DEVELOPMENT OF AN EFFICIENT PLATFORM FOR PRODUCTION OF SOLUBLE MONOCLONAL ANTIBODY FRAGMENT USING MICROBIAL EXPRESSION SYSTEM

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SEPTEMBER 2024

Development of an efficient platform for production of soluble monoclonal antibody fragment using microbial expression system

by

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Submitted

in fulfilment of the requirements of the degree of Doctor of Philosophy
to the

Department of Chemical Engineering
Indian Institute of Technology Delhi

SEPTEMBER 2024

Dedicated to my family...

CERTIFICATE

This is to certify that the thesis entitled **"Development of an efficient platform for production of soluble monoclonal antibody fragment using microbial expression system"** being submitted by **Preeti Saroha** to the **Indian Institute of Technology, Delhi** for the award of the degree of **Doctor of Philosophy** is a record of the original bonafide research work carried out by him under my guidance and supervision. The results contained in this thesis have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma. I certify that he has pursued the prescribed course of research.

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Acknowledgements

I express my deepest gratitude to my PhD advisor, Prof Anurag S Rathore, for his guidance, support and encouragement throughout the course of the current research. His insightful feedback and criticism to my thesis development have been instrumental and unwaverred.

I am also profoundly grateful to the members of my research committee, Prof .James Gomes, Prof. Anupam Shukla and Prof. Vikram Singh, for their periodic assessments, constructive comments, and contributions that significantly enhanced the quality of this work.

I would like to acknowledge the financial support from IIT Delhi, without which this research would not have been possible.

I extend my heartfelt appreciation to the non-technical and administrative staff of the lab, for their continuous support and for ensuring that all logistical aspects of my research were handled efficiently.

Special thanks goes to my family, Priyanka and Tushar whose camaraderie and assistance made the research process enjoyable and enriching. Their support and collaboration were vital in overcoming the many challenges encountered during this study. I thank my colleagues for their kind support and co-operation during this work.

I am deeply indebted to my family especially my parents Mr. Narender Kumar and Mrs Vedvanti and my extended family for their unwavering love, patience, and encouragement. Their belief in me provided the motivation to persevere through the demanding periods of my research journey.

Thank you to everyone who contributed, directly or indirectly, to the successful completion of this thesis. Your support has been invaluable, and I am truly grateful.

PREETI SAROHA

Abstract

Recombinant proteins dominate the pharmaceutical pipelines today with microbial host systems continuing to be a major contributor towards their production. *E. coli* is a versatile host for recombinant protein expression due to its simplicity, affordability, and ability to be cultivated at high cell density. It is particularly suitable for non-glycosylated proteins and small proteins. Despite the aforementioned benefits, use of *E.coli* as the host for the synthesis of recombinant antibody fragments often suffer from low yield and reduced activity. In most cases, proteins are expressed as inclusion bodies and need to undergo refolding to achieve their active forms and this refolding step is generally low yielding. In this thesis, we report the various approaches that researchers have taken for enhancing production of recombinant antibody fragments in *E.coli*. Molecular biology-oriented approaches such as cloning, chaperone-mediated folding, and host cell screening as well as process optimization involving examination of process parameters, media, and feeding have been addressed.

In the first objective of this research, various strategies have been developed such as changes at DNA level (codon optimization), fusion with soluble tags and variations in process parameters (temperature) and inducer concentration. However, there is no "one size fits all" strategy. The most used approach involves induction at low temperature, as reducing the temperature during cultivation has been reported to increase bioactive protein production in *E.coli.* We examine the impact of various process parameters such as temperature and inducer concentration, as well as high plasmid copy number vector for achieving enhanced soluble expression of TNFα inhibitor Fab. An interaction amongst these parameters has been observed and their optimization has been demonstrated to result in expression of 30 ±3 mg/L antibody fragment using *E.coli*.

Predominantly, recombinant expression of soluble Fabs in *E.coli* is commonly acknowledged as a challenging endeavour, characterized by low expression levels. Typically, efforts involve attempting the translocation of proteins from the reductive cytoplasmic environment to the slightly oxidative periplasmic space or spent media to achieve desired soluble protein production in *E.coli*. We have investigated the use of additives to enhance the expression of recombinant enzymes containing signal peptides. These additives include surfactants, amino acids, and osmolytes, both individually and in combination. We report the usage of dose dependent concentration of additives namely Triton X 100, sucrose, and glycine, for achieving enhanced expression of recombinant Tumour Necrosis Factor (TNFα) inhibitor Fab

in *E.coli*. Interestingly, we found the enhanced expression (1-3 fold) using all the three additives. Most optimal results were obtained with Triton X 100, which improved yield of periplasmic expression by 3.2-fold, demonstrating significant role of these additives delivering enhanced production of difficult to express products such as the TNFα inhibitor Fab in the *E.coli* system.

E.coli strain development has witnessed major advancements over the past decade to manufacture affordable biotherapeutics. The goal of the third objective was to perform *E. coli* strain screening for enhanced expression of TNFα inhibitor Fab. The commonly used T7 based expression host system has been compared with 10 other advanced strains. We observe that derivatives of BL21(DE3) exhibit about 3-fold increased expression of soluble TNFα inhibitor Fab than other systems that were evaluated. The host strains, One shot BL21 (DE3)* and BL21 AI, offered the highest specific protein productivity (3.5-fold and 3.2-fold, respectively) in the bioreactor.

To sustain stability and bioactivity of the expressed proteins within the cell, co-expression of multiple genes was examined in the fourth objective. Combination of plasmids such as multiple promoters on single vectors or single promoter-based vectors with different antibiotic genes were co-transformed into *E.coli*. To investigate the enhancement of desired recombinant TNFα inhibitor Fab, we co-transformed the dual promoter construct carrying gene of interest with single promoter's plasmid clone bearing individual chains of the same. The expression doubled when heavy chain was co-expressed with the main clone and 1.7-fold enhanced expression was observed in the case of light chain co-expression. The study demonstrates that co-expression of same genes or subunits of desired proteins offers a potent strategy for enhancing the soluble expression of recombinant proteins without any internal genetic modifications within the host.

Overall, the current study, illustrates how process optimization can contribute towards making biotherapeutics affordable and could be very beneficial for the large-scale, economical extracellular production of biotherapeutics, particularly those without any glycosylation or other post-translational modification. The results of the study would be of interest to researchers who work in the field of protein expression in microbial hosts.

अमूर्त (Abstract)

आज दवा निर्माण में रिकॉम्बिनेंट प्रोटीन का बोलबाला है, तथा माइक्रोबियल होस्ट सिस्टम उनके उत्पादन में प्रमुख योगदानकर्ता बने हुए हैं। ई. कोली अपनी सरलता, सामर्थ्य और उच्च कोशिका घनत्व पर विकसित होने की क्षमता के कारण रिकॉम्बिनेंट प्रोटीन अभिव्यक्ति के लिए एक बहुमुखी होस्ट है। यह गैर-ग्लाइकोसिलेटेड प्रोटीन और छोटे प्रोटीन के लिए विशेष रूप से उपयुक्त है। उपर्युक्त लाभों के बावजूद, पुनः संयोजक एंटीबॉडी अंशों के संश्लेषण के लिए मेजबान के रूप में ई.कोली का उपयोग अक्सर कम उपज और कम गतिविधि से ग्रस्त होता है। अधिकांश मामलों में, प्रोटीन को समावेशन निकायों के रूप में व्यक्त किया जाता है और उनके सक्रिय रूपों को प्राप्त करने के लिए उन्हें फिर से मोड़ने की आवश्यकता होती है और यह फिर से मोड़ने का चरण आम तौर पर कम उपज वाला होता है। इस थीसिस में, हम उन विभिन्न तरीकों की रिपोर्ट करते हैं जो शोधकर्ताओं ने ई.कोली में पुनः संयोजक एंटीबॉडी अंशों के उत्पादन को बढ़ाने के लिए अपनाए हैं। क्लोनिंग, चैपरोन-मध्यस्थ फोल्डिंग और होस्ट सेल स्क्रीनिंग जैसे आणविक जीव विज्ञान-उन्मुख दृष्टिकोणों के साथ-साथ प्रक्रिया मापदंडों, मीडिया और फीडिंग की जांच से जुड़े प्रक्रिया अनुकूलन को संबोधित किया गया है।

इस शोध के पहले उद्देश्य में, डीएनए स्तर पर परिवर्तन (कोडन अनुकूलन), घुलनशील टैग के साथ संलयन और प्रक्रिया मापदंडों (तापमान) और प्रेरक सांद्रता में बदलाव जैसी विभिन्न रणनीतियाँ विकसित की गई हैं। हालाँकि, कोई "एक आकार सभी के लिए उपयुक्त" रणनीति नहीं है। सबसे अधिक इस्तेमाल किया जाने वाला दृष्टिकोण कम तापमान पर प्रेरण शामिल है, क्योंकि खेती के दौरान तापमान कम करने से ई.कोली में बायोएक्टिव प्रोटीन उत्पादन में वृद्धि होने की सूचना मिली है। हम तापमान और प्रेरक सांद्रता जैसे विभिन्न प्रक्रिया मापदंडों के प्रभाव की जाँच करते हैं, साथ ही ट्यूमर नेक्रोसिस फैक्टर अल्फा अवरोधक एंटीबॉडी टुकड़े की बढ़ी हुई घुलनशील अभिव्यक्ति को प्राप्त करने के लिए उच्च प्लास्मिड कॉपी संख्या वेक्टर भी। इन मापदंडों के बीच एक अंतःक्रिया देखी गई है और उनके अनुकूलन के परिणामस्वरूप ई.कोली का उपयोग करके 30 ± 3 मिलीग्राम / एल एंटीबॉडी टुकड़े की अभिव्यक्ति का प्रदर्शन किया गया है।

मुख्य रूप से, ई.कोली में घुलनशील एंटीबॉडी टुकड़े (एफएबीएस) की पुनः संयोजक अभिव्यक्ति को आमतौर पर एक चुनौतीपूर्ण प्रयास के रूप में स्वीकार किया जाता है, जिसकी विशेषता निम्न अभिव्यक्ति स्तर है। आम तौर पर, प्रयासों में ई.कोली में वांछित घुलनशील प्रोटीन उत्पादन को प्राप्त करने के लिए रिडक्टिव साइटोप्लाज्मिक वातावरण से प्रोटीन को थोड़ा ऑक्सीडेटिव पेरिप्लास्मिक स्पेस या स्पेंड मीडिया में स्थानांतरित करने का प्रयास शामिल होता है। हमने सिग्नल पेप्टाइड्स युक्त

पुनः संयोजक एंजाइमों की अभिव्यक्ति को बढ़ाने के लिए एडिटिव्स के उपयोग की जांच की है। इन एडिटिव्स में सर्फेक्टेंट, अमीनो एसिड और ऑस्मोलाइट्स शामिल हैं, दोनों व्यक्तिगत रूप से और संयोजन में। हम ई.कोली में पुनः संयोजक ट्यूमर नेक्रोसिस फैक्टर (ट्यूमर नेक्रोसिस फैक्टर अल्फा) अवरोधक एंटीबॉडी टुकड़े की बढ़ी हुई अभिव्यक्ति प्राप्त करने के लिए ट्राइटन एक्स 100, सुक्रोज और ग्लाइसिन जैसे एडिटिव्स की खुराक पर निर्भर सांद्रता के उपयोग की रिपोर्ट करते हैं। दिलचस्प बात यह है कि हमने तीनों एडिटिव्स का उपयोग करके बढ़ी हुई अभिव्यक्ति (1-3 गुना) पाई। ट्राइटॉन एक्स 100 के साथ सर्वाधिक इष्टतम परिणाम प्राप्त हुए, जिससे पेरिप्लास्मिक अभिव्यक्ति की उपज में 3.2 गुना सुधार हुआ, तथा ई.कोली प्रणाली में टीएनएफ α अवरोधक फैब जैसे व्यक्त करने में कठिन उत्पादों के उत्पादन में इन योजकों की महत्वपूर्ण भूमिका प्रदर्शित हुई।

पिछले दशक में ई.कोली स्ट्रेन विकास में किफायती बायोथेरेप्यूटिक्स के निर्माण के लिए बड़ी प्रगति देखी गई है। तीसरे उद्देश्य का लक्ष्य ट्यूमर नेक्रोसिस फैक्टर अल्फा अवरोधक एंटीबॉडी टुकड़े की बढ़ी हुई अभिव्यक्ति के लिए ई.कोली स्ट्रेन स्क्रीनिंग करना था। आमतौर पर इस्तेमाल किए जाने वाले T7 आधारित अभिव्यक्ति होस्ट सिस्टम की तुलना 10 अन्य उन्नत स्ट्रेन से की गई है। हमने देखा कि बीएल21(डीई3) के व्युत्पन्न अन्य मूल्यांकन किए गए सिस्टम की तुलना में घुलनशील ट्यूमर नेक्रोसिस फैक्टर अल्फा अवरोधक एंटीबॉडी टुकड़े की अभिव्यक्ति में लगभग 3 गुना वृद्धि दर्शाते हैं। होस्ट स्ट्रेन, वन शॉट बीएल21(डीई3)* और बीएल21 एआई, ने बायोरिएक्टर में उच्चतम विशिष्ट प्रोटीन उत्पादकता (क्रमशः 3.5 गुना और 3.2 गुना) की पेशकश की।

कोशिका के भीतर व्यक्त प्रोटीन की स्थिरता और जैव सक्रियता को बनाए रखने के लिए, चौथे उद्देश्य में कई जीनों की सह-अभिव्यक्ति की जांच की गई। एकल वेक्टर पर कई प्रमोटर या अलग-अलग एंटीबायोटिक जीन वाले एकल प्रमोटर-आधारित वेक्टर जैसे प्लास्मिड के संयोजन को ई.कोली में सह-रूपांतरित किया गया। वांछित पुनः संयोजक ट्यूमर नेक्रोसिस फैक्टर अल्फा अवरोधक एंटीबॉडी टुकड़े की वृद्धि की जांच करने के लिए, हमने रुचि के जीन को ले जाने वाले दोहरे प्रमोटर निर्माण को उसी की अलग-अलग श्रृंखलाओं वाले एकल प्रमोटर के प्लास्मिड क्लोन के साथ सह-रूपांतरित किया। जब भारी श्रृंखला को मुख्य क्लोन के साथ सह-अभिव्यक्त किया गया तो अभिव्यक्ति दोगुनी हो गई और हल्की श्रृंखला सह-अभिव्यक्ति के मामले में 1.7 गुना बढ़ी हुई अभिव्यक्ति देखी गई। अध्ययन दर्शाता है कि वांछित प्रोटीन के समान जीन या उप-इकाइयों की सह-अभिव्यक्ति मेजबान के भीतर किसी भी आंतरिक आनुवंशिक संशोधन के बिना पुनः संयोजक प्रोटीन की घुलनशील अभिव्यक्ति को बढ़ाने के लिए एक शक्तिशाली रणनीति प्रदान करती है।

कुल मिलाकर, वर्तमान अध्ययन यह दर्शाता है कि प्रक्रिया अनुकूलन बायोथेरेप्यूटिक्स को किफ़ायती बनाने में कैसे योगदान दे सकता है और बायोथेरेप्यूटिक्स के बड़े पैमाने पर, किफायती बाह्यकोशिकीय उत्पादन के लिए बहुत फायदेमंद हो सकता है, विशेष रूप से बिना किसी ग्लाइकोसिलेशन या अन्य पोस्ट-ट्रांसलेशनल संशोधन के। अध्ययन के परिणाम उन शोधकर्ताओं के लिए रुचिकर होंगे जो माइक्रोबियल होस्ट में प्रोटीन अभिव्यक्ति के क्षेत्र में काम करते हैं।

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List of Abbreviations

E.coli	*Escherichia coli*
bGH	Bovine growth hormone
IB	Inclusion bodies
DsB	Di-sulphide bonds
araBAD	Arabinose (*araBAD*) based promoter
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
GST	Glutathione S-transferase
MBP	Maltose binding protein
His6	Hexahistadine
NusA	N-utilizing substance A protein
Trx	Thioredoxin
SUMO	Small-ubiquitin-like modifier
EDTA	Ethylenediamine tetra acetic acid
PEI	Polyethyleneimine
DO	Dissolved oxygen
TNFα	Tumor necrosis factor
Fab	Antigen binding fragment
dAbs	Domain antibody fragments
scFvs	Single-chain variable fragments
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
BCA	Bicinchoninic acid
Omp A	Outer membrane protein A
PCR	Polymerase chain reaction
RPM	Rotation per minute
SRP	Signal recognition peptide
SDS PAGE	Sodium dodecyl sulphate –polyacrylamide gel electrophoresis
SEC-HPLC	Size-exclusion high-performance liquid chromatography
DTT	Dithiothreitol

OD	Optical density
PTMs	Post translational modifications
IgG	Immunoglobulin
Mm	Millimolar
L/m	Litre per minute
TFA	Trifluoroacetic acid
GFP	Green fluorescent protein

List of Symbols

α	Alpha
°C	Centigrade
s	Seconds
µ	Micro
µg	Microgram
mg	Milligram
L	Litre
mL	Millilitre
%	Percentage
w/v	Weight by volume
µL	Micro litre
V	Voltage
m/z	Mass by ion
um	Micro molar
w/w	Weight by weight
M	Molar
mg/L	Milligram per litre
Da	Dalton
nM	Nanometer
β	Beta
pH	Measurement of hydroxide ion
µ max	Micro max