

**STRATEGIES FOR ENHANCED PRODUCTION
OF VIRUS LIKE PARTICLES FOR
THERAPEUTIC USE**

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**SCHOOL OF INTERDISCIPLINARY RESEARCH
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by

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Submitted

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To The



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Dedicated to my family...

CERTIFICATE

This is to certify that the thesis entitled “**STRATEGIES FOR ENHANCED PRODUCTION OF VIRUS LIKE PARTICLES FOR THERAPEUTIC USE**” being submitted by **ABHILASHA KUMARI RANI** 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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ABHILASHA KUMARI RANI

Abstract

Virus-like particles (VLPs) have emerged as a promising vaccine platform due to their ability to mimic native viral structures while being non-infectious, offering high safety and immunogenicity. This thesis work involved the development and optimization of scalable production strategies for VLPs targeting two major viral pathogens: Chikungunya virus (CHIKV) and Human papillomavirus (HPV). Global burden posed by CHIKV highlights the urgent need for effective prophylactic solutions. A stable serum-free suspension culture of HEK293T cells expressing CHIK-VLPs was established to overcome scalability issues in CHIK-VLP production. In parallel, HPV, a leading cause of cervical cancer, was addressed through the development of a robust, cost-effective expression platform in *Pichia pastoris* expressing HPV-VLPs. Optimizations at the level of strain promoter combinations, process parameters through Design of Experiments (DoE), targeted micronutrient supplementation, and fed-batch fermentation strategies led to enhanced VLP yield, improved structural integrity, and enhance process scalability. Implementation across both shake flasks and lab-scale bioreactors demonstrated the potential for efficient VLP-based vaccine production, laying a strong foundation for future large-scale manufacturing and commercialization.

The production of CHIK-VLPs using traditional HEK293T adherent cell culture relies on supplementation of fetal bovine serum (FBS), which introduces variability, contamination risks, high costs, and scalability challenges for vaccine manufacturing. To address this challenge, transition of adherent to suspension cell culture in a serum-free medium presents a promising strategy for large-scale VLPs production, in compliance with the quality and safety standards followed in the biopharmaceutical industry. In the first objective (Chapter 3), we established a stable HEK293T cell line pool capable of producing CHIK-VLPs and subsequently transitioned these cells into suspension culture to improve scalability. We verified the successful production of CHIK-VLPs through batch cultivation in 1000 mL Erlenmeyer flasks, incorporating a temperature shift during the production phase of process to enhance yield. CHIK-VLPs expression was confirmed through various analytical techniques including SDS-PAGE, western blotting, mass spectrometry and the morphology of VLPs produced by both stable adherent and suspension-adapted HEK293 cells was assessed by transmission electron microscopy. A 5-fold enhancement in VLP production was achieved in suspension-adapted culture using a temperature shift as quantified by absorbance-based method. This establishment of stable HEK293T cell lines capable of efficient CHIK-VLPs secretion provides a versatile and scalable method for VLPs production that is free from the need for antibiotics

and FBS. This process represents a valuable contribution to the biopharmaceutical industry, offering potential for large-scale VLPs-based vaccine development.

Large-scale production of HPV-VLPs requires a robust expression system. One of the preferred choices is yeast expression system due to their cost-effectiveness, rapid growth, and high levels of protein expression. In the next study (Chapter 4), we specifically aimed to screen optimal promoters, strains, and cultivation conditions to enhance large-scale production of HPV-VLPs in *P. pastoris* using BioLector XT microbioreactor system. The analysis reveals that the GS115 strain under the inducible alcohol oxidase promoter exhibited highest VLP yield of 20.50 mg/L as compared to X33 (11.07 mg/L) and KM71H (6.49 mg/L) strains. HPV-VLP production was confirmed through SDS-PAGE, western blotting, HPLC, intact mass analysis, and peptide mapping. Structural characterization with electron microscopy validated the homogeneity, colloidal stability, and structural similarity to native HPV. Optimization of *Pichia* cell lysis conditions was achieved to improve intracellular VLP recovery while maintaining particle integrity. This study offers a viable path for using the GS115 strain for efficient and cost-effective large-scale production of VLPs. The study offers a comprehensive approach to optimize HPV-VLP production in *P. pastoris*, highlighting the importance of promoter choice, strain optimization, and cultivation techniques in achieving high yields of L1 protein and structurally intact HPV-VLPs for possible vaccine development.

The third study (Chapter 5) demonstrates how optimal addition of micronutrients enhances VLP yield and quality. In this study, we have done a 3-step design of experiments to screen and optimize impact of seven metal and seven vitamins on the production of HPV-VLPs in *P. pastoris*. First, a one-factor-at-a-time (OFAT) screening was performed to evaluate the impact of seven metal ions (Fe, Zn, Ba, Ni, Cd, Li, Ca) and seven vitamins on cell growth and VLPs yield. Next, a Plackett-Burman based DoE study confirmed Fe, Zn, nicotinic acid, and D-calcium pantothenate as essential micronutrients for VLPs yield. Finally, a response surface design (RSM) was performed to optimize the interactions between selected micronutrients to determine the best conditions for maximum biomass and production of VLPs. Optimal concentration of these micronutrients was found to be 1 mM Zn, 1 mM Fe, 2.04 mM nicotinic acid, and 4 mM D-calcium pantothenate, as determined by RSM prediction model with $R^2 = 0.90$ and Root Mean Square Error (RMSE) = 1.4 for biomass and $R^2 = 0.94$ and RMSE = 0.98 for HPV-L1 protein titer. Optimized metal ions and vitamins enhanced HPV-VLPs L1 protein production, achieving 1.46-fold higher biomass (24.1 g/L) and 2.07-fold higher protein titer (33.2 mg/L) in shake flasks. In bioreactor, a 1.44-fold (101.1 g/L) enhancement in biomass and

1.54-fold (37.1 mg/L) enhancement in the VLPs yield was observed when compared to control. This study highlights the significance of micronutrient optimization for large-scale production of HPV-VLPs.

In the fourth study (Chapter 6), fed-batch fermentation was employed to optimize HPV16 L1 VLP production in *P. pastoris* GS115 (Mut⁺), aiming to reduce methanol inhibition and enhance VLPs yield. Four methanol-based feeding strategies were evaluated during the induction phase, incorporating both pulse and linear feeding patterns: 100% methanol (M100), 70% methanol + 30% glycerol (M70+G30), 70% methanol + 30% sorbitol (M70+S30), 70% methanol + 15% glycerol + 15% sorbitol (M70+G15+S15). Key process parameters, including cell growth (optical density and dry cell weight), metabolic activity (dissolved oxygen levels), and protein expression, were monitored to assess process performance. All strategies were first evaluated in micro-scale fed-batch cultivations for HPV VLP production, and the most effective approach was further validated in a 1L lab-scale bioreactor to confirm scalability and performance. Among the strategies examined, the M70 + G30 (Linear feeding) approach demonstrated the highest productivity in microbioreactor cultivations, achieving an OD₆₀₀ of 210.9, a DCW of 90.36 g/L, and a product titer of 42.92 mg/L. This strategy was further validated in a 1L lab-scale bioreactor, where it achieved even higher yields, with an optical density (OD) of ~320, a DCW of 161 g/L, and a protein titer of 49 mg/L, confirming its scalability and effectiveness. This approach enhanced cell growth, protein expression, and cost efficiency while reducing methanol consumption, making methanol-glycerol co-feeding a promising strategy for scalable HPV VLP production.

सारांश

वायरस जैसे कण (वीएलपी) देशी वायरल संरचनाओं की नकल करने की अपनी क्षमता के कारण एक आशाजनक वैक्सीन प्लेटफॉर्म के रूप में उभरे हैं, जबकि वे गैर-संक्रामक होते हैं, उच्च सुरक्षा और प्रतिरक्षात्मकता प्रदान करते हैं। इस थीसिस कार्य में दो प्रमुख वायरल रोगजनकों को लक्षित करने वाले वीएलपी के लिए स्केलेबल उत्पादन रणनीतियों का विकास और अनुकूलन शामिल था: चिकनगुनिया वायरस (CHIKV) और ह्यूमन पेपिलोमावायरस (HPV)। CHIKV द्वारा उत्पन्न वैश्विक बोझ प्रभावी रोगनिरोधी समाधानों की तत्काल आवश्यकता को उजागर करता है। CHIK-VLPs को व्यक्त करने वाली HEK293T कोशिकाओं की एक स्थिर सीरम-मुक्त निलंबन संस्कृति CHIK-VLP उत्पादन में स्केलेबिलिटी के मुद्दों को दूर करने के लिए स्थापित की गई थी। समानांतर में, HPV, गर्भाशय ग्रीवा के कैंसर का एक प्रमुख कारण, HPV-VLPs को व्यक्त करने वाले पिचिया पास्टोरिस में एक मजबूत, लागत प्रभावी अभिव्यक्ति प्लेटफॉर्म के विकास के माध्यम से संबोधित किया गया था। स्ट्रेन प्रमोटर संयोजनों के स्तर पर अनुकूलन, प्रयोगों के डिजाइन (DoE) के माध्यम से प्रक्रिया पैरामीटर, लक्षित सूक्ष्म पोषक पूरकता और फीड-बैक किण्वन रणनीतियों ने वीएलपी उपज में वृद्धि, संरचनात्मक अखंडता में सुधार और प्रक्रिया स्केलेबिलिटी को बढ़ाया। शेक फ्लास्क और लैब-स्केल बायोरिएक्टर दोनों में कार्यान्वयन ने कुशल वीएलपी-आधारित वैक्सीन उत्पादन की क्षमता का प्रदर्शन किया, जिसने भविष्य के बड़े पैमाने पर विनिर्माण और व्यावसायीकरण के लिए एक मजबूत नींव रखी। पारंपरिक HEK293T एडहेरेंट सेल कल्चर का उपयोग करके CHIK-VLPs का उत्पादन भ्रूण गोजातीय सीरम (FBS) के पूरक पर निर्भर करता है, जो वैक्सीन निर्माण के लिए परिवर्तनशीलता, संदूषण जोखिम, उच्च लागत और स्केलेबिलिटी चुनौतियों का परिचय देता है पहले उद्देश्य (अध्याय 3) में, हमने एक स्थिर HEK293T सेल लाइन पूल की स्थापना की जो CHIK-VLPs का उत्पादन करने में सक्षम है और बाद में इन कोशिकाओं को स्केलेबिलिटी में सुधार करने के लिए निलंबन संस्कृति में स्थानांतरित किया। हमने 1000 एमएल एर्लेनमेयर फ्लास्क में बैच कल्चर के माध्यम से CHIK-VLPs के सफल उत्पादन की पुष्टि की, जिसमें उपज बढ़ाने के लिए प्रक्रिया के दौरान तापमान में बदलाव शामिल था। SDS-PAGE, वेस्टर्न ब्लॉटिंग, मास स्पेक्ट्रोमेट्री सहित विभिन्न विश्लेषणात्मक तकनीकों के माध्यम से CHIK-VLPs अभिव्यक्ति की पुष्टि की गई और स्थिर अनुयाई और निलंबन-अनुकूलित HEK293 कोशिकाओं द्वारा उत्पादित VLPs की आकृति विज्ञान का ट्रांसमिशन इलेक्ट्रॉन माइक्रोस्कोपी द्वारा मूल्यांकन किया गया। अवशोषण-आधारित विधि द्वारा निर्धारित तापमान बदलाव का उपयोग करके निलंबन-अनुकूलित संस्कृति में VLP उत्पादन में 5 गुना वृद्धि हासिल की गई। कुशल CHIK-VLPs स्राव में सक्षम स्थिर HEK293T सेल लाइनों की यह स्थापना VLPs उत्पादन के लिए एक बहुमुखी और स्केलेबल विधि प्रदान करती है जो एंटीबायोटिक दवाओं और FBS की आवश्यकता से मुक्त है। यह प्रक्रिया बायोफार्मासिटिकल उद्योग में एक मूल्यवान योगदान का प्रतिनिधित्व करती है, जो बड़े पैमाने पर VLPs-आधारित वैक्सीन विकास की क्षमता प्रदान करती है। HPV-VLPs के बड़े पैमाने पर उत्पादन के लिए एक मजबूत अभिव्यक्ति प्रणाली की आवश्यकता होती है। पसंदीदा विकल्पों में से एक उनकी लागत-प्रभावशीलता, तेजी से विकास और प्रोटीन अभिव्यक्ति के उच्च स्तर के कारण खमीर अभिव्यक्ति प्रणाली है। अगले अध्ययन (अध्याय 4) में, हमने विशेष रूप से बायोलैक्टर एक्सटी माइक्रोबायोरिएक्टर सिस्टम का उपयोग करके पी। पास्टोरिस में एचपीवी-वीएलपी के बड़े पैमाने पर उत्पादन को बढ़ाने के लिए इष्टतम प्रमोटरों, उपभेदों और बायोप्रोसेस स्थितियों को स्क्रीन करने का लक्ष्य रखा। विश्लेषण से पता चलता है कि प्रेरित अल्कोहल ऑक्सीडेज प्रमोटर के तहत GS115 स्ट्रेन ने X33 (11.07 mg/L) और KM71H (6.49 mg/L) स्ट्रेन की तुलना में 20.50 mg/L की उच्चतम VLP उपज प्रदर्शित की। HPV-VLP उत्पादन की पुष्टि SDS-PAGE, वेस्टर्न ब्लॉटिंग, HPLC, इंटेक्ट मास एनालिसिस और पेप्टाइड मैपिंग के माध्यम से की गई। इलेक्ट्रॉन माइक्रोस्कोपी के साथ संरचनात्मक लक्षण वर्णन ने मूल HPV के लिए समरूपता, कोलाइडल स्थिरता और संरचनात्मक समानता को मान्य किया। कण अखंडता को बनाए रखते हुए इंटरसेल्युलर VLP रिकवरी में सुधार करने के लिए पिचिया सेल लिसिस स्थितियों का अनुकूलन प्राप्त किया गया था। यह अध्ययन VLP के कुशल और लागत प्रभावी

बड़े पैमाने पर उत्पादन के लिए GS115 स्ट्रेन का उपयोग करने के लिए एक व्यवहार्य मार्ग प्रदान करता है। यह अध्ययन पी. पास्टोरिस में एचपीवी-वीएलपी उत्पादन को अनुकूलित करने के लिए एक व्यापक दृष्टिकोण प्रदान करता है, जो संभावित टीका विकास के लिए संरचनात्मक रूप से बरकरार एचपीवी-वीएलपी की उच्च पैदावार प्राप्त करने में प्रमोटर विकल्प, तनाव अनुकूलन और खेती तकनीकों के महत्व पर प्रकाश डालता है। तीसरा अध्ययन (अध्याय 5) दर्शाता है कि सूक्ष्म पोषक तत्वों का इष्टतम जोड़ वीएलपी उपज और गुणवत्ता को कैसे बढ़ाता है। इस अध्ययन में, हमने पी. पास्टोरिस में एचपीवी-वीएलपी के उत्पादन पर सात धातुओं और सात विटामिनों के प्रभाव को स्क्रीन करने और अनुकूलित करने के लिए प्रयोगों का 3-चरणीय डिज़ाइन किया है। सबसे पहले, सेल विकास और वीएलपी उपज पर सात धातु आयनों (Fe, Zn, Ba, Ni, Cd, Li, Ca) और सात विटामिनों के प्रभाव का मूल्यांकन करने के लिए एक-कारक-एक-समय (OFAT) स्क्रीनिंग की गई थी। इसके बाद, प्लैकेट-बर्मन आधारित DoE अध्ययन ने VLPs उपज के लिए आवश्यक सूक्ष्म पोषक तत्वों के रूप में Fe, Zn, निकोटिनिक एसिड और D-कैल्शियम पैटोथेनेट की पुष्टि की। अंत में, अधिकतम बायोमास और VLPs के उत्पादन के लिए सर्वोत्तम स्थितियों को निर्धारित करने के लिए चयनित सूक्ष्म पोषक तत्वों के बीच परस्पर क्रिया को अनुकूलित करने के लिए एक प्रतिक्रिया सतह डिज़ाइन (RSM) किया गया। इन सूक्ष्म पोषक तत्वों की इष्टतम सांद्रता 1 mM Zn, 1 mM Fe, 2.04 mM निकोटिनिक एसिड और 4 mM D-कैल्शियम पैटोथेनेट पाई गई, जैसा कि RSM पूर्वानुमान मॉडल द्वारा बायोमास के लिए $R^2 = 0.90$ और रूट मीन स्क्वायर एरर (RMSE) = 1.4 और HPV-L1 प्रोटीन टिटर के लिए $R^2 = 0.94$ और RMSE = 0.98 के साथ निर्धारित किया गया था। अनुकूलित धातु आयनों और विटामिनों ने HPV-VLPs L1 प्रोटीन उत्पादन को बढ़ाया, जिससे शेक फ्लास्क में 1.46 गुना अधिक बायोमास (24.1 ग्राम/ली) और 2.07 गुना अधिक प्रोटीन टिटर (33.2 मिलीग्राम/ली) प्राप्त हुआ। बायोरिएक्टर में, नियंत्रण की तुलना में बायोमास में 1.44 गुना (101.1 ग्राम/ली) वृद्धि और VLPs उपज में 1.54 गुना (37.1 मिलीग्राम/ली) वृद्धि देखी गई। यह अध्ययन HPV-VLPs के बड़े पैमाने पर उत्पादन के लिए सूक्ष्म पोषक तत्व अनुकूलन के महत्व पर प्रकाश डालता है। चौथे अध्ययन (अध्याय 6) में, पी. पास्टोरिस GS115 (Mut⁺) में HPV16 L1 VLP उत्पादन को अनुकूलित करने के लिए फीड-बैच किण्वन का उपयोग किया गया था, जिसका उद्देश्य मेथनॉल अवरोध को कम करना और VLPs उपज को बढ़ाना था। प्रेरण चरण के दौरान चार मेथनॉल-आधारित फीडिंग रणनीतियों का मूल्यांकन किया गया, जिसमें पल्स और रैखिक फीडिंग पैटर्न दोनों शामिल थे: 100% मेथनॉल (M100), 70% मेथनॉल + 30% ग्लिसरॉल (M70+G30), 70% मेथनॉल + 30% सोर्बिटोल (M70+S30), 70% मेथनॉल + 15% ग्लिसरॉल + 15% सोर्बिटोल (M70+G15+S15)। प्रक्रिया प्रदर्शन का आकलन करने के लिए सेल वृद्धि (ऑप्टिकल घनत्व और शुष्क कोशिका भार), चयापचय गतिविधि (घुलित ऑक्सीजन स्तर) और प्रोटीन अभिव्यक्ति सहित प्रमुख प्रक्रिया मापदंडों की निगरानी की गई। सभी रणनीतियों का मूल्यांकन पहले HPV VLP उत्पादन के लिए माइक्रो-स्केल फ़ेड-बैच खेती में किया गया था, और सबसे प्रभावी दृष्टिकोण को स्केलेबिलिटी और प्रदर्शन की पुष्टि करने के लिए 1L लैब-स्केल बायोरिएक्टर में आगे मान्य किया गया था। जांच की गई रणनीतियों में से, M70 + G30 (रैखिक फीडिंग) दृष्टिकोण ने माइक्रोबायोरिएक्टर खेती में उच्चतम उत्पादकता का प्रदर्शन किया, जिसमें 210.9 का OD₆₀₀, 90.36 g/L का DCW और 42.92 mg/L का उत्पाद टिटर प्राप्त हुआ। इस रणनीति को 1L लैब-स्केल बायोरिएक्टर में आगे मान्य किया गया, जहाँ इसने ~320 के ऑप्टिकल घनत्व (OD), 161 g/L के DCW और 49 mg/L के प्रोटीन टिटर के साथ और भी अधिक उपज प्राप्त की, जिससे इसकी मापनीयता और प्रभावशीलता की पुष्टि हुई। इस दृष्टिकोण ने मेथनॉल की खपत को कम करते हुए सेल वृद्धि, प्रोटीन अभिव्यक्ति और लागत दक्षता को बढ़ाया, जिससे मेथनॉल-ग्लिसरॉल सह-फीडिंग स्केलेबल HPV VLP उत्पादन के लिए एक आशाजनक रणनीति बन गई।

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List of Abbreviations

AOX	Alcohol oxidase promoter
ATF	Alternating tangential flow
BCA	Bicinchoninic acid
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BSA	Bovine serum albumin
CAI	Codon adaptation index
CCD	Central composite design
CHO	Chinese hamster ovary
CHIKV	Chikungunya virus
CQAs	Critical quality attributes
DCW	Dry cell weight
DMEM	Dulbecco modified eagle medium
DNA	Deoxyribonucleic acid
DOE	Design of experiments
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FACs	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FMDV	Foot-and-mouth disease virus
GAP	Glyceraldehyde-3-phosphate dehydrogenase promoter
GMP	Good manufacturing practices
GuHCL	Guanidinium hydrochloride
HCL	Hydrochloric acid
HEK cells	Human embryonic kidney cells
HPV	Human papillomavirus
LB	Luria–Bertani
OFAT	One factor at a time

Opti-MEM	Optimized minimal essential medium
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PTM	Post-translational modifications
RMSE	Root Mean Square Error
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase high-performance liquid chromatography
RSM	Response surface design
SEM	Scanning electron microscopy
SFM	Serum-free medium
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
STI	Sexually transmitted infection
SV40	Simian virus 40
TEM	Transmission electron microscopy
TNE	Tris, NaCl, EDTA buffer
TBST	Tris-buffered saline with 0.1% Tween 20
VCC	Viable cell count
VLP	Virus-like particles
YPD	Yeast extract peptone dextrose