

**MANIPULATING CELL CULTURE MEDIA
COMPONENTS AND USE OF ADDITIVES TO
ENHANCE THE PRODUCTION OF MONOCLONAL
ANTIBODIES**

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By

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Submitted

**in fulfillment of the requirements of the degree of Doctor of Philosophy
to the**



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Dedicated to My Mother

CERTIFICATE

This is to certify that the thesis entitled “MANIPULATING CELL CULTURE MEDIA COMPONENTS AND USE OF ADDITIVES TO ENHANCE PRODUCTION OF THE MONOCLONAL ANTIBODIES” being submitted by GEETANJALI BASAVARAJ HUBLI to the Indian Institute of Technology Delhi for the award of the degree of Doctor of Philosophy is a record of the original bonified research work carried out by her under my guidance and supervision. The results contained in this thesis have not been submitted part or 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
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Geetanjali Hubli

Abstract

Biotherapeutics have revolutionized the treatment of complex diseases such as cancer, rheumatoid arthritis, and psoriasis. However, the high cost associated with its production significantly limits their accessibility, particularly in the developing countries. As large number of biosimilars receive regulatory approval in markets each year, intensifying the competition amongst manufacturers to reduce the production costs. Manufacturers are under growing pressure from both governmental bodies and patient advocacy groups to enhance product yield and lower expenses in order to reduce the cost. Government initiatives, including regulatory modernization, expedited approval processes, reimbursement reforms, and standardization of clinical trials are fostering a supportive environment for innovation in “Upstream Process Technologies”. Currently, an estimated 400 million people worldwide rely on protein-based therapies, often for chronic conditions requiring lifelong treatment. Thus, the development of improved manufacturing processes for biosimilars represents an urgent and ongoing need.

Chinese hamster ovary (CHO) cell growth and productivity remain a critical focus, as high- yield production strategies are integral to the affordability of biologics and biosimilars. Implementing such strategy is only feasible during the development of upstream processes, where key factors include cell line development, media optimization, the use of cell culture additives, and the adoption of perfusion continuous manufacturing techniques are performed. A subtle alteration in media composition and feeding strategies can significantly influence the critical quality attributes (CQAs) of therapeutic proteins. The industry relies on the Design of Experiments (DOE) and multivariate statistical methods to identify critical process parameters and their relationships with product yield and quality attributes. Recent advances in Process Analytical Technology (PAT) now enable real-time monitoring of cell physiology, critical process parameters (CPPs), and CQAs, which supports the quality-by-design (QbD) approach in upstream bioprocessing. In this work, some of the major upstream challenges to produce therapeutic proteins have been addressed to improve protein production The use of QbD and DOE approaches. for media component optimization, and real-time monitoring of 23 cell culture process parameters are thoroughly studied.

The first objective investigates the application of pyruvate dehydrogenase kinase (PDK) inhibitors to enhance monoclonal antibody (mAb) production in mammalian cell cultures by mitigating the "Warburg effect," characterized by high glucose uptake and lactate accumulation. Four PDK

inhibitors dichloroacetate (DCA), metformin (M), (S)-3,3,3- trifluoro-2-hydroxy-2-methyl propionic acid (TPP), and 4-phenylbutyrate (PB)—were screened for their effects on lactate reduction. Optimal yields were achieved with PB (0.5 mM) and TPP (0.5-1 mM), both showing nearly complete lactate inhibition, while DCA reduced lactate by 36%. A combination of 5 mM DCA and 1 mM TPP led to a two-fold increase in monoclonal antibody (mAb) yield (1.20 g/L on day 15) with a significant lactate reduction. Metabolic flux analysis revealed a 19.5-fold increase in pyruvate flux, along with two- and four-fold increases in acetyl CoA and lactate, respectively. Importantly, critical quality attributes such as charge variants, aggregation, and glycosylation remained unaffected. Amino acid consumption profiles showed specific depletion patterns, and proteomic analysis revealed significant changes in cellular protein expression. These findings suggest that PDK inhibitors can improve cell viability and mAb yield by reducing lactate accumulation in fed-batch cultures without compromising product quality.

The second objective focuses on improving non-perfusion N-1 intensified fed-batch yields with Sodium Butyrate and n-valeric Acid. Recent optimizations in cell culture processes have illustrated the impact of using additives in enhancing the inoculation cell densities in the N – 1 stage, leading to higher volumetric productivity and shorter production culture durations. In this work, we present a DOE approach to optimize sodium butyrate and valeric acid composition in the media to improve protein titers and regulate the CQAs in the desired range. Around 2.8-fold increase in trastuzumab yield, from 0.62 mg/ml to 1.8 mg/ml, was achieved in intensified fed-batch cultures using a combination of sodium butyrate and valeric acid (0.25 mM each) over 6 days. Individually, sodium butyrate enhanced the product titer by 2.17-fold, while n-valeric acid contributed to a 1.9-fold increase during high-throughput screening. Hence, the combined effect is better than the individual butyrate and valeric acid supplements. Butyrate (1 mM) addition promotes the formation of the acidic variant in trastuzumab. This effect was mitigated when butyrate (0.25 mM) and n-valeric (0.25 mM) were simultaneously added to the media and feed. Their combination has negligible impact on glycosylation, decreases glucose uptake, and prolonged viability by 24 hours. Arginine, Serine, Valine Threonine, Tryptophan, Aspartic Acid, and Tyrosine were found to be possible feed targets by our amino acid analysis. Lysine, methionine, phenylalanine, Leucine, Valine, and Isoleucine uptake is slower; hence, their concentration can be minimized in feed optimization. Hence the application of sodium butyrate and valeric acid along with feed regimen and seeding density optimization can produce 2.8-fold higher protein than the typical fed-batch process.

Later, the study focuses on developing PAT for monitoring CPPs of mammalian cell culture. For this, the development of a robust fourier transform near-infrared (FT-NIR) spectroscopy- based method for the quantification of all 20 amino acids (0–24 mM), along with glucose (0– 6.7 mg/mL), lactate (0–2.7 mg/mL), and trastuzumab (0–2.5 mg/mL) concentrations in CHO cell cultures. Near-infrared spectra (4000–11,000 cm^{-1}) were acquired, and pre-processing techniques such as smoothing and derivatives were employed to enhance signal detection. High-performance liquid chromatography with pre-column derivatization served as an orthogonal method for validation. Calibration models were developed using principal component analysis and partial least squares regression, yielding strong coefficients of determination for calibration ($R_c^2 = 0.94\text{--}0.99$) and prediction ($R_p^2 = 0.83\text{--}0.98$), with high RPD values (>3) for most components. The model was validated through external testing ($REV^2 = 0.89\text{--}0.99$, $RMSE = 0.04\text{--}1.04$) and successfully applied for at-line monitoring of two 10 L perfusion runs. The demonstration of NIR spectroscopy for the measurement of 20 amino acids for the first time have been tested for the mammalian cell cultures, which is offering a valuable tool for biopharmaceutical manufacturers implementing continuous processing and process analytical technology (PAT)-based control. This study also highlights the amino acid consumption patterns and optimized feeding strategies, supported by process analytical tools such as near-infrared (NIR) monitoring, Amino acids impact the growth, viability, and productivity of CHO cells in biopharmaceutical production Optimizing amino acid feed concentrations and combinations, supported by NIR monitoring, significantly enhances CHO cell performance by improving viability and productivity by 150 percent. The study identified that concentrated amino acid stocks (150-200 mM) prepared with sodium hydroxide were cytotoxic, leading to the development of a modified Krebs bicarbonate buffer to mitigate toxicity. This approach enabled stable amino acid solutions at physiological pH, contributing to improved cell culture outcomes, while preventing batch failures through rapid feedback on process perturbations. This also demonstrates feasibility of Near-infrared spectroscopy for high throughput process optimization of CHO cell culture.

सारांश

बायोथेरेप्यूटिक्स (जैविक उपचार) ने कैंसर, रूमेटॉइड आर्थराइटिस और सोरायसिस जैसी जटिल बीमारियों के उपचार में क्रांतिकारी परिवर्तन लाया है। हालांकि, इसके उत्पादन से जुड़ी उच्च लागत इसकी उपलब्धता, खासकर विकासशील देशों में, गंभीर रूप से सीमित करती है। हर वर्ष बड़ी संख्या में बायोसिमिलर उत्पाद बाजार में नियामक स्वीकृति प्राप्त करते हैं, जिससे निर्माताओं के बीच लागत कम करने की प्रतिस्पर्धा बढ़ रही है। सरकारों और रोगी सहायता समूहों की ओर से उत्पाद की मात्रा बढ़ाने और खर्च घटाने का दबाव लगातार बढ़ रहा है। सरकार द्वारा की जा रही पहल, जैसे कि नियामक सुधार, शीघ्र अनुमोदन प्रक्रियाएं, रिइम्बर्समेंट सुधार और क्लिनिकल ट्रायल्स का मानकीकरण, "अपस्ट्रीम प्रोसेस टेक्नोलॉजी" में नवाचार के लिए अनुकूल वातावरण बना रहे हैं। वर्तमान में लगभग 400 मिलियन लोग प्रोटीन-आधारित उपचारों पर निर्भर हैं, जिनमें से अधिकांश को आजीवन इलाज की आवश्यकता होती है। इस प्रकार, बायोसिमिलर के बेहतर उत्पादन के लिए उन्नत निर्माण प्रक्रियाओं का विकास समय की आवश्यकता बन गया है।

चीनी हैमस्टर ओवरी (CHO) कोशिकाओं की वृद्धि और उत्पादकता पर विशेष ध्यान केंद्रित किया गया है, क्योंकि उच्च उत्पादकता रणनीतियाँ जैविक उत्पादों और बायोसिमिलर्स की सुलभता में अहम भूमिका निभाती हैं। ऐसी रणनीतियाँ केवल *अपस्ट्रीम प्रक्रिया विकास* के दौरान ही लागू की जा सकती हैं, जिसमें कोशिका लाइन का विकास, मीडिया का अनुकूलन, सेल कल्चर एडिटिव्स का उपयोग और परफ्यूज़न कंटिन्युअस मैन्युफैक्चरिंग तकनीकों को अपनाना शामिल है। मीडिया की संरचना और फीडिंग रणनीति में सूक्ष्म बदलाव भी चिकित्सीय प्रोटीन की गुणवत्ता को गहराई से प्रभावित कर सकते हैं। उद्योग में डिज़ाइन ऑफ़ एक्सपेरिमेंट्स (DOE) और मल्टीवैरिएट सांख्यिकीय विधियों का उपयोग प्रमुख प्रक्रिया मापदंडों की पहचान और उत्पाद की गुणवत्ता से उनके संबंध को समझने के लिए किया जाता है। हाल ही में, प्रोसेस एनालिटिकल टेक्नोलॉजी (PAT) में प्रगति से कोशिका भौतिकी, प्रक्रिया मापदंडों और उत्पाद की गुणवत्ता का रीयल-टाइम निगरानी संभव हो गया है, जिससे क्वालिटी-बाय-डिज़ाइन (QbD) दृष्टिकोण को समर्थन मिला है। इस अध्ययन में चिकित्सीय प्रोटीन उत्पादन में आने वाली मुख्य *अपस्ट्रीम चुनौतियों* का समाधान किया गया है, जिसमें QbD और DOE दृष्टिकोणों के माध्यम से मीडिया घटकों का अनुकूलन और 23 सेल कल्चर प्रक्रिया मापदंडों की रीयल-टाइम निगरानी शामिल है।

पहला उद्देश्य pyruvate dehydrogenase kinase (PDK) इनहिबिटर्स के उपयोग द्वारा माउंटोक्लोनल एंटीबॉडी (mAb) उत्पादन को बढ़ाने की संभावना की जांच करना था। यह "Warburg effect" को कम करके किया गया, जो अधिक ग्लूकोज़ खपत और लैक्टेट संचयन की

विशेषता है। चार PDK इनहिबिटर्स—डाइक्लोरोएसेटेट (DCA), मेटफॉर्मिन (M), (S)-TPP और 4-फिनाइलब्यूटायरेट (PB)—को लैक्टेट कम करने के प्रभावों के लिए जांचा गया। PB (0.5 mM) और TPP (0.5–1 mM) के साथ अधिकतम उत्पादकता प्राप्त हुई, जिन्होंने लगभग पूरी तरह लैक्टेट को अवरुद्ध कर दिया, जबकि DCA ने 36% तक लैक्टेट कम किया। 5 mM DCA और 1 mM TPP के संयोजन से mAb उत्पादन में दो गुना वृद्धि (15वें दिन 1.20 g/L) हुई, और लैक्टेट में काफी कमी देखी गई। मेटाबॉलिक फ्लक्स विश्लेषण में पाइरूवेट फ्लक्स में 19.5 गुना वृद्धि और एसिटाइल-CoA व लैक्टेट में 2-4 गुना वृद्धि देखी गई। महत्वपूर्ण गुणवत्ता विशेषताएं—जैसे कि चार्ज वेरिएंट्स, एग्रीगेशन और ग्लाइकोसाइलेशन—प्रभावित नहीं हुईं। एमिनो एसिड उपयोग पैटर्न और प्रोटीओमिक विश्लेषण ने कोशिकीय प्रोटीन अभिव्यक्ति में महत्वपूर्ण परिवर्तन दिखाए। यह संकेत देते हैं कि PDK इनहिबिटर्स से लैक्टेट को कम करके कोशिका की जीवन क्षमता और mAb उत्पादन को बेहतर बनाया जा सकता है, उत्पाद की गुणवत्ता प्रभावित किए बिना।

दूसरे उद्देश्य में सोडियम ब्यूटायरेट और n-वैलेरिक एसिड के साथ N-1 स्टेज पर इन्टेंसिफाइड फेड-बैच उत्पादकता को बेहतर बनाने पर ध्यान केंद्रित किया गया है। हाल के अनुकूलन प्रयासों ने दिखाया है कि ये एडिटिव्स उच्च इनोकुलेशन सेल डेंसिटी प्राप्त करने में मदद करते हैं, जिससे कम समय में अधिक उत्पादन संभव होता है। इस अध्ययन में, DOE दृष्टिकोण का उपयोग कर सोडियम ब्यूटायरेट और वैलेरिक एसिड की उपयुक्त संरचना निर्धारित की गई, जिससे प्रोटीन टाइटर्स बढ़े और CQAs नियंत्रित रहे। ट्रास्टुजुमैब की मात्रा 0.62 mg/ml से बढ़कर 1.8 mg/ml (2.8 गुना) हो गई जब 0.25 mM ब्यूटायरेट और 0.25 mM वैलेरिक एसिड का संयोजन उपयोग किया गया। व्यक्तिगत रूप से, ब्यूटायरेट से 2.17 गुना और n-वैलेरिक एसिड से 1.9 गुना वृद्धि हुई। ब्यूटायरेट (1 mM) के उपयोग से ट्रास्टुजुमैब में अम्लीय वेरिएंट बनता है, जो संयुक्त उपयोग (0.25 mM ब्यूटायरेट + 0.25 mM वैलेरिक) से रोका जा सकता है। यह संयोजन ग्लाइकोसाइलेशन को नहीं बदलता, ग्लूकोज़ खपत घटाता है और जीवन क्षमता को 24 घंटे तक बढ़ाता है। अमीनो एसिड विश्लेषण के आधार पर आर्जिनिन, सेरीन, वेलीन, थ्रेओनिन, ट्रिप्टोफान, ऐस्पार्टिक एसिड, और टायरोसीन संभावित फीड लक्ष्य बने। लाइसिन, मेथियोनिन, फिनाइलएलनिन, ल्यूसीन, वेलीन, और आइसोल्यूसिन की धीमी खपत को देखते हुए फीड में इनकी मात्रा घटाई जा सकती है।

बाद में, अध्ययन का फोकस PAT विकास पर रहा, जो स्तनधारी कोशिका कल्चर में CPPs की निगरानी के लिए है। इसमें FT-NIR spectroscopy आधारित विधि विकसित की गई, जिससे 20 अमीनो एसिड (0–24 mM), ग्लूकोज़ (0–6.7 mg/mL), लैक्टेट (0–2.7 mg/mL), और ट्रास्टुजुमैब (0–2.5 mg/mL) की मात्रात्मकता की जा सके। NIR स्पेक्ट्रा (4000–11,000 cm⁻¹) एकत्र किए गए और

सिग्नल बेहतर करने के लिए प्री-प्रोसेसिंग तकनीकें अपनाई गईं। HPLC का उपयोग मान्यता के लिए किया गया। PCA और PLS रिग्रेशन द्वारा कैलिब्रेशन मॉडल बनाए गए, जिनके R_c^2 और R_p^2 मानक काफी उच्च (0.94–0.99 और 0.83–0.98) रहे, और RPD >3 रहा। मॉडल को बाहरी परीक्षणों ($REV^2 = 0.89–0.99$) में सत्यापित किया गया और दो 10 L परफ्यूजन रन के दौरान प्रयोग में लाया गया। यह पहली बार है कि 20 अमीनो एसिड की माप NIR spectroscopy से स्तनधारी कोशिका कल्चर में की गई है, जो PAT और निरंतर प्रोसेसिंग के लिए एक महत्वपूर्ण उपकरण सिद्ध हो सकता है। इस अध्ययन ने यह भी दर्शाया कि अमीनो एसिड की खपत, कोशिका वृद्धि, जीवन क्षमता और उत्पादकता पर असर डालती है। NIR आधारित निगरानी द्वारा अमीनो एसिड फीड को अनुकूलित करने से CHO कोशिकाओं की उत्पादकता 150% तक बढ़ सकती है। सोडियम हाइड्रॉक्साइड के साथ बनाए गए अमीनो एसिड स्टॉक्स (150–200 mM) कोशिकाओं के लिए विषैले पाए गए, जिसे सुधारते हुए Krebs बाइकार्बोनेट बफर विकसित किया गया। इस दृष्टिकोण ने स्थिर pH पर अमीनो एसिड समाधान उपलब्ध कराया और कोशिका कल्चर की गुणवत्ता में सुधार किया, साथ ही बैच फेल्योर की संभावना भी कम की।

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List of Abbreviations

| Abbreviation | Full form |
|--------------|--|
| 2D-FS | Fluorescence spectroscopy |
| 2DG | 2-deoxy-D-glucose |
| 5TG | 5-thio-D-glucose |
| Aas | Amino acids |
| ATF | Alternating tangential flow filtration |
| ATP | Adenosine triphosphate |
| AVV | Adeno-Associated Virus |
| BV | Intensified fed-batch, 3×10^6 cells ml ⁻¹ seeding density, feeding of 10 % every 12 hrs up to culture viability of 90%. Substituted with sodium butyrate and n- valeric acid (0.25 mM each) in media and feed. |
| BIPS | Bioelectrical impedance spectroscopy |
| CHO | Chinese hamster ovary cells |
| CO2 | Carbon dioxide |
| CQA | Critical quality attribute |
| Cu | Copper |
| DCA | Dichloroacetate |
| DS | Dielectric spectroscopy |
| F | Sum Amino acid from each feed every 12 hr |
| FB | Typical fed-batch, seeding density of 0.5×10^6 cells ml ⁻¹ , Feeding of 10 % feed on day 3,5,7,9,11. |
| FC | Flow cytometry |
| Fe | Iron |
| FT-NIR | Fourier transform near-infrared spectroscopy |
| GSH | Glutathione |
| iFB | Intensified fed-batch, 3×10^6 cells ml ⁻¹ seeding density, feeding of 10 % every 12 hrs up to culture viability of 90%. |
| K | Potassium |

| | |
|----------------|---|
| M | Metformin |
| | Amino acid in culture media |
| M | |
| mAb | Monoclonal antibody |
| Mn | Manganese |
| MS | Mass spectroscopy |
| MVDA | Multivariate data analyses |
| N | number of observations in data set |
| Na | Sodium |
| NIR | Near infrared spectroscopy |
| NMR | Nuclear magnetic resonance |
| O ₂ | Oxygen |
| PAT | Process analytical technology |
| PB | 4 –phenylbutyrate |
| PDK | Pyruvate dehydrogenase Kinase |
| PLS | partial least squares |
| PRESS | Prediction Residual Error Sum of Squares |
| RMSECV | Root mean square error of cross-validation |
| RMSEEV | Root mean square error of external validation |
| RMSEP | Root mean square error of prediction |
| ROS | reactive oxygen species |
| RPD | Residual predictive deviation |
| RS | Raman spectroscopy |
| T | Total amino acid-fed |
| TCA | Tricarboxylic acid cycle |
| TCR | Total amino acid rate |
| TFF | Tangential flow filtration |
| TPP | 3,3,3-trifluoro-2-hydroxy-2-methyl propionic acid |
| VVD | Variable volume per day |
| Zn | Zinc |

| | |
|-------------------------|--|
| ΔCAA | Where ΔCAA is the change in amino acid concentration, Δt is the time interval over which the change in amino acid concentration is measured. TCR is the total (cells mL ⁻¹). The units of CSCR will typically be in terms of $\mu\text{mol cell}^{-1} \text{h}^{-1}$ amino acid consumption rate (e.g., $\mu\text{mol L}^{-1} \text{h}^{-1}$). Cell Density is the number of cells per milliliter |
| $\Delta CAA / \Delta t$ | Cell-specific consumption rate |
| LDH | Lactate dehydrogenase |
| PDH | Pyruvate dehydrogenase |
| Y_i | Where is the predicted value by the model for the i th sample |
| SD | Standard deviation |
| y_i | y_i is the actual/reference value |
| \bar{y} | \bar{y} is the mean of the actual values and n is the number of samples. |