Postprint: Optimization and Control of PCV2 VLPs Production Process Based on PAT

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Abstract

The insect cell-baculovirus expression vector system (BEVS) is an ideal production platform for virus-like particle (VLP) subunit vaccines. Process analytical technology (PAT) for animal cell culture focuses on acquiring additional process parameters related to cellular physiological states, as well as achieving online characterization of process-sensitive parameters and online determination of critical time points for process control, thereby guiding process optimization and control. Through batch and fed-batch suspension culture of insect Sf9 cells, this study identified a correlation between cellular metabolic activity and online characteristic frequency (fc). Using fc as an online indicator of cellular metabolic activity, feeding was initiated prior to the decline in cellular metabolic activity, which resulted in a 1.75-fold increase in maximum viable cell density on the basis of significantly enhanced metabolic activity in Sf9 cells. Through correlation analysis of cellular physiological characteristic parameters in batch and fed-batch culture processes, it was discovered that specific capacitance growth rate ( ) correlates with the proportion of S-phase cells in the culture system. As an online indicator parameter of cellular proliferation activity state, can serve as a basis for determining optimal virus infection timing. Furthermore, the study found that the online detection parameter capacitance ( ) correlates with maximum vaccine yield and can serve as an online characterization parameter for optimal vaccine harvest timing. By employing online fc value as an indicator for feeding time, online value as an indicator for virus infection time, and online value as an indicator for virus harvest time, efficient production of Porcine circovirus type 2 (PCV2) VLPs was achieved. Compared with batch culture, the PAT-based production process increased vaccine volumetric productivity by 76% and shortened the production cycle by 24 h. This study provides a novel efficient production model for large-scale manufacturing of PCV2 virus-like particle subunit vaccines.

Full Text

Preamble

Process Control and Optimization of PCV2 VLPs Production Based on PAT

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Abstract

The baculovirus expression vector system (BEVS) is an ideal production platform for virus-like particle (VLP) subunit vaccines. Process analytical technology (PAT) in animal cell culture focuses on acquiring process parameters related to cell physiological status, enabling online characterization of process-sensitive parameters and online determination of critical control time points, thereby guiding process optimization and control. Through batch and fed-batch suspension culture of insect Sf9 cells, this study identified a correlation between cell metabolic activity and the online characteristic frequency (fc). Using fc as an online indicator of cell metabolic activity and implementing feeding before metabolic activity declined, the peak viable cell density increased by 1.75-fold with significantly enhanced metabolic activity.

Correlation analysis of cell physiological parameters between batch and fed-batch cultures revealed a relationship between specific capacitance growth rate ( ) and the proportion of S-phase cells in the culture system. As an online indicator of cell proliferation activity, can serve as a criterion for determining optimal infection time. Additionally, the study found a correlation between the online capacitance parameter ( ) and maximum vaccine yield, enabling online characterization of the optimal harvest time. By using online fc as the feeding time indicator, online as the infection time indicator, and online as the harvest time indicator, efficient production of porcine circovirus type 2 (PCV2) VLPs was achieved. Compared to batch culture, the PAT-based production process increased vaccine volumetric productivity by 76% and shortened the production cycle by 24 hours. This study provides a novel and efficient production model for large-scale manufacturing of PCV2 VLP subunit vaccines.

Keywords: VLPs, Baculovirus Expression Vector System, PAT

Introduction

Porcine circovirus type 2 (PCV2) is a DNA virus belonging to the family *Circoviridae* and genus *Circovirus*, and serves as the causative agent for multiple swine diseases including porcine dermatitis and nephropathy syndrome (PDNS), porcine necrotizing pneumonia (PNP), and postweaning multisystemic wasting syndrome (PMWS) [1-4]. With mortality rates ranging from 10% to 30%,

PCV2 infection causes substantial losses to the global swine industry. Vaccination represents the primary preventive measure, and VLPs constitute the third-generation vaccine against this pathogen that has been successfully commercialized. These vaccines possess the immunogenicity of wild-type viruses while lacking their infectious properties, with their advantages widely reported in the literature [5-7]. Consequently, VLP-based vaccines are gaining increasing traction in the industry.

BEVS serves as an ideal production platform for VLPs. As a eukaryotic protein expression system, it provides post-translational modification capabilities such as glycosylation and disulfide bond formation [8]. The system has been employed for structural and functional studies of therapeutic proteins as well as for animal and human vaccine production [9-11].

Optimizable parameters in the BEVS culture system include process variables such as cell seeding density, multiplicity of infection (MOI), time of infection (TOI), time of feeding (TOF), time of harvest (TOH), and culture temperature, as well as cultivation mode optimizations including batch [12], fed-batch [13], and continuous perfusion culture [14-15]. Traditional optimization approaches primarily rely on design of experiments (DOE) to identify optimal parameter combinations, but suffer from numerous experimental runs and lengthy cycles.

The advent of Quality by Design (QbD) and PAT concepts has imposed higher demands on process optimization and control, with the application of advanced sensors strongly facilitating QbD and PAT implementation. For instance, Zeiser et al. [16] utilized online capacitance electrodes to monitor viral infection processes in BEVS, while infrared CO analysis systems enabled online monitoring of viral release timing [17]. Qiu J et al. [18] employed near-infrared spectroscopy for real-time monitoring of cell density, glucose, and lactate concentrations in insect cell culture systems, providing control basis for process management. Guided by PAT principles and based on online analysis using viable cell sensors, this study investigates the reactor-based production of PCV2 VLP subunit vaccines using insect Sf9 cells. The research explores correlation patterns among various physiological and metabolic parameters during VLP vaccine production, identifies online characterization parameters for optimal TOF, TOI, and TOH, and establishes an efficient PAT-based PCV2 VLPs production process to lay the foundation for large-scale reactor manufacturing of PCV2 vaccines.

Materials and Methods

1.1 Online and Offline Viable Cell Counting, Characteristic Frequency Determination, and Specific Capacitance Growth Rate Calculation

Offline viable cell density and viability were determined using a hemocytometer. Culture broth was mixed 1:1 with trypan blue staining solution and loaded onto a hemocytometer for offline measurement. When cell concentration was excessively high, appropriate dilution was performed.

The online viable cell sensor (model BM220) was operated at a measurement frequency of 608 kHz with LowPass set to 30. Specific capacitance growth rate was calculated as follows:

ln − ln
t − t

1.2 Offline Specific Oxygen Consumption Rate (qO) Measurement

Specific oxygen consumption rate was measured using an OROBOROS Oxygraph 2K. Each measurement chamber was filled with 2.1 mL fresh medium to calibrate the dissolved oxygen electrode saturation point. Cultured cells were diluted to 1.0 × 10 cells/mL with fresh medium, and 2.1 mL of the diluted cell suspension was added for measurement to plot the dissolved oxygen versus time curve.

1.3 Mitochondrial Membrane Potential Depolarization Ratio Measurement

One million cultured cells were harvested and centrifuged at 1000 r/min for 5 minutes to remove supernatant. The cell pellet was resuspended in 500 L ice-cold PBS, mixed with 500 L JC-1 staining working solution, and incubated at 37°C for 20 minutes. JC-1 staining buffer was prepared during incubation and immediately placed on ice. After incubation, cells were centrifuged and resuspended in JC-1 staining buffer. Fluorescence intensity was detected using a dual-wavelength fluorescence spectrophotometer. JC-1 monomer green fluorescence was measured at excitation/emission wavelengths of 490 nm/530 nm, while JC-1 aggregate red fluorescence was measured at 525 nm/590 nm, with detection temperature set at 27°C.

1.4 Cell Cycle Analysis

Five hundred thousand cultured insect cells were centrifuged at 1000 r/min for 5 minutes, resuspended in 1 mL PBS, and washed twice. Cells were then resuspended in 1 mL 70% ice-cold ethanol, fixed overnight at 4°C, and stored at -20°C until analysis. After sample collection, cells were washed with 1 mL PBS and incubated with 500 L staining working solution at 37°C for 30 minutes. Red fluorescence was detected using flow cytometry at an excitation wavelength of 488 nm.

1.5 Specific Nutrient Consumption Rate Calculation

Appropriate volumes of cell culture broth were centrifuged to remove cells. The supernatant was diluted to approximately 1 g/L based on predicted sugar concentration, and the diluted sample was injected into an SBA-60 system (Shandong Institute of Life Science) for sugar concentration measurement. Specific glucose consumption rate was calculated according to the following formula:

Glu = 2(鵠�near　− 鵠�near　)
1 + 2

1.6 Cell Revival, Passage, Scale-up, and Fermenter Setup

Frozen Sf9 cells were rapidly thawed at 27°C, centrifuged, and resuspended in fresh SF II medium for culture at 27°C. When cell density reached (3.0–4.0) × 10 cells/mL, cells were passaged or scaled up at a density of 1.0 × 10 cells/mL. Upon reaching appropriate density, cells were inoculated into fermenters at the same density under the following conditions: agitation speed 65 rpm, culture temperature 27°C, pH monitored but not controlled, dissolved oxygen automatically controlled at 40% using pure oxygen.

1.7 PCV2 Protein Particle Quantification by ELISA

Microplates were coated with standards at concentrations of 1000, 500, 250, 125, 62.5, and 31.5 ng/mL (100 L) or test samples. After overnight incubation at 4°C, plates were washed three times with PBST and blocked with 5% skim milk at 37°C for 1 hour (200 L/well), followed by three washes. Mouse anti-PCV2 Cap monoclonal antibody diluted 2000-fold was added and incubated at 37°C for 1 hour. Horseradish peroxidase-labeled goat anti-mouse antibody diluted 2000-fold (100 L/well) was added and incubated at 37°C for 1 hour, followed by four washes. TMB solution (100 L/well) was added and incubated at 37°C for 15 minutes. The reaction was terminated with 2 M sulfuric acid, and absorbance was read at 450 nm using a microplate reader.

1.8 Recombinant Baculovirus Culture

The recombinant virus used in this study was developed in-house. Recombinant viral particles from the same production batch were used, with the multiplicity of infection (MOI) uniformly controlled at 0.5.

Results

2.1 Physiological and Metabolic Changes in Batch Culture and Determination of Optimal Feeding Time

Viable cell density is a critical state variable in large-scale cell culture, as changes in cell physiological status and implementation of operational variables are largely associated with viable cell density. Using a viable cell sensor for online monitoring of Sf9 cell density during culture, we found that electrode signals correlated positively with viable cell density during the lag, exponential, and stationary phases (Figure 1 [Figure 1: see original paper]). Linear fitting yielded a coefficient of determination $R^2 = 0.99$, enabling real-time online detection of viable cell density changes before cell viability decline.

Prior to apoptosis onset, cell membrane properties and cytoplasmic conductivity remain relatively constant, serving as significant factors affecting viable cell

capacitance signals. When cells enter apoptosis, membrane function begins to deteriorate with increased permeability, leading to leakage of intracellular substances, particularly charged ions. Additionally, cell diameter expands during apoptosis, increasing biomass while viable cell density remains constant or declines. Consequently, capacitance signals increase while viable cell density stays unchanged or decreases. Nevertheless, during the lag, exponential, and plateau phases, online capacitance signals correlate positively with viable cell density, enabling online characterization of viable cell density in the culture system.

Cell metabolic activity status represents another important state variable in cell culture. Precise online analysis of cell metabolic activity facilitates more accurate process control. In practical manufacturing, feeding operations are implemented to increase cell density and improve equipment utilization. Appropriate feeding replenishes nutrients and enhances VLPs production capacity, but simultaneously alters osmotic pressure and other environmental parameters, imposing stress on cells. Therefore, feeding time is a critical control node that must occur at an appropriate moment. The optimal feeding timing is considered to be when cells exhibit maximum metabolic activity and environmental adaptability. Mitochondria serve as cellular energy metabolism factories, and their functional status more accurately represents metabolic activity than cell viability alone. This study introduced analysis of mitochondrial membrane potential depolarization ratio (mmp) to characterize cell metabolic activity status and better understand physiological metabolic changes during culture. Specific glucose consumption rate (qGlu) and specific oxygen consumption rate (qO) are important physiological parameters related to cell metabolism. These two parameters were used as offline criteria for cell metabolic activity and compared with online parameters to identify the optimal metabolic activity point and its online indicator.

As shown in Figure 3 [Figure 3: see original paper], during the initial culture phase, the mitochondrial membrane potential depolarization ratio was relatively high due to cellular adaptation to the environment. As cells adapted, this ratio gradually decreased. At 40 hours, cells entered the exponential growth phase with vigorous metabolism, and the depolarization ratio stabilized at a relatively low level. After 120 hours, cell viability and growth rate began to decline, entering the deceleration phase, while the depolarization ratio increased rapidly, demonstrating high correlation with cell viability.

Since complete glucose oxidation occurs in mitochondria, changes in mitochondrial membrane potential depolarization ratio affect glucose and oxygen consumption. During mid-exponential phase, cells exhibit vigorous metabolism with relatively low depolarization ratio and high qO and qGlu values. When cells enter the deceleration phase, the depolarization ratio increases, indicating declining metabolic activity, accompanied by significant reductions in qO and qGlu values. Notably, the rapid qO decline preceded that of qGlu. The metabolic activity decline occurred significantly earlier than the viability decline, suggesting that monitoring only cell count, viability, and glucose metabolism

exhibits lag time, whereas qO changes provide more timely and accurate indication of metabolic activity status.

To enable online analysis of cell metabolic activity, this study introduced online characteristic frequency (fc) analysis of Sf9 cell culture using viable cell sensors. Analysis of Figure 4 [Figure 4: see original paper] revealed that when fc reached its minimum, qO increased rapidly, indicating peak metabolic activity. As fc rose significantly from its minimum, qO increase weakened, and with rapid fc elevation, qO declined sharply. The high correlation between qO changes and online fc values enables their use as indicators for cell metabolic activity and feeding operations. Since viral replication depends on host cell metabolic activity, the online fc parameter can also serve as an auxiliary criterion for selecting optimal viral infection timing.

2.2 Comparison of Physiological and Metabolic Changes Between Batch and Fed-Batch Cultures

Specific growth rate is an important physiological parameter characterizing cell proliferation status. Due to the high correlation between online capacitance values and cell mass, online specific capacitance ( ) calculated from online capacitance values also correlates highly with specific growth rate (data not shown). Batch culture results demonstrated that online values correlate strongly with cell growth proliferation status, enabling online characterization of proliferation changes. Based on combined online analysis of fc and , criteria for optimal feeding time were established (Figure 5 [Figure 5: see original paper]).

At 24 hours of culture, fc stabilized with a viable cell density of 12×10 cells/mL. Between 24 and 58 hours, fc remained near its minimum of 350 Hz while maintained high levels, indicating optimal growth and metabolic activity. Feeding was implemented at approximately 60 hours, before fc increased and decreased. Compared to batch culture, cells entered logarithmic growth after a brief adaptation period post-feeding. The fed-batch culture achieved a maximum viable cell density of 21×10 cells/mL, representing a 1.7-fold increase over batch culture (Figure 6 [Figure 6: see original paper]a).

The substantial increase in peak viable cell density correlated with enhanced proliferative activity. Comparison of specific growth rates between batch and fed-batch cultures revealed that feeding significantly increased and maintained specific growth rates during the later culture period (Figure 6 [Figure 6: see original paper]b). The post-feeding decline in specific growth rate also confirmed the stress imposed by feeding on cells.

Further analysis indicated that the increased viable cell density and improved specific growth rate maintenance were associated with elevated S-phase cell proportions (Figure 6 [Figure 6: see original paper]c). The nutrient-rich feed promoted cell division and proliferation, increasing the S-phase cell proportion and thereby enhancing specific growth rate and viable cell density. S-phase cells exhibit active macromolecular synthesis and possess the enzymatic machinery

for viral replication, creating favorable conditions for viral propagation.

VLPs production requires both the biosynthetic machinery and energy metabolism of insect cells. Therefore, efficient VLPs expression necessitates not only increased viable cell density but also enhanced energy metabolic activity. Glucose serves as the primary carbon source for cell growth and proliferation, while cellular respiration is the main ATP-generating pathway. Specific oxygen consumption rate (qO) is a physiological parameter directly related to metabolic status, and viral replication is entirely dependent on host cell energy, showing high correlation with cellular respiratory intensity. Using qGlu and qO as primary indicators, we investigated changes in cellular energy metabolic activity. Prior to feeding, batch and fed-batch cultures exhibited similar qGlu trends, though batch culture showed more dramatic changes with rapid post-peak decline, likely due to rapid depletion of specific nutrients. In fed-batch culture, feeding caused a slight qGlu decrease, but cells subsequently maintained high qGlu levels until the later culture period, indicating that feeding enhanced and prolonged metabolic activity. Similar to qGlu patterns, feeding significantly promoted oxygen consumption throughout the culture, demonstrating that feeding operations substantially enhanced cellular energy metabolic activity and extended the duration of vigorous metabolic status (Figures 6d and 6e).

2.3 Online Indicators for Viral Infection Time in Batch and Fed-Batch Cultures

Viral proliferation and replication depend on the host cell enzymatic system, making viral infection reliant on the cell division cycle. S-phase cells possess DNA replication-related enzymes and represent a prerequisite for early viral protein expression. Viral infection efficiency is highest when the S-phase cell proportion is high and cells are arrested in S-phase. This study employed offline cell cycle analysis to identify related online parameters. Data demonstrated high correlation between S-phase cell proportion and online values in both batch and fed-batch cultures, enabling online characterization of S-phase status (Figures 7 [Figure 7: see original paper] and 8 [Figure 8: see original paper]). Since viral replication depends on host cell proliferation and metabolic activity, combined analysis of offline S-phase proportion, qO values, and online and fc values identified online as the primary criterion and online fc as the auxiliary criterion. Considering that S-phase proportion changes precede online changes, optimal infection times were determined to be approximately 36 hours for batch culture and 96 hours for fed-batch culture.

2.4 Online Indicators for Viral Harvest Time in Batch and Fed-Batch Cultures

Online monitoring of the critical process parameter—maximum vaccine yield time point—would significantly improve vaccine production control efficiency and maximize product recovery. Batch and fed-batch data (Figures 9 [Figure

9: see original paper] and 10 [Figure 10: see original paper]) demonstrated high correlation between maximum vaccine yield time points and maximum online capacitance signals, enabling online characterization of peak vaccine production timing.

The capacitance signal from online viable cell sensors results from multiple interacting variables, with cell membrane polarity being the most sensitive and critical factor. During the later culture period, cells trigger apoptosis due to viral infection and nutrient depletion. During the transition to cell death, cell membrane polarity changes significantly, with complete polarity loss leading to capacitance signal decline. Subsequent cell lysis releases hydrolytic enzymes, causing vaccine yield reduction. Therefore, the capacitance peak serves as an online indicator for the critical time point of maximum vaccine yield.

2.5 Comparison Between PAT-Based and Traditional Production Processes

Comparison with traditional optimization processes revealed several bottlenecks: First, using viable cell density and viability as process control criteria lacks real-time capability, and cell counting operations suffer from large errors and poor reliability. Second, traditional processes struggle to reduce inter-batch variability. Due to inherent characteristics of cell culture, cell expansion, inoculation density, and initial culture volume cannot be completely consistent across batches, making it difficult to precisely correlate process control standards—whether based on viable cell density or culture time—with real-time cell metabolic status. Moreover, traditional processes using offline viable cell density as the control indicator lack real-time capability. Offline viable cell density cannot be obtained continuously, and hemocytometer-based measurements are highly operator-dependent, easily causing yield and quality variations between batches and compromising product consistency and repeatability.

This study employed advanced viable cell sensing technology to investigate the PCV2 VLPs production process and established an efficient PAT-based PCV2 VLPs manufacturing process. The PAT-based PCV2 VLPs vaccine production achieved a 1.7-fold increase in maximum cell density compared to batch culture (Figure 11 [Figure 11: see original paper]), a 76% increase in vaccine yield (Figure 12 [Figure 12: see original paper]), and a 24-hour reduction in culture duration, improving equipment utilization efficiency. Furthermore, the PAT-based optimization approach is more efficient than traditional methods, significantly reducing process development time. Using online parameters to guide and control production ensures better batch-to-batch consistency and repeatability.

This study investigated PCV2 VLPs vaccine production using insect Sf9 cells, aiming to identify correlation patterns among physiological and metabolic parameters and establish online indicators for optimal feeding time, viral infection time, and vaccine harvest time. Correlation analysis of various physiological and metabolic parameters in Sf9 batch culture revealed that online values cor-

relate with viable cell density and can characterize viable cell density. Online changes correlate with cell growth proliferation rate and can characterize proliferation status. Online qO and fc changes correlate with metabolic activity status and can characterize metabolic activity changes. Additionally, online and fc changes correlate with cell viability status and can characterize viability changes.

Based on correlation analysis of cell physiological parameters from batch culture, using fc changes as the online indicator for optimal feeding time successfully increased maximum viable cell density to 21 × 10 cells/mL in fed-batch culture, representing a 1.7-fold increase over the 1.2 × 10 cells/mL achieved in batch culture, enabling high-density Sf9 cell culture in bioreactors. Comparative analysis of cell proliferation and metabolic activity between batch and fed-batch cultures demonstrated that feeding not only substantially increased viable cell density but also enhanced cell proliferation status and metabolic activity.

Using characteristic frequency (fc) changes as the online feeding indicator, specific capacitance ( ) as the online infection indicator, and maximum as the online harvest indicator, efficient PCV2 VLPs production was achieved. The PAT-based PCV2 VLPs vaccine production increased peak vaccine yield by 76% compared to batch culture, shortened production cycle by 24 hours, and significantly improved equipment utilization efficiency. Moreover, the PAT-based optimization approach is more efficient than traditional methods, substantially reducing process development time. Acquisition of various online physiological parameters facilitates manufacturing operations and ensures batch-to-batch consistency, repeatability, and product quality.

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Note: Figure translations are in progress. See original paper for figures.