

UPSTREAM PROCESS OPTIMIZATION FOR BIOTHERAPEUTICS PRODUCTION

RUCHA SUHAS PATIL



**DEPARTMENT OF CHEMICAL ENGINEERING
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UPSTREAM PROCESS OPTIMIZATION FOR BIOTHERAPEUTICS PRODUCTION

By

RUCHA SUHAS PATIL

Submitted

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to the**



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Dedicated to my family...!

CERTIFICATE

This is to certify that the thesis entitled “**UPSTREAM PROCESS OPTIMIZATION FOR BIOTHERAPEUTICS PRODUCTION**” being submitted by **RUCHA SUHAS PATIL** 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 she has pursued the prescribed course of research.

Prof. Anurag S. Rathore

Department of Chemical Engineering

Indian Institute of Technology Delhi

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Abstract

Microbial host systems remain the most efficient and cost-effective choice for biotherapeutics production. *E. coli* is often the preferred host due to ease of cloning, scale-up, high product yields, and most importantly, cost-effective production. However, *E. coli* often experiences difficulties in producing biologically active therapeutics such as antibody fragment (Fab) molecules. Low Fab expression and high production costs present major challenges for manufacturers. To enhance process economics, an optimized upstream process is essential. This study employs a systematic approach to upstream process development of the Fab fragment, ranibizumab, in *E. coli*. Initially, key process variables were optimized by using a two-step Design of Experiments (DOE) strategy. Key process parameters—including post-induction temperature, post-induction time, inducer concentration, agitation speed, media pH, and cell density at induction (induction OD) were evaluated for their effects on biomass concentration, protein yield, impurity levels, and light-to-heavy chain ratio. Fractional factorial screening design identified induction OD, inducer concentration, pH, and temperature as critical parameters. Response surface methodology further determined the optimal values of induction OD, inducer concentration, media pH and post-induction temperature as 1.2 OD, 1 mM inducer concentration, pH 7.4, and 35°C, respectively. The optimized parameters yielded 0.051 g protein/g inclusion bodies, 4.99 g/L biomass concentration, a balanced light-to-heavy chain ratio (LC:HC = 0.98), and an impurity level of 8.2%. Notably, temperature and induction OD significantly influenced LC:HC balance and impurity formation, underscoring the need for tight process control.

Overproduction of heterologous protein often causes excessive consumption of ATPs and other precursors, especially amino acids, and this can disrupt cell metabolism, thereby leading to impaired cell growth and reduced protein yields. This study further explores the role of amino acid supplementation in alleviating this metabolic strain. A Plackett-Burman DOE screening identified critical amino acids, followed by a targeted supplementation approach based on amino acid consumption patterns. The strategy resulted in a 28% increase in dry cell weight, a 40% improvement in protein yield per gram of inclusion bodies, a 23% rise in specific growth rate, and a 37% enhancement in substrate uptake. These findings highlight amino acid supplementation as an effective approach to enhance process productivity.

Production of Fab molecules in *E. coli* as a host presents a significant challenge due to low protein expression and poor yields. However, overexpression often leads to the formation of

insoluble protein aggregates known as inclusion bodies (IBs). Downstream processing of Fab fragments remains a bottleneck due to challenges in IBs recovery and refolding efficiency. To improve product yield and purity, we optimized lysis conditions, IB recovery, and IB washing steps. DOE-driven buffer composition optimization achieved 0.069 g protein/g IB, 61% IB purity, and 87% lysis efficiency. Homogenization using two passes at 1000 bar increased lysis efficiency to 93.5%, maintaining 60% IB purity. Furthermore, optimized IB washing using 1% Triton X-100 and 2M urea for 30 minutes at room temperature resulted in 84.53% IB recovery and 75% IB purity. These optimizations demonstrated that refining lysis and washing parameters significantly enhances IBs quality, thereby improving refolding yields and downstream purification.

IBs are typically more stable and contain a higher proportion of the target protein compared to the soluble fraction. However, quality and stability of IBs play a crucial role in determining the efficiency of refolding and subsequent purification of the target protein. IBs storage conditions, including temperature, duration, and freeze-thaw cycles, were systematically analyzed to determine their impact on stability. IBs stored at -80°C exhibited superior stability compared to those stored at -20°C, with degradation increasing over time and with repeated freeze-thaw cycles. IBs stored at -20°C and -80°C for 45 days showed a 51% and 30% decrease in refolding yield, respectively. Lyophilized IBs exhibited 40% and 20% loss under the same conditions. Lyophilized IBs demonstrated improved stability under all tested conditions, outperforming conventional IBs. Moreover, an increase in light chain fragmentation was observed over time, potentially contributing to reduced refolding yields and complications in downstream processing. Additionally, IBs formed during high-cell-density cultivation contained host protein impurity, potentially interfering with purification. Based on these findings, lyophilized IBs and IBs stored at -80°C with minimal freeze-thaw cycles were identified as optimal conditions for preserving IB integrity and maximizing recombinant protein yield.

This study presents a comprehensive approach to optimizing Fab molecule production in *E. coli*, addressing key challenges in upstream processing, metabolic burden, downstream purification, and IBs stability. The findings emphasize the importance of precise control over induction parameters, targeted amino acid supplementation, and optimized IBs handling to enhance overall process efficiency. Implementing these strategies can significantly improve ranibizumab Fab protein yields, reduce impurities, and streamline biopharmaceutical manufacturing.

सारांश

माइक्रोबियल होस्ट सिस्टम बायोथेरेप्यूटिक्स उत्पादन के लिए सबसे कुशल और लागत प्रभावी विकल्प बने हुए हैं। क्लोनिंग, स्केल-अप, उच्च उत्पाद पैदावार और सबसे महत्वपूर्ण रूप से, लागत प्रभावी उत्पादन की आसानी के कारण एस्चेरिचिया कोली (ई. कोली) अक्सर पसंदीदा होस्ट होता है। हालाँकि, ई. कोली को अक्सर एंटीबॉडी फ्रेगमेंट (Fab) अणुओं जैसे जैविक रूप से सक्रिय चिकित्सीय उत्पादन में कठिनाइयों का सामना करना पड़ता है। कम Fab उत्पादन और उच्च उत्पादन लागत निर्माताओं के लिए बड़ी चुनौतियाँ पेश करती हैं। प्रक्रिया अर्थशास्त्र को बढ़ाने के लिए, एक अनुकूलित अपस्ट्रीम प्रक्रिया आवश्यक है। यह अध्ययन ई. कोली में Fab फ्रेगमेंट, रैनिबिजुमैब के अपस्ट्रीम प्रक्रिया विकास के लिए एक व्यवस्थित दृष्टिकोण का उपयोग करता है। प्रारंभ में, दो-चरणीय डिज़ाइन ऑफ़ एक्सपेरीमेंट (DOE) रणनीति का उपयोग करके प्रमुख प्रक्रिया चर को अनुकूलित किया गया था। मुख्य प्रक्रिया पैरामीटर - जिसमें प्रेरण के बाद का तापमान, प्रेरण के बाद का समय, प्रेरक सांद्रता, आंदोलन की गति, मीडिया पीएच और प्रेरण पर कोशिका घनत्व (प्रेरण ओडी) शामिल हैं - का मूल्यांकन बायोमास सांद्रता, प्रोटीन उपज, अशुद्धता के स्तर और हल्के से भारी श्रृंखला अनुपात पर उनके प्रभावों के लिए किया गया था। आंशिक कारक स्क्रीनिंग डिज़ाइन ने प्रेरण ओडी, प्रेरक सांद्रता, पीएच और तापमान को महत्वपूर्ण मापदंडों के रूप में पहचाना। प्रतिक्रिया सतह पद्धति ने प्रेरण ओडी, प्रेरक सांद्रता, मीडिया पीएच और प्रेरण के बाद के तापमान के इष्टतम मूल्यों को क्रमशः 1.2 ओडी, 1 एमएम प्रेरक सांद्रता, पीएच 7.4 और 35 डिग्री सेल्सियस के रूप में निर्धारित किया। अनुकूलित मापदंडों ने 0.051 ग्राम प्रोटीन/जी समावेशन निकाय, 4.99 ग्राम/एल बायोमास सांद्रता, एक संतुलित हल्के से भारी श्रृंखला अनुपात (एलसी:एचसी = 0.98) और 8.2% का अशुद्धता स्तर प्राप्त किया। उल्लेखनीय रूप से, तापमान और प्रेरण OD ने LC:HC संतुलन और अशुद्धता गठन को महत्वपूर्ण रूप से प्रभावित किया, जिससे सख्त प्रक्रिया नियंत्रण की आवश्यकता को रेखांकित किया गया। विषम प्रोटीन का अधिक उत्पादन अक्सर एटीपी और अन्य अग्रदूतों, विशेष रूप से अमीनो एसिड की अत्यधिक खपत का कारण बनता है, और यह कोशिका चयापचय को बाधित कर सकता है, जिससे कोशिका वृद्धि बाधित होती है और प्रोटीन की पैदावार कम होती है। यह अध्ययन इस चयापचय तनाव को कम करने में अमीनो एसिड पूरकता की भूमिका का आगे पता लगाता है। प्लैकेट-बर्मन डीओई स्क्रीनिंग ने महत्वपूर्ण अमीनो एसिड की पहचान की, इसके बाद अमीनो एसिड खपत पैटर्न के आधार पर लक्षित पूरकता दृष्टिकोण अपनाया गया। इस रणनीति के परिणामस्वरूप सूखी कोशिका भार में 28% की वृद्धि, समावेशन निकायों के प्रति ग्राम प्रोटीन उपज में 40% सुधार, विशिष्ट विकास दर में 23% की वृद्धि और सबस्ट्रेट अपटेक में 37% वृद्धि हुई।

हालांकि, अधिक अभिव्यक्ति अक्सर अघुलनशील प्रोटीन समुच्चय के निर्माण की ओर ले जाती है जिसे समावेशन निकाय (IB) के रूप में जाना जाता है। IBs रिकवरी और रीफोल्डिंग दक्षता में चुनौतियों के कारण Fab टुकड़ों की डाउनस्ट्रीम प्रोसेसिंग एक अड़चन बनी हुई है। उत्पाद की उपज और शुद्धता में सुधार करने के लिए, हमने लाइसिस की स्थिति, IB रिकवरी और IB वॉशिंग चरणों को अनुकूलित किया। DOE द्वारा संचालित बफर संरचना अनुकूलन ने 0.069 ग्राम प्रोटीन/g IB, 61% IB शुद्धता और 87% लाइसिस दक्षता हासिल की। 1000 बार पर दो पास का उपयोग करके होमोजेनाइजेशन ने लाइसिस दक्षता को 93.5% तक बढ़ा दिया, जिससे 60% IB शुद्धता बनी रही। इसके अलावा, कमरे के तापमान पर 30 मिनट के लिए 1% ट्राइटन X-100 और 2M यूरिया का उपयोग करके अनुकूलित IB वॉशिंग के परिणामस्वरूप 84.53% IB रिकवरी और 75% IB शुद्धता हुई। इन अनुकूलन ने प्रदर्शित किया कि रिफाइनिंग लाइसिस और वॉशिंग पैरामीटर IBs की गुणवत्ता को महत्वपूर्ण रूप से बढ़ाते हैं, जिससे रीफोल्डिंग उपज और डाउनस्ट्रीम शुद्धिकरण में सुधार होता है। आईबी आमतौर पर अधिक स्थिर होते हैं और घुलनशील अंश की तुलना में लक्ष्य प्रोटीन का उच्च अनुपात रखते हैं। हालांकि, आईबी की गुणवत्ता और स्थिरता लक्ष्य प्रोटीन के रीफोल्डिंग और उसके बाद के शुद्धिकरण की दक्षता निर्धारित करने में महत्वपूर्ण भूमिका निभाते हैं। तापमान, अवधि और फ्रीज-थॉ चक्रों सहित आईबी भंडारण स्थितियों का व्यवस्थित रूप से विश्लेषण किया गया ताकि स्थिरता पर उनके प्रभाव को निर्धारित किया जा सके। - 80 डिग्री सेल्सियस पर संग्रहीत आईबी ने -20 डिग्री सेल्सियस पर संग्रहीत आईबी की तुलना में बेहतर स्थिरता प्रदर्शित की, समय के साथ और बार-बार फ्रीज-थॉ चक्रों के साथ गिरावट बढ़ गई। 45 दिनों के लिए -20 डिग्री सेल्सियस और -80 डिग्री सेल्सियस पर संग्रहीत आईबी ने क्रमशः रीफोल्डिंग उपज में 51% और 30% की कमी दिखाई। लाइओफिलाइज्ड आईबी ने समान परिस्थितियों में 40% और 20% की हानि प्रदर्शित की। लाइओफिलाइज्ड आईबी ने सभी परीक्षण स्थितियों के तहत बेहतर स्थिरता का प्रदर्शन किया, जो पारंपरिक आईबी से बेहतर प्रदर्शन करता है। इसके अलावा, समय के साथ लाइट चेन विखंडन में वृद्धि देखी गई, जो संभावित रूप से रीफोल्डिंग उपज और डाउनस्ट्रीम प्रसंस्करण में जटिलताओं को कम करने में योगदान दे सकती है। इसके अतिरिक्त, उच्च-कोशिका-घनत्व संवर्धन के दौरान बनने वाले IBs में होस्ट प्रोटीन अशुद्धता होती है, जो संभावित रूप से शुद्धिकरण में बाधा उत्पन्न करती है। इन निष्कर्षों के आधार पर, लाइओफिलाइज्ड IBs और न्यूनतम फ्रीज-थॉ चक्रों के साथ -80°C पर संग्रहीत IBs को IB अखंडता को संरक्षित करने और पुनः संयोजक प्रोटीन उपज को अधिकतम करने के लिए इष्टतम स्थितियों के रूप में पहचाना गया।

यह अध्ययन ई. कोलाई में फैब अणु उत्पादन को अनुकूलित करने के लिए एक व्यापक दृष्टिकोण प्रस्तुत करता है, जो अपस्ट्रीम प्रसंस्करण, चयापचय बोझ, डाउनस्ट्रीम शुद्धिकरण और आईबी स्थिरता में प्रमुख

चुनौतियों का समाधान करता है। निष्कर्ष प्रेरण मापदंडों पर सटीक नियंत्रण, लक्षित अमीनो एसिड पूरकता और समग्र प्रक्रिया दक्षता को बढ़ाने के लिए अनुकूलित आईबी हैंडलिंग के महत्व पर जोर देते हैं। इन रणनीतियों को लागू करने से रैनिबिजुमैब फैब प्रोटीन की पैदावार में काफी सुधार हो सकता है, अशुद्धियों को कम किया जा सकता है और बायोथेरेप्यूटिक विनिर्माण को सुव्यवस्थित किया जा सकता है।

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List of Abbreviations

Fab	Antigen-binding fragment
LC	Light chain of fab fragment
HC	Heavy chain of fab fragment
rHu	Recombinant Humanized
LB	Luria-Bertani medium
AA	Amino acid
DCW	Dry cell weight
DOE	Design of Experiments
CDM	Chemically defined media
OUR	Oxygen Uptake Rate
CER	Carbon Dioxide Evolution Rate
RSM	Response surface Methodology
CCD	Central composite design
IBs	Inclusion bodies
RP-HPLC	Reversed-phase high-performance liquid chromatography
rpm	Rotations per minute
EDTA	Ethylenediaminetetraacetic acid
IPTG	Isopropyl- β -D-thiogalactoside
OD	Optical density
PBS	Phosphate Buffered Saline
HCPs	Host cell proteins
RT	Room temperature
NaCl	Sodium Chloride
DTT	Dithiothreitol
TFA	Trifluoroacetic acid
ppGpp	Guanosine 3', 5' biphosphate
pppGpp	Guanosine 5'-triphosphate 3' diphosphate
mM	Millimolar
mg	Milligram
μ g	Microgram
g	Gram

L	Litre
mL	Millilitre
h	Hour
min	Minute
°C	degree Celsius
pH	Potential of Hydrogen
DO	Dissolved oxygen
PAGE	Polyacrylamide gel electrophoresis
pET	Plasmid for expression by T7 RNA polymerase
RMSE	Root mean square error

List of Symbols

$Y_{P/S}$: Product yield coefficient on substrate

$Y_{X/S}$: Yield coefficient of biomass on substrate

Greek letters

μ : Specific growth rate (h^{-1})

μ_{max} : Maximum specific growth rate (h^{-1})