

**BIOPROCESS DEVELOPMENT FOR ENHANCING
RECOMBINANT PROTEIN PRODUCTION
IN ESCHERICHIA COLI**

JASHWANT KUMAR



**DEPARTMENT OF CHEMICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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IN ESCHERICHIA COLI**

by

JASHWANT KUMAR

DEPARTMENT OF CHEMICAL ENGINEERING

Submitted

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

to the



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Dedicated to my family...!

CERTIFICATE

This is to certify that the thesis entitled “**BIOPROCESS DEVELOPMENT FOR ENHANCING RECOMBINANT PROTEIN PRODUCTION IN *ESCHERICHIA COLI*”** being submitted by **JASHWANT KUMAR** 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.

Prof. Anurag S. Rathore

Department of Chemical Engineering
Indian Institute of Technology Delhi

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JASHWANT KUMAR

Abstract

Escherichia coli (*E. coli*) is the industrial workhorse for recombinant protein production (RPP). However, *E. coli* based RPP has its own share of challenges, fuelling continued research into topics such as foreign protein expression as insoluble aggregates, protein toxicity to host, stress induced by amino acid starvation, stress induced by strong overexpression of heterologous gene, and accumulation of toxic by-product mainly acetate. In this thesis work, some of the major limitations of *E. coli* bioprocess have been addressed to improve recombinant protein production.

The work's first objective (Chapter 3) presents a novel amino acids (AAs) supplementation strategy for improving recombinant protein production in *E. coli* using a chemically defined medium. During recombinant protein production, AAs starvation result in a global stress response known as stringent-like response. This stress response results in sudden cell growth arrest and halt of protein synthesis, which compromises process productivity. Initially, in this study effect of all 20 AAs supplementation on cellular growth and RPP was investigated. Based on the consumption profile of amino acids, few AAs were categorized as growth-promoting (GP1) and few as recombinant protein promoting (GP2). Different feeding strategies of GP1 and GP2 AAs were tested in shake flask, followed by scale up of best strategy in the bioreactor. Optimized AAs supplementation strategy resulted in a 40% increment of recombinant pramlintide multimer (rPramlintide) titer. These results signify that the external supply of critical AAs decreases the cellular stress during RPP and improves process productivity.

Through the second objective (Chapter 4) of the thesis, a lactose-based induction strategy in fed-batch cultivation of recombinant *E. coli* was developed to overcome the limitation of isopropyl-D-1-thiogalactopyranoside (IPTG) as an inducer. In this study, we have initially demonstrated that supplementation of critical amino acids (AAs) improves glycerol and lactose uptake in *E. coli* BL21(DE3) in defined medium. A feeding strategy of mixed glycerol-lactose feed along with supplement of critical AAs was developed enhancing rPramlintide titer. High cell density cultivation of *E. coli* using mixed glycerol-lactose feed and critical AAs supplement resulted in a final cell density of 52.2 ± 0.90 g/L and rPramlintide titer of 7.8 g/L. We hypothesize that supplementation of critical AAs serves a dual purpose: i) faster assimilation of carbon sources ii) combating metabolic stress arises due to AAs starvation.

The Process Analytical Technology (PAT) framework outlined by US FDA has highlighted the need for real-time monitoring and control of critical process parameters in biopharmaceutical

manufacturing. Feed and acetate control are two critical parameters in *E. coli* based upstream process that decide the process productivity. Through this objective (Chapter 5), we have demonstrated the application of Near Infrared Spectroscopy (NIRS) as a PAT tool for real-time control of feed and acetate in fed-batch cultivation of recombinant *E. coli*. NIRS models were developed using a calibration library of bioreactor samples spectra to quantify the three key analytes changing over the course of fed-batch production phase, namely lactose, acetate, and galactose. A novel feeding strategy was implemented using real-time analysis of the NIRS spectra to control the glycerol and lactose feed pumps. Acetate concentration was controlled in real-time by automatically reducing the glycerol feed rate in a stage-wise manner whenever acetate concentrations were observed to exceed certain fixed thresholds. Production of rPramlintide was enhanced by 58% in the PAT controlled process compared to the conventional process.

Specific growth rate (μ) is a key process parameter that affects cell growth and recombinant protein expression in *E. coli*. Process productivity depends upon the careful selection of pre- and post-induction μ during fed-batch cultivation. However, post-induction μ has a direct effect on heterologous protein expression. Through the last objective (Chapter 6) of the thesis work, we investigated the effect of different (low to high) post-induction μ on the production of pramlintide multimer (rPramlintide) and ranibizumab. An exponential feeding strategy was used to control pre- and post-induction μ . Results demonstrated that low post-induction μ improves the expression of heterologous protein. The study highlighted a correlation between μ and protein production, thereby elucidates the importance of controlling post-induction specific growth rate.

Overall, this thesis work aims to address some of the key challenges accompanying *E. coli* based recombinant bioprocess. The proposed bioprocess strategies and NIRS based PAT application showed enhanced recombinant protein yield when applied on *E. coli* based process.

सार

एस्चेरिचिया कोलाई (ई. कोलाई) रेकॉम्बीनेंट प्रोटीन उत्पादन (आरपीपी) के लिए औद्योगिक वर्कहॉर्स है। हालांकि, ई. कोलाई आधारित रेकॉम्बीनेंट प्रोटीन उत्पादन की अपनी चुनौतियाँ हैं, जिसके फलस्वरूप निरंतर काफी विषयों में अनुसंधान को बढ़ावा मिल रही है, जैसे की फॉरेन प्रोटीन का अघुलनशील अग्रेगेट्स की तरह एक्सप्रेसन, होस्ट पर प्रोटीन टॉक्सिसिटी का प्रभाव, अमीनो एसिड्स भुखमरी से प्रेरित तनाव, हेटेरोलोगोस जीन की अत्यधिक एक्सप्रेसन से प्रेरित तनाव और विषकारक सह-उत्पाद का संग्रह मुख्य रूप से एसीटेट। इस थीसिस कार्य में, ई. कोलाई बायोप्रोसेस की कुछ प्रमुख चुनौतियाँ का समाधान के द्वारा रेकॉम्बीनेंट प्रोटीन उत्पादन में वृद्धि कराने का प्रयास किया गया है।

शोधकार्य का पहले उद्देश्य (अध्याय ३) में रासायनिक रूप से परिभाषित माध्यम का उपयोग करके ई. कोलाई में रेकॉम्बीनेंट प्रोटीन उत्पादन में सुधार के लिए एमिनो एसिड्स सप्लिमेंटेशन की रणनीति प्रस्तुत किया गया है। रेकॉम्बीनेंट प्रोटीन उत्पादन के दौरान, अमीनो एसिड्स भुखमरी के परिणामस्वरूप वैश्विक तनाव प्रतिक्रिया होती है जिसे स्ट्रिंजेंट-लाइक रिस्पांस के रूप में जाना जाता है। इस तनाव प्रतिक्रिया के परिणामस्वरूप अचानक कोशिका वृद्धि और प्रोटीन उत्पादन रुक जाता है, जो प्रोसेस की उत्पादकता से समझौता करता है। प्रारंभ में, इस अध्ययन में सेलुलर विकास और आरपीपी पर सभी २० एमिनो एसिड्स सप्लिमेंटेशन के प्रभाव की जांच की गई। अमीनो एसिड्स की खपत प्रोफाइल के आधार पर, कुछ अमीनो एसिड्स को कोशिका विकास को बढ़ावा देने वाले (जीपी १) के रूप में और कुछ को रेकॉम्बीनेंट प्रोटीन को बढ़ावा देने वाले (जीपी २) के रूप में वर्गीकृत किया गया। जीपी १ और जीपी २ अमीनो एसिड्स की विभिन्न फीडिंग रणनीतियों का शेक फ्लास्क में परीक्षण किया गया, इसके बाद बायोरिएक्टर में सर्वश्रेष्ठ रणनीति का विस्तार किया गया। ऑप्टिमाइज़्ड अमीनो एसिड्स सप्लिमेंटेशन रणनीति के परिणामस्वरूप रेकॉम्बीनेंट प्रोटीन प्राम्लिंटाइड मल्टीमर के उत्पादन में ४०% की वृद्धि हुई। ये परिणाम दर्शाते हैं कि महत्वपूर्ण अमीनो एसिड्स की बाहरी सप्लिमेंटेशन से रेकॉम्बीनेंट प्रोटीन उत्पादन के दौरान सेलुलर तनाव को कम करती है और प्रोसेस की उत्पादकता में सुधार लाती है।

शोधकार्य के दूसरे उद्देश्य (अध्याय ४) के माध्यम से रेकॉम्बीनेंट ई. कोलाई की फेड-बैच कल्टीवेशन में लैक्टोज-आधारित इंडक्शन रणनीति को विकसित किया गया जो आइसोप्रोपिल-डी-1-थियोगैलेक्टोपायरोसाइड (आईपीटीजी) की लिमिटेशन को दूर करती है। मिश्रित ग्लिसरॉल-लैक्टोज फीड और महत्वपूर्ण अमीनो एसिड्स सप्लिमेंटेशन का उपयोग करके ई. कोलाई की हाई सेल डेंसिटी कल्टीवेशन के परिणामस्वरूप सेल डेंसिटी 42.2 ± 0.90 ग्राम प्रति लीटर और प्राम्लिंटाइड अनुमापंक ७.८ ग्राम प्रति लीटर प्राप्त हुई। हम परिकल्पना करते हैं कि

महत्वपूर्ण अमीनो एसिड सप्लिमेंटेशन दो उद्देश्य की पूर्ति करता है: i) कार्बन स्रोतों का तेजी से आत्मसात करना ii) अमीनो एसिड्स भुखमरी के कारण मेटाबोलिक तनाव का मुकाबला करना ।

यू एस एफडीए द्वारा उल्लिखित प्रोसेस एनालिटिकल टेक्नोलॉजी (पीएटी) रूपरेखा में बायोफार्मासिटिकल उत्पादन के द्वारा प्रोसेस की वास्तविक समय में निगरानी और क्रिटिकल प्रोसेस पैरामीटरस की आवश्यकता पर प्रकाश डाला है। ई. कोलाई आधारित अपस्ट्रीम प्रोसेस में फीड और एसीटेट नियंत्रण दो महत्वपूर्ण पैरामीटरस हैं जो प्रोसेस की उत्पादकता तय करते हैं। शोधकार्य के इस उद्देश्य (अध्याय ५) के माध्यम से, हमने रेकॉम्बीनेंट ई. कोलाई की फेड-बैच कल्टीवेशन में फीड और एसीटेट के वास्तविक समय नियंत्रण के लिए नियर इन्फ्रारेड स्पेक्ट्रोस्कोपी (एनआईआरएस) को पीएटी उपकरण के रूप में अनुप्रयोग का प्रदर्शन किया है। फेड-बैच उत्पादन चरण के द्वारा तीन प्रमुख मेटाबॉलिट्स जो निरन्तर बदलते हैं अर्थात् लैक्टोज, एसीटेट, और गैलेक्टोज, उनका विश्लेषण करने के लिए बायोरिएक्टर सैम्पल्स के स्पेक्ट्रा के कैलिब्रेशन लाइब्रेरी द्वारा एनआईआरएस मॉडल विकसित किया गया। ग्लिसरॉल और लैक्टोज फीड पंपों को नियंत्रित करने के लिए एनआईआरएस स्पेक्ट्रा के वास्तविक समय में विश्लेषण करके एक नई फीडिंग रणनीति लागू की गई । जब भी एसीटेट कंसंट्रेशन कुछ निश्चित थ्रेशहोल्ड से अधिक देखी गई, तो स्टेज वाइज तरीके से ग्लिसरॉल फीड दर को स्वचालित रूप से कम करके वास्तविक समय में एसीटेट कंसंट्रेशन को नियंत्रित किया गया। पारंपरिक प्रोसेस की तुलना में पीएटी नियंत्रित प्रोसेस में प्रोमिल्टाइड का उत्पादन ५८ % बढ़ा पाया गया।

विशिष्ट विकास दर (μ) एक प्रमुख प्रोसेस पैरामीटर है जो ई. कोलाई में कोशिका वृद्धि और रेकॉम्बीनेंट प्रोटीन उत्पादन को प्रभावित करता है। प्रोसेस की उत्पादकता फेड-बैच कल्टीवेशन के दौरान प्री- और पोस्ट-इंडक्शन के विशिष्ट विकास दर (μ) के सावधानीपूर्वक चयन पर निर्भर करती है। हालांकि, पोस्ट-इंडक्शन μ का रेकॉम्बीनेंट प्रोटीन उत्पादन पर सीधा प्रभाव पड़ता है । थीसिस शोधकार्य के अंतिम उद्देश्य (अध्याय ६) के माध्यम से, हमने प्रोमिल्टाइड मल्टीमर और रानीबिजुमैब के उत्पादन पर विभिन्न (निम्न से उच्च) पोस्ट-इंडक्शन μ के प्रभाव की जांच की। प्री- और पोस्ट-इंडक्शन μ नियंत्रित करने के लिए एक्सपोजेक्शन फीडिंग रणनीति का उपयोग किया गया। परिणामों ने प्रदर्शित किया कि निम्न पोस्ट-इंडक्शन μ रेकॉम्बीनेंट प्रोटीन उत्पादन में सुधार करता है। अध्ययन ने विशिष्ट विकास दर और प्रोटीन उत्पादन के बीच एक संबंध पर प्रकाश डाला, जिससे पोस्ट-इंडक्शन के μ को नियंत्रित करने के महत्व को स्पष्ट किया ।

कुल मिलाकर, इस थीसिस शोधकार्य का उद्देश्य ई. कोलाई आधारित रेकॉम्बीनेंट बायोप्रोसेस के साथ आने वाली कुछ प्रमुख चुनौतियाँ का समाधान करना था । प्रस्तावित बायोप्रोसेस रणनीतियाँ और एनआईआरएस आधारित पीएटी अनुप्रयोग ने ई. कोलाई आधारित प्रोसेस के रेकॉम्बीनेंट प्रोटीन उपज में वृद्धि दिखाई।

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List of Abbreviations

AAs	Amino acids
ACN	Acetonitrile
ANOVA	Analysis of variance
CCR	Carbohydrate catabolic repression
CDM	Chemically defined medium
DCU	Digital control unit
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GCSF	Granulocyte-colony stimulating factor
GUI	Graphical user interface
HPLC	High performance liquid chromatography
HSP	Heat shock protein
IB	Inclusion body
IPTG	Isopropyl-D-1-thiogalactopyranoside
NIRS	Near infrared spectroscopy
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PAT	Process analytical technology
PEP	Phosphoenolpyruvate
PLS	Partial least square
pET	Plasmid for expression by T7 RNA polymerase
ppGpp	Guanosine 3', 5' biphosphate
pppGpp	Guanosine 5'-triphosphate 3' diphosphate
PTMs	Post translational modifications
PTS	Phosphotransferase system
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNAP	RNA polymerase
RPP	Recombinant protein production
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
rDNA	Recombinant deoxyribonucleic acid
TCA	Tricarboxylic acid
tRNA	Transfer RNA
UPLC	Ultra-performance liquid chromatography

Symbols

C_S	Substrate concentration in feed reservoir	[gL ⁻¹]
C_{XL}	Cell density	[gL ⁻¹]
F_0	initial start rate of peristaltic pump	[Lh ⁻¹]
F_R	Exponential feeding rate	[Lh ⁻¹]
Gal	Galactose	[gL ⁻¹]
Glu	Glucose	[gL ⁻¹]
Gly	Glycerol	[gL ⁻¹]
$k_{X/OD}$	Strain specific constant factor	[gL ⁻¹ AU ⁻¹]
Lac	Lactose	[gL ⁻¹]
n	Dilution factor	[-]
OD_{600nm}	Optical density at 600 nm	[AU]
pO_2	Relative oxygen partial pressure in the liquid phase	[%]
q_s	Specific substrate consumption rate	[C-mmol gDCW ⁻¹ h ⁻¹]
q_s/x_m	Specific maintenance rate	[h ⁻¹]
V_L	Volume of the reactor	[L]
xCO_2	Mole fraction of carbon dioxide	[-]
$Y_{P/S}$	Product yield coefficient on substrate	[-]
$Y_{X/S}$	Yield coefficient of biomass on substrate	[-]

Greek symbols

μ	Specific growth rate	[h ⁻¹]
μ_{max}	Maximum specific growth rate	[h ⁻¹]
μ_{set}	Set point for specific growth rate	[h ⁻¹]

Publications

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