma, 25(4), 181-192.

Ghilardi, N., Pappu, R., Arron, J. R., & Chan, A. C. (2020). 30 Years of biotherapeutics development—what have we learned?. Annual Review of Immunology, 38, 249-287.

P, Surat. (2019, March 28). What are Recombinant Proteins?. News-Medical. Retrieved on July 30, 2023 from https://www.news-medical.net/life-sciences/What-are-Recombinant-Proteins.aspx

Johnson, D. E. (2018). Biotherapeutics: challenges and opportunities for predictive toxicology of monoclonal antibodies. International journal of molecular sciences, 19(11), 3685.

Murphy, K., & Weaver, C. (2016). Janeway's immunobiology. Garland science.

Forthal, D. N. (2014). Functions of antibodies. Microbiology spectrum, 2(4), 2-4.

Glockshuber, R., Malia, M., Pfitzinger, I., & Plueckthun, A. (1990). A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry, 29(6), 1362-1367.

Huston, J. S., George, A. J., Adams, G. P., Stafford, W. F., Jamar, F., Tai, M. S., ... & Weiner, L. M. (1996). Single-chain Fv radioimmunotargeting. The quarterly journal of nuclear medicine: official publication of the Italian Association of Nuclear Medicine (AIMN)[and] the International Association of Radiopharmacology

Jin, S., Sun, Y., Liang, X., Gu, X., Ning, J., Xu, Y., ... & Pan, L. (2022). Emerging new therapeutic antibody derivatives for cancer treatment. Signal Transduction and Targeted Therapy, 7(1), 39.

Colcher, D., Pavlinkova, G., Beresford, G., & Booth, B. J. M. (1998). Pharmacokinetics and biodistribution of genetically-engineered antibodies. The Quarterly Journal of Nuclear Medicine and Molecular Imaging, 42(4), 225.

Hudson, P. J. (1999). Recombinant antibody constructs in cancer therapy. Current opinion in immunology, 11(5), 548-557.

Chester, K., Pedley, B., Tolner, B., Violet, J., Mayer, A., Sharma, S., ... & Begent, R. (2004). Engineering antibodies for clinical applications in cancer. Tumor Biology, 25(1-2), 91-98.

Kontermann, R. E., Wing, M. G., & Winter, G. (1997). Complement recruitment using bispecific diabodies. Nature biotechnology, 15(7), 629-631.

Luka, J., Arlen, P. M., & Bristol, A. (2011). Development of a serum biomarker assay that differentiates tumor-associated MUC5AC (NPC-1C ANTIGEN) from normal MUC5AC. BioMed Research International, 2011.

Nelson, P. N., Reynolds, G. M., Waldron, E. E., Ward, E., Giannopoulos, K., & Murray, P. G. (2000). Demystified…: monoclonal antibodies. Molecular pathology, 53(3), 111.

Griswold, K. E., & Bailey-Kellogg, C. (2016). Design and engineering of deimmunized biotherapeutics. Current opinion in structural biology, 39, 79-88.

Coker, V. (2012). Biotherapeutics outpace conventional therapies. BioPharm International, 2012(1).

Kipriyanov, S. M. (2002). High-level Periplasmic expression and purification of scFvs. Antibody Phage Display: Methods and Protocols, 333-341.

Gupta, V., Sudhakaran, I. P., Islam, Z., Vaikath, N. N., Hmila, I., Lukacsovich, T., ... & El-Agnaf, O. M. (2020). Expression, purification and characterization of α-synuclein fibrillar specific scFv from inclusion bodies. Plos one, 15(11), e0241773.

Licari, G., Martin, K. P., Crames, M., Mozdzierz, J., Marlow, M. S., Karow-Zwick, A. R., ... & Bauer, J. (2022). Embedding Dynamics in Intrinsic Physicochemical Profiles of Market-Stage Antibody-Based Biotherapeutics. Molecular Pharmaceutics, 20(2), 1096-1111.