

STRATEGIES FOR IMPROVING THE PRODUCTION OF BIOTHERAPEUTIC PROTEIN

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by

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.....Dedicated to my daughter

Certificate

This is to certify that the thesis entitled “**Strategies for improving the production of biotherapeutic protein**” being submitted by **PRIYANKA** 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 her under our 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.

We certify that she has pursued the prescribed course of research.

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PRIYANKA

Abstract

This thesis documents a study of how process efficiency can be increased by the combined effect of molecular biology, media development, process optimization, and implementing advanced control schemes. The bioprocess outcome (critical quality attributes) is affected by many factors, including the type of microorganism used, medium composition, and operating conditions and control system employed (critical process parameters).

Molecular biology has seen significant advancements over the past few decades. The Assembly of multiple DNA copies and essential transcriptional and translational entities has enabled us to produce complex biotherapeutic products in microbial systems, earlier constrained to mammalian systems. The first objective presents a novel strategy for achieving efficient expression of antibody fragment in *Escherichia coli*. Ranibizumab, a humanized monoclonal antibody fragment that is used for treatment of age-related macular degeneration and macular edema, has been chosen as a case study. Most manufacturers express it as inclusion bodies in BL21 DE3 *E. coli* expression system. The gene encoding for Ranibizumab has previously been cloned but the reported processes were either very tedious or vector and host-specific. In our proposed strategy, the whole antibody fragment has been single step cloned in a pET series vector belonging to a prokaryotic host along with an efficient promoter and extra ribosome binding site to cater for equal expression of the light and heavy chains. Another advantage is that the whole expression unit could be excised and placed in any vector choice. Heterologous expression was found to be doubled, which has been confirmed both using Tricine PAGE level as well as RP-HPLC. The reduced antibody fragment has also been analytically characterized using LC-MS. Besides, the role of clone selection and process optimization to enhance expression yield has been explored. We think that the proposed strategy would be of interest to those seeking production of antibody fragments in microbial hosts.

Upstream process development has a significant impact on overall process yield. Most manufacturers currently express it in *Escherichia coli strain* BL21 (DE3), where low-level expression of this complex protein has been acknowledged to result in higher production cost. This paper aims to present a novel strategy involving use of additives for enhancing expression of Ranibizumab in *E. coli*. Media additives, identified based on risk analysis, were screened for impact on productivity. Glutamine, proline, arginine, and ethanol exhibited an increase in protein

production compared to control. Based on process economics and scale-up considerations, ethanol was chosen as the optimal medium additive. The effect of ethanol addition on cell size (control 1.82 μm and optimized 2 μm), cellular physiology (no cell swelling or shrinkage observed) and cellular viability (better in optimized sample) were examined. A two-fold improvement in expression has been demonstrated (from 0.23 mg/mL to 0.48 mg/mL in single copy clone and 0.4 mg/mL to 0.72 mg/mL in double copy clone) using the optimized conditions vis-a-vis the control, thereby demonstrating the efficacy of the proposed approach. Transcriptomic analysis and RT-qPCR validation indicated that changes in membrane properties and DNA synthesis result in gene amplification and enhance synthesis of inducible proteins in case of the optimized medium.

Recombinant protein production by various host organisms is strictly regulated, which is determined mainly by host cell genetics and maintenance of process variables within desirable limits in a reactor. Since the process dynamics typically exhibit non-linear interactions amongst the critical process variables, the commonly employed controllers are suboptimal for control of most bioprocesses. Through the third objective, we present the implementation of an augmented advanced controller for improving the production of recombinant human serum albumin using *Pichia pastoris*. The augmented controller consisted of a decoupled adaptive control (DAC) and a decoupled input-output linearizing control (DIOLC) in a combined architecture that permits simultaneous control of substrate concentration and dissolved oxygen concentration, sampled at different rates during fed-batch fermentation. The performance of augmented controller was compared to the performance of standalone implemented individually on the process. Our results showed that the augmented controller increases productivity with better regulation of substrate and DO along the desired control trajectory compared to individual DAC and DIOLC controllers. The performance of the augmented DAC-DIOLC controller showed deviations of only 0.021 g/L from substrate set point compared to 0.09 g/L in PID and 0.031 g/L in DIOLC, and a 1.12% deviation from DO set point compared to 11.86% in PID and 2.58% in DAC. Overall, better control was achieved using the augmented controller and resulted in a 1.5-fold increase in rHSA production.

The Process Analytical Technology (PAT) initiative from US FDA has highlighted the need for robust monitoring tools and effective control systems capable of receiving information on critical process parameters in real time and execute immediate response as soon as there are deviations in

process parameters. A typical bioreactor uses a supervisory control and data acquisition (SCADA) system for control, a combination of software and hardware tools designed to gather real-time data, analyze and control using a mix of hardware and soft sensors. However, when the process is disrupted by utility or instrumentation failure, typical process controllers may be unable to reinstate normal operating conditions before the cells in the reactor shift to unfavorable metabolic regimes. The objective of this study was to examine how the response of a controller affects process recovery when disruptive incidences occur under a Process Analytical Technology (PAT) framework. The DIOLC is an advanced controller that is derived using methods of global linearization and input-output decoupling. The performance of the DIOLC was compared to a PID controller subjected to the same conditions. As a case study, the disruptions were introduced manually and the effect of controller action on process recovery and Lethal Toxin Neutralizing Factor (LTNF) synthesis was measured in terms of product quality and concentration. It was observed that DIOLC performs better after reinstating operating conditions and results in a meaningful performance improvement. The data presented in this study illustrates the importance of a control scheme to generate optimal process performance.

सार

यह थीसिस दस्तावेज का अध्ययन करता है कि आणविक जीव विज्ञान, मीडिया विकास, प्रक्रिया अनुकूलन और संयुक्त नियंत्रण योजनाओं को लागू करने के संयुक्त प्रभाव से प्रक्रिया दक्षता कैसे बढ़ाई जा सकती है। बायोप्रोसेस परिणाम (महत्वपूर्ण गुणवत्ता गुण) कई कारकों से प्रभावित होता है, जिसमें प्रयुक्त सूक्ष्मजीव का प्रकार, मध्यम संरचना, और संचालन की स्थिति और नियोजित प्रणाली (महत्वपूर्ण प्रक्रिया पैरामीटर) शामिल हैं। पिछले कुछ दशकों में आणविक जीवविज्ञान ने महत्वपूर्ण प्रगति देखी है। कई डीएनए प्रतियों और आवश्यक ट्रांसक्रिप्शनल और ट्रांसलेशनल इकाइयों की असेंबली ने हमें माइक्रोबियल सिस्टम में जटिल जैव-चिकित्सीय उत्पादों का उत्पादन करने में सक्षम किया है, जो पहले स्तनधारी प्रणालियों के लिए विवश थे। पहला उद्देश्य एस्चेरिचिया कोलाई में एंटीबॉडी टुकड़े की कुशल अभिव्यक्ति प्राप्त करने के लिए एक उपन्यास रणनीति प्रस्तुत करता है। रानीबिजुमाब, एक मानवकृत मोनोक्लोनल एंटीबॉडी टुकड़ा जो कि उम्र से संबंधित धब्बेदार अधः पतन और धब्बेदार एडिमा के उपचार के लिए उपयोग किया जाता है, को केस स्टडी के रूप में चुना गया है। अधिकांश निर्माता इसे BL21 DE3 E. कोली एक्सप्रेसन सिस्टम में शामिल किए जाने वाले निकाय के रूप में व्यक्त करते हैं। रानीबिजुमाब के लिए जीन एन्कोडिंग पहले से क्लोन की गई है, लेकिन रिपोर्ट की गई प्रक्रियाएं बहुत थकाऊ या वेक्टर और होस्ट-विशिष्ट थीं। हमारी प्रस्तावित रणनीति में, प्रकाश और भारी श्रृंखलाओं की समान अभिव्यक्ति के लिए एक कुशल प्रमोटर और अतिरिक्त राइबोसोम बाइंडिंग साइट के साथ एक प्रोकेरियोटिक होस्ट से संबंधित पीईटी श्रृंखला वेक्टर में पूरे एंटीबॉडी टुकड़े को एकल चरण क्लोन किया गया है। एक और लाभ यह है कि पूरी अभिव्यक्ति इकाई को किसी भी वेक्टर पसंद में रखा जा सकता है। Heterologous व्यंजक को दोगुना पाया गया, जिसकी पुष्टि Tricine PAGE स्तर के साथ-साथ RP-HPLC दोनों का उपयोग करके की गई है। कम एंटीबॉडी टुकड़ा भी LC-MS का उपयोग करके विश्लेषणात्मक रूप से विशेषता है। इसके अलावा, अभिव्यक्ति की उपज बढ़ाने के लिए क्लोन चयन और प्रक्रिया अनुकूलन की भूमिका का पता लगाया गया है। हमें लगता है कि प्रस्तावित रणनीति माइक्रोबियल होस्ट में एंटीबॉडी टुकड़े के उत्पादन की मांग करने वालों के लिए दिलचस्पी की होगी।

अपस्ट्रीम प्रोसेस डेवलपमेंट का समग्र प्रोसेस यील्ड पर महत्वपूर्ण प्रभाव पड़ता है। अधिकांश निर्माता वर्तमान में इसे एस्चेरिचिया कोलाई स्ट्रेन BL21 (DE3) में व्यक्त करते हैं, जहां इस जटिल प्रोटीन की निम्न-स्तरीय अभिव्यक्ति को उच्च उत्पादन लागत के परिणामस्वरूप स्वीकार किया गया है। इस पत्र का उद्देश्य ई. कोलाई में रानीबिजुमाब की अभिव्यक्ति को बढ़ाने के लिए योजक के उपयोग से संबंधित एक उपन्यास रणनीति प्रस्तुत करना है। मीडिया एडिटिक्स, जो जोखिम विश्लेषण के आधार पर पहचाने जाते हैं, उत्पादकता पर प्रभाव के लिए जांच की गई। ग्लूटामाइन, प्रोलिन, आर्जिनिन और इथेनॉल ने नियंत्रण की तुलना में प्रोटीन उत्पादन में वृद्धि का प्रदर्शन किया। प्रक्रिया अर्थशास्त्र और स्केल-अप विचारों के आधार पर, इथेनॉल को इष्टतम मध्यम योजक के रूप में चुना गया था। सेल आकार (नियंत्रण 1.82 माइक्रोन और अनुकूलित 2 माइक्रोन) पर इथेनॉल के अलावा के प्रभाव, सेलुलर शरीर क्रिया विज्ञान (कोई सेल सूजन या संकोचन नहीं देखा गया) और सेलुलर व्यवहार्यता (अनुकूलित नमूने में

बेहतर) की जांच की गई। अभिव्यक्ति में दो गुना सुधार का प्रदर्शन किया गया है (एकल कॉपी क्लोन में 0.23 मिलीग्राम / एमएल से 0.48 मिलीग्राम / एमएल और डबल कॉपी क्लोन में 0.4 मिलीग्राम / एमएल से 0.72 मिलीग्राम / एमएल) का उपयोग करके अनुकूलित परिस्थितियों में विज़-ए-विज़ का उपयोग किया गया है नियंत्रण, जिससे प्रस्तावित दृष्टिकोण की प्रभावकारिता प्रदर्शित होती है। ट्रांसक्रिप्टामिक विश्लेषण और आरटी-क्यूपीसीआर सत्यापन ने संकेत दिया कि झिल्ली गुणों और डीएनए संश्लेषण में परिवर्तन के परिणामस्वरूप जीन प्रवर्धन होता है और अनुकूलित माध्यम के मामले में अमिट प्रोटीन के संश्लेषण को बढ़ाता है।

विभिन्न मेजबान जीवों द्वारा पुनः संयोजक प्रोटीन उत्पादन को सख्ती से विनियमित किया जाता है, जो मुख्य रूप से मेजबान सेल आनुवंशिकी और एक रिएक्टर में वांछनीय सीमा के भीतर प्रक्रिया चर के रखरखाव द्वारा निर्धारित किया जाता है। चूंकि प्रक्रिया की गतिशीलता आम तौर पर महत्वपूर्ण प्रक्रिया चर के बीच गैर-रेखीय इंटरैक्शन का प्रदर्शन करती है, आमतौर पर नियोजित नियंत्रक अधिकांश बायोप्रोसेस के नियंत्रण के लिए उप-रूपी होते हैं। तीसरे उद्देश्य के माध्यम से, हम *Pichia pastoris* का उपयोग कर पुनः संयोजक मानव सीरम एल्बुमिन के उत्पादन में सुधार के लिए एक उन्नत उन्नत नियंत्रक के कार्यान्वयन को प्रस्तुत करते हैं। संवर्धित नियंत्रक में एक संयुक्त आर्किटेक्चर में एक डिकूड एडेप्टिव कंट्रोल (डीएसी) और एक डिकूड इनपुट-आउटपुट लाइनराइजिंग कंट्रोल (DIOLC) शामिल है जो सबस्ट्रेट एकाग्रता और भंग ऑक्सीजन एकाग्रता के एक साथ नियंत्रण की अनुमति देता है, जो फेड-बैच किण्वन के दौरान अलग-अलग दरों पर सैंपल देता है। संवर्धित नियंत्रक के प्रदर्शन की तुलना प्रक्रिया पर व्यक्तिगत रूप से लागू किए गए स्टैंडअलोन के प्रदर्शन से की गई थी। हमारे परिणामों से पता चला है कि संवर्धित नियंत्रक सबस्ट्रेट और डीओ के बेहतर विनियमन के साथ उत्पादकता बढ़ाता है और व्यक्तिगत डीएसी और डीओओएलसी नियंत्रकों की तुलना में वांछित नियंत्रण प्रक्षेपवक्र के साथ होता है। संवर्धित DAC-DIOLC नियंत्रक के प्रदर्शन ने सबस्ट्रेट सेट बिंदु से केवल 0.021 g / L के विचलन और PID में 0.09 ग्राम / लीटर और DIOLC में 0.031 ग्राम / लीटर की तुलना में दिखाया, और 11.86% की तुलना में DO सेट बिंदु पर 1.12% विचलन किया। पीआईडी में और 2.58% डीएसी में। कुल मिलाकर, संवर्धित नियंत्रक का उपयोग करके बेहतर नियंत्रण हासिल किया गया और परिणामस्वरूप आरएचएसए उत्पादन में 1.5 गुना वृद्धि हुई।

यूएस एफडीए से प्रोसेस एनालिटिकल टेक्नोलॉजी (पीएटी) पहल ने वास्तविक समय में महत्वपूर्ण प्रक्रिया मापदंडों की जानकारी प्राप्त करने में सक्षम मजबूत निगरानी उपकरण और प्रभावी नियंत्रण प्रणालियों की आवश्यकता पर प्रकाश डाला है और प्रक्रिया मापदंडों में विचलन होते ही तत्काल प्रतिक्रिया निष्पादित की है। एक विशिष्ट बायोरिएक्टर नियंत्रण के लिए एक पर्यवेक्षी नियंत्रण और डेटा अधिग्रहण (SCADA) प्रणाली का उपयोग करता है, हार्डवेयर और सॉफ्ट सेंसर के मिश्रण का उपयोग करके वास्तविक समय के डेटा को इकट्ठा करने, विश्लेषण और नियंत्रण करने के लिए डिज़ाइन किए गए सॉफ्टवेयर और हार्डवेयर टूल का संयोजन। हालाँकि, जब प्रक्रिया उपयोगिता या इंस्ट्रुमेंटेशन विफलता से बाधित होती है, तो विशिष्ट प्रक्रिया नियंत्रक प्रतिकूल मेटाबॉलिक शासनों के लिए रिएक्टर शिफ्ट में कोशिकाओं से पहले सामान्य परिचालन स्थितियों को बहाल करने में असमर्थ हो सकते हैं। इस अध्ययन का उद्देश्य यह जांचना था कि एक प्रक्रिया की विश्लेषणात्मक प्रक्रिया (पैट) ढांचे के तहत

विघटनकारी घटना होने पर नियंत्रक की प्रतिक्रिया प्रक्रिया वसूली को कैसे प्रभावित करती है। DIOLC एक उन्नत नियंत्रक है जो वैश्विक रैखिककरण और इनपुट-आउटपुट डिकम्पलिंग के तरीकों का उपयोग करके प्राप्त किया जाता है। DIOLC के प्रदर्शन की तुलना समान परिस्थितियों के अधीन PID नियंत्रक से की गई थी। एक मामले के अध्ययन के रूप में, व्यवधानों को मैनुअल रूप से पेश किया गया था और प्रक्रिया वसूली और घातक टॉक्सिन न्यूट्राइजिंग फैक्टर (एलटीएनएफ) संश्लेषण पर नियंत्रक कार्रवाई के प्रभाव को उत्पाद की गुणवत्ता और एकाग्रता के संदर्भ में मापा गया था। यह देखा गया कि DIOLC ऑपरेटिंग परिस्थितियों और परिणामों को एक सार्थक प्रदर्शन में सुधार करने के बाद बेहतर प्रदर्शन करता है। इस अध्ययन में प्रस्तुत डेटा इष्टतम प्रक्रिया प्रदर्शन उत्पन्न करने के लिए एक नियंत्रण योजना के महत्व को दर्शाता है।

Contents

Certificate	i
Acknowledgments	ii
Abstract.....	iii
Contents	vi
List of Figures.....	xii
List of Tables	xvii
Abbreviations.....	xix
Chapter 1: Introduction and Objectives.....	1
1.1 Motivation and background	2
1.2 Problem definition, scope and objective of the research	4
1.2.1 Complex biotherapeutic production at microbial platform	4
1.2.2 Cost-effective process development for enhanced recombinant protein expression	5
1.2.3 Improvement over existing advanced controller	6
1.2.4 Process analytical implementation (PAT) using advanced controller	7
Chapter 2: Literature Review	8
2.1 Molecular biology approach	10
2.1.1 Cloning strategies	11
2.1.2 Codon optimization	11
2.1.3 Host Selection	12
2.2 Upstream process development approach.....	16
2.2.1 Medium screening and optimization	16
2.2.2 Process parameter optimization	17
2.3 Process monitoring.....	21

2.4 Process control approach	23
2.4.1 Open loop controller.....	24
2.4.2 Closed loop control system	25
2.4.3 Advanced control strategies	26
Chapter 3: A novel strategy for efficient expression of an antibody fragment in <i>E. coli</i> : Ranibizumab as a case study	32
3.1 Introduction.....	33
3.2 Material and methods.....	35
3.2.1 Strains, plasmids and media	35
3.2.2 Synthesis of the codon optimized Ranibizumab gene and construction of the expression vector.....	35
3.2.3 Transformation of the positive construct in expression host and screening of transformants with high expression capacity	38
3.2.4 Optimization of cell growth and recombinant Ranibizumab production in a shake flask	39
3.2.5 Screening of growth medium	39
3.2.6 Temperature optimization	40
3.2.7 Offline analysis	40
3.2.8 Tricine-PAGE and HPLC analysis.....	40
3.2.9 LCMS analysis for intact mass analysis.....	41
3.3 Results and discussions.....	41
3.3.1 Cloning of Ranibizumab into the expression plasmid	41
3.3.2 Preliminary expression check and intact mass confirmation	42
3.3.3 Medium optimization	47
3.3.4 Temperature optimization	49
3.3.5 Double copy expression to enhance protein expression.....	50

3.4 Conclusions.....	53
Chapter 4: Medium optimization for enhancing expression of antibody fragment in microbial cells: Impact on cellular physiology and transcriptome.....	54
4.1 Introduction.....	55
4.2 Experimental Procedure.....	56
4.2.1 Materials.....	56
4.2.2 Strains, plasmids, and media	56
4.2.3 Cloning and expression of Ranibizumab gene	56
4.2.4 Optimization of the initial expression	57
4.2.5 Screening of chemical additives.....	57
4.2.6 Economic analysis.....	60
4.2.7 Effect of ethanol on cell physiology	60
4.2.8 Zeta Sizer measurements.....	61
4.2.9 Confocal microscopy.....	61
4.2.10 Scanning electron microscopy (SEM).....	61
4.2.11 Transcriptome analysis.....	61
4.2.12 RNA isolation and RT-qPCR.....	62
4.2.13 Offline analysis	63
4.2.14 Tricine-PAGE, HPLC and LCMS analysis.....	63
4.3 Results and discussions.....	64
4.3.1 Cloning of Ranibizumab into expression plasmid	64
4.3.2 Effect of chemical additives on protein production	64
4.3.3 Economic analysis of positive medium additive.....	67
4.3.4 Final optimized conditions	67
4.3.5 Effect of the optimized condition on protein enhancement at shake flask level.....	68

4.3.6 Confocal Microscopy	70
4.3.7 Scanning Electron Microscopy (SEM)	70
4.3.8 Transcriptomics analysis	72
4.3.9 Process implication on double copy clone	78
4.4 Conclusions.....	79
Chapter 5: Comparison and implementation of different control strategies for improving the production of rHSA using <i>Pichia pastoris</i>	80
5.1 Introduction.....	81
5.2 Theoretical development.....	83
5.2.1 Model development.....	83
5.2.2 Derivation of Decoupled Adaptive Controller (DAC).....	83
5.2.3 Derivation of Decoupled Input-Output Linearizing Controller (DIOLC)	87
5.2.4 Implementation of Augmented Control Scheme using DAC & DIOLC	90
5.4 Materials and methods	90
5.4.1 Strain	90
5.4.2 Media and cultivation conditions	91
5.4.3 Offline analysis	92
5.4.4 Modeling	92
5.5 Result and discussions	93
5.5.1 Simulation results.....	93
5.5.2 Process development.....	95
5.5.3 Experimental validation	96
5.6 Overview.....	103
5.7 Conclusions.....	103
Chapter 6: Implementing Process Analytical Technology for Production of Recombinant Proteins	

in <i>E. coli</i> Using Advanced Controller Scheme	105
6.1 Introduction.....	106
6.2 Materials and methods	108
6.2.1 Microorganism culture and maintenance	108
6.2.2 Culture Media.....	109
6.2.3 Inoculum preparation	109
6.2.4 Bioreactor operation.....	109
6.2.5 Analytical methods.....	110
6.2.6 Interfacing and data acquisition	110
6.2.7 System and model selection	111
6.2.8 Estimation of kinetic parameters.....	111
6.2.9 Derivation of decoupled input-output linearizing controller (DIOLC) for <i>E. coli</i> system	112
6.2.10 PAT implementation for PID and DIOLC	114
6.2.11 Cell disruption, inclusion body generation, and solubilisation	114
6.2.12 LTNF expression and estimation	114
6.3 Results and discussions.....	115
6.3.1 Comparative analysis of PID and DIOLC controller as PAT tools	115
6.3.2 Recombinant LTNF protein analysis	121
6.4 Conclusions.....	124
Chapter 7: Conclusions and Future Perspective	125
7.1 Conclusions.....	126
7.2 Future Scopes.....	128
7.2.1 Regarding cloning and expression of Ranibizumab.....	128
7.2.2 Regarding process controller and PAT implementation	128

7.2.3 Utilization of advanced online monitoring tool and concepts:	129
7.2.4 Advocating continuous bioprocessing and implementing plant-wide control schemes	129
References	131
Appendix	156
Publications.....	159
Personal data.....	160

List of Figures

Figure 2.1 Flowchart depicting generalized strategies for improving production of biotherapeutic protein.....	9
Figure 2.2 Flowchart depicting upstream considerations for selection and optimization of host cell, vector and growth condition for production of recombinant proteins using microbial expression system (information adapted from Sørensen & Mortensen 2005; Rosano & Ceccarelli 2014).....	10
Figure 2.3 Importance of process understanding and controller for process development.....	22
Figure 2.4 Schematic showing advances in process monitoring for measurement of critical process parameter	23
Figure 2.5 Quality by design (QbD) philosophy for bioprocess development using PAT.....	27
Figure 3.1. Illustration of the biology of VEGF and VEGF receptor on endothelial cell membrane (A) and of the effector mechanism (B). Adapted from Zou et al. 2011.	34
Figure 3.2 A) Schematic of the Ranibizumab enterokinase gene cassette. B) Schematic view of the Ranibizumab periplasmic gene cassette. C) Schematic view of the 2:2 clone of Ranibizumab periplasmic gene cassette. D) The scheme involved three steps i) Expression cassette synthesis, ii) Restriction digestion of the coding sequence and expression vector with respective enzyme and its gel purification, and iii) Ligation and transformation (zoomed view of final constructs are in Fig 3.3, 3.10 (A & B)).....	37
Figure 3.3 Expression vector construct with A) Ranibizumab with enterokinase signal sequence B) Ranibizumab with periplasmic signal sequence.....	42
Figure 3.4 A) Periplasmic clone confirmation using double restriction digestion; B) Protein expression confirmation of periplasmic clone (Rani pelB) using Tricine-PAGE; C) Enterokinase clone confirmation using double restriction digestion; D) Enterokinase clone (Rani ent) expression confirmation; E) Localization study to access the protein expression in Rani ent; F) Localization study to access the protein expression in Rani pelB.....	44
Figure 3.5 Intact mass confirmation using LC-MS of A) Periplasmic clone heavy chain; B)	

Periplasmic clone light chain; C) Innovator light chain confirmation; D) Innovator heavy chain confirmation; E) Enterokinase heavy and light chain confirmation..... 47

Figure 3.6 Amount of biomass produced in various growth mediums..... 48

Figure 3.7 Tricine-PAGE Ranibizumab expression of A) Rani enterokinase clone and B) Rani pelb clone under various growth medium (indicates the product of interest). 49

Figure 3.8 Impact of temperature on biomass content produced..... 49

Figure 3.9 Impact of temperature on biomass content produced A) for Rani pelB clone B) Rani entero clone. CE: cytoplasmic extract, IB: Inclusion bodies..... 50

Figure 3.10 A) Single copy construct under single promoter B) Duet vector for the expression of light and heavy chain gene of a rHu Ranibizumab in 2:2 ratio under Duet T7 promoter; C) Cloning confirmation using restriction digestion of 2:2 clone D) Reducing Tricine-PAGE; E) RP-HPLC profile of single copy pelB clone vs innovator; F) RP-HPLC profile of single copy (control) vs double copy (clone 1). 52

Figure 4.1 A) Biomass and protein content after additive supplementation. B) Tricine-PAGE analysis of all process conditions (lane 1: marker, lane 2: standard, lane 3: glutamine treatment without pH maintained, lane 4: thiamine treatment, lane 5: proline treatment, lane 6: sorbitol treatment, lane 7: autoinduction medium, lane 8: arginine treatment with pH maintained, lane 9: glutamine treatment with pH maintained, lane 10: glutamic acid treatment, lane 11: marker, lane 12: ethanol at the time of induction, lane 13: repeat of autoinduction medium, lane 14: glutamine treatment in autoinduction medium, lane 15: glutamine and ethanol (at the time of induction), lane 16: ethanol (at the time of inoculation), lane 17: induction at 37 °C for 4hr in autoinduction medium, lane 18: glutamine + betaine + ethanol (at the time of induction), lane 19: betaine treatment in autoinduction medium, lane 20: marker, lane 21: control for SOC medium, lane 22: betaine + salt treatment, lane 23: ethanol at the time of induction, lane 24: blank , lane 25: repeat of SOC medium). C) Tricine-PAGE analysis for optimizing ethanol concentration. Lane 1: protein marker, lane 2 & 3: empty, lane 4: control, medium supplemented with 1% ethanol, lane 5: control, medium supplemented with 1% ethanol, lane 6: medium supplemented with 3% ethanol, lane 7: medium supplemented with 3% ethanol, lane 8: medium supplemented with 4% ethanol, lane 9:

medium supplemented with 5% ethanol.....	66
Figure 4.2 A) Reveals the economic potential comparison of the cases which showed higher yield. B) Actual process cost comparison of all the cases.....	67
Figure 4.3 Overall schematic of the process developed.	68
Figure 4.4 A) RP HPLC analysis and B) Tricine-PAGE comparison of protein expression between optimized processes and control conditions.	69
Figure 4.5 Comparative confocal analysis for determining cells viability in control vs. optimized.	71
Figure 4.6 SEM analysis for determining cell structure and size in ethanol-treated (optimized) and untreated cells (control).	71
Figure 4.7 Illustration showing A) Single copy construct under single promoter; B) Duet vector for the expression of light and heavy chain gene of a rHu Ranibizumab in 2:2 ratio under duet T7 promoter; C) HPLC data of single and double copy titer of Ranibizumab produced; D) Reducing Tricine-PAGE; lane 1: protein marker, lane 2: reduced innovator standard rHu Ranibizumab, lane 3: un-induced E. coli cells, lane 4: gene expressing single copy antibody fragment (optimized conditions), lane 5: gene expressing double copy antibody fragment (optimized conditions). ...	79
Figure 5.1 Schematic illustration of the Decoupled Adaptive Control (DAC) structure.	86
Figure 5.2 Schematic representation of the Decoupled Input Output Linearizing Control (DIOLC) structure.	89
Figure 5.3 DAC-DIOLC augmented control structure schematic representation.	90
Figure 5.4 Illustration of regulation of DO and substrate in the simulated process for the initial half hour after start of fed-batch (to capture maximum offset) using different control schemes a) DO control at 30% by PID b) Substrate control at 0.25 g/L by PID c) DO control at 30% by DIOLC d) Substrate control at 0.25 g/L by DIOLC e) DO control at 30% by DAC f) Substrate control at 0.25 g/L by DAC g) DO control at 30% by DAC-DIOLC h) Substrate control at 0.25 g/L by DAC- DIOLC	95

Figure 5.5 Comparison of the process attributes obtained using two different feed medium composition for <i>Pichia</i> fed-batch fermentation. Results obtained using feed medium consisting of only methanol and mixed feed medium consisting of sorbitol and methanol is presented. (a) Biomass production profiles, (b) rHSA production profiles.	95
Figure 5.6 Illustration of regulation of DO and substrate in fed-batch fermentation of <i>P. pastoris</i> using different control schemes a) DO control at 30% by PID controller b) Substrate control at 0.25 g/L by PID controller c) DO control at 30% by DIOLC d) Substrate control at 0.25 g/L by DIOLC e) DO control at 30% by DAC f) Substrate control at 0.25 g/L by DAC g) DO control at 30% by DAC-DIOLC h) Substrate control at 0.25 g/L by DAC-DIOLC	99
Figure 5.7 Using standard Box-Plot representation for comparing the performance of different control structures, i.e., PID, DIOLC, DAC, and DAC-DIOLC obtained through simulation and experiment respectively.....	101
Figure 5.8 Using Residual plots for qualitatively comparing the performance of different control structures, i.e., PID, DIOLC, DAC, and DAC-DIOLC obtained through simulation and experiment respectively.....	102
Figure 5.9 Comparison of the various process attributes of <i>Pichia</i> fed-batch fermentation under different control schemes including traditional PID controller, DIOLC, DAC, and DAC-DIOLC. Data illustrated includes a) Biomass profiles b) Protein production in fed-batch fermentation (protein production starts in fed-batch fermentation after methanol induction).	103
Figure 6.1 Displaying schematic diagram showing the flow of process information from the reactor to the controller, and the return of calculated control and its implementation. The states x become the measured values y_m . The error is the difference between the set point y_d and y_m . The control algorithm uses this information to compute the control action u which is implemented on the reactor.	107
Figure 6.2 Standard curve of densitometer profiles for known concentration of the recombinant LTNF protein.....	115
Figure 6.3 Experimental results showing the time course profiles of the control action of PID and DIOLC under normal operating conditions, and during air supply and substrate feeding	

disruptions. A & B) PID control action under normal operation for controlling the DO and substrate concentrations at the desired set points. C) PID control action when air supply to the process is cut off for 5 min durations. The down arrow ↓ in the inset figure marks the instances when the air supply is cut off. D) PID control action when the substrate feeding pump is manually switched off. The down arrow ↓ in the inset figure marks the instances when the pump is switch off. E & F) are the parallel experiments to A & B performed using DIOLC under normal operation DO and substrate concentration control. G & H) are the parallel experiments to C & D performed using DIOLC when the air supply and substrate feeding is manually disrupted. In G & H, the down arrow ↓ in the inset figure marks the instances when the air supply is cut off and the when the substrate feeding pump is manually switched off. (Here blue colour lines presents the process value of the controlled variable while green and orange lines are manipulating variable and red colour line shows the set point of controlled variable)..... 120

Figure 6.4 Tricine SDS PAGE analysis for LTNF in experiment using PID controller (A) and DIOLC (B) with and without disruptions. A) Lane M1 is a prestained marker 10–250 kDa (Thermo Fisher: #26619). Lanes 1–4: hourly samples taken post induction from the fed-batch reactor under normal operating conditions. Lane M2 is a low-range unstained protein marker 3.4–100 kDa (Thermo Fisher: #26632). Lanes 5-8: hourly samples taken post induction from the fed-batch reactor during disruption experiments. B) Lane M2 is the same as used in A1. Lanes 1–4 are the hourly samples taken post induction from the fed-batch reactor under normal operating conditions and lanes 5-8 are the hourly samples taken post induction from the fed-batch reactor during disruption experiments (C) Comparison of LTNF protein profiles post induction using different controller (D) Specific production rate profiles post induction during different controller runs (E) Residual glucose concentration profiles (F) Biomass growth profile of different controller run. 122

Figure 6.5 Representative analytical RP- HPLC chromatogram..... 123

List of Tables

Table 2.1 Strategies for N terminal methionine removal.....	11
Table 3.1 Primers. Desalted oligonucleotides from IDT used for cloning	36
Table 3.2 Key components of the different media that were screened for Fab production.	39
Table 3.3 RP-HPLC gradient for analysis of Ranibizumab samples.....	41
Table 3.4 Representative signal sequence used for therapeutic protein expression in microbial host.....	42
Table 3.5 Comparison of present study with published literature	51
Table 4.1 List of various chemical additives used in literature for enhancing the recombinant protein production.....	58
Table 4.2 Optimized concentration of additives based on the literature.....	59
Table 4.3 Oligonucleotides used for RT-qPCR validation study	63
Table 4.4 RP-HPLC gradient time point method for Ranibizumab.....	64
Table 4.5 Comparative study of control vs optimized at shake flask level	69
Table 4.6. List of differentially expressed genes in optimized culture as compared to control...	74
Table 4.7 RT-qPCR validation of differentially expressed genes.....	76
Table 5.1 Model parameters description for <i>Pichia pastoris</i> fed-batch cultivation	84
Table 5.2 Comparison of normalized root mean square error values (NRMSE) for PID controller, DIOLC controller, DAC controller, and DAC-DIOLC controller (DO set point at 30% and substrate set point at 0.25 g/L) obtained from the simulation studies span over initial 2 hours in fed-batch mode.	93
Table 5.3 Comparison of normalized root mean square error values (NRMSE) for PID controller, DIOLC controller, DAC controller, and DAC-DIOLC controller (DO set point at 30% and substrate set point at 0.25 g/L) span over 48 fed-batch hours.	100

Table 6.1 Tuning parameters used for PID and DIOLC	111
Table 6.2 Comparison of root mean square error values (RMSE) of substrate and DO for PID controller, PID with manual disturbance, DIOLC and DIOLC controller with manual disturbance (DO set point at 20% and substrate set point at 0.03/L).....	117
Table 6.3 Interpolated graph of RP-HPLC chromatogram showing the percentage of the total area of eluted peaks occupied by LTNF and its closely related variant.....	124
Table A1 Commercially available engineered <i>E.coli</i> strains for recombinant protein production	

Abbreviations

AOX1	: Alcohol Oxidase 1
cDNA	: complementary DNA
CPP	: Critical Process Parameters
CQA	: Critical Quality Attributes
DCW	: Dry cell weight
DNA	: Deoxy ribo nucleic acid
DO	: Dissolved oxygen
DS	: Dielectric spectroscopy
FDA	: Food and Drug administration
Fab	: Fragment of antibody
HPLC	: High-Performance Liquid Chromatography
IPTG	: isopropyl β -d-1-thiogalactopyranoside
Ig	: Immunoglobulin
Mab	: Monoclonal antibody
MOO	: Multi objective optimization
MV	: Manipulating variable
OD	: Optical density
PAT	: Process Analytical Technology
pH	: Potential of hydrogen
QbD	: Quality by design
RP-HPLC	: Reversed phase High-Performance Liquid Chromatography

RT PCR : Real time polymerase chain reaction

SCADA : Supervisory Control and Data acquisition

SOC : Super optimal broth with catabolite repression

TFA : Trifluoroacetic acid

Nomenclature

Area : Area of the fermenter (m^2)

Air_{in} : Air flow rate (L h^{-1})

A, B, C: Correlation constants for volumetric mass transfer coefficient (-)

C_L : Dissolved oxygen concentration (g L^{-1})

C_L^* : Dissolved oxygen concentration at equilibrium with the gas phase (g L^{-1})

D_i : Impeller diameter (m)

DO : Dissolved oxygen concentration (%)

F : Substrate flow rate in fed-batch phase (L h^{-1})

h : Henry's constant (L kPa g^{-1})

k_{La} : Volumetric mass transfer coefficient (h^{-1})

k_{O_2} : Monod saturation constant for oxygen (g L^{-1})

k_{S_b} : Monod saturation constant for substrate in batch phase (g L^{-1})

k_{S_f} : Monod saturation constant for substrate in fed-batch phase (g L^{-1})

m_{O_2} : Maintenance coefficient of oxygen ($\text{g oxygen g}^{-1} \text{biomass h}^{-1}$)

m_{S_b} : Maintenance coefficient of substrate in batch phase ($\text{g substrate g}^{-1} \text{biomass h}^{-1}$)

m_{S_f} : Maintenance coefficient of substrate in fed-batch phase ($\text{g substrate g}^{-1} \text{biomass h}^{-1}$)

N_i : Stirrer speed (h^{-1})

N_p : Power number (-)

O_2 : Oxygen in gas phase (%)

$O_{2,\text{in}}$: Pure oxygen input (%)

p : Pressure (kPa)

P : Product concentration (g L^{-1})

P_T : Power (watt)

- $q_{O_{2,b}}$: Specific oxygen consumption rate in batch phase (g oxygen g⁻¹ biomass h⁻¹)
 $q_{O_{2,f}}$: Specific oxygen consumption rate in fed-batch phase (g oxygen g⁻¹ biomass h⁻¹)
 q_P : Specific product production rate (g product g⁻¹ biomass h⁻¹)
 q_{S_b} : Specific substrate consumption rate for substrate in batch phase (g substrate g⁻¹ biomass h⁻¹)
 q_{S_f} : Specific substrate consumption rate for substrate in fed-batch phase (g substrate g⁻¹ biomass h⁻¹)
 R : Gas constant (K⁻¹ kPa L mol⁻¹)
 S_b : Substrate concentration in batch phase (g L⁻¹)
 S_f : Substrate concentration in fed-batch phase (g L⁻¹)
 $S_{f,in}$: Substrate concentration in the feed in fed - batch phase (g L⁻¹)
 T : Temperature (K)
 u_g : Superficial gas velocity (m h⁻¹)
 V : Volume of the broth (L)
 V_g : Gas volume (L)
 V_T : Total reactor volume (L)
 X : Biomass concentration (g L⁻¹)
 X_{in} : Biomass concentration in the feed (g L⁻¹)
 y_{X/S_b} : Biomass yield coefficient from substrate in batch phase (g biomass g⁻¹ substrate)
 y_{X/S_f} : Biomass yield coefficient from substrate in fed-batch phase (g biomass g⁻¹ substrate)
 $y_{X/O_{2,b}}$: Biomass yield coefficient from oxygen utilized in batch phase (g oxygen g⁻¹ biomass)
 $y_{X/O_{2,f}}$: Biomass yield coefficient from oxygen utilized in fed-batch phase (g oxygen g⁻¹ biomass)

Greek

- α : Growth associated product yield coefficient (g product g⁻¹ biomass)
 β : Non-growth associated product yield coefficient (g product g⁻¹ biomass h⁻¹)
 μ_b : Specific growth rate of batch phase (h⁻¹)
 μ_f : Specific growth rate of fed-batch phase (h⁻¹)
 μ_{m_b} : Maximum specific growth rate of batch phase (h⁻¹)
 μ_{m_f} : Maximum specific growth rate of fed-batch phase (h⁻¹)