



REVIEW ARTICLE

Bioprocessing of Avian Influenza VLP Vaccine using Baculovirus-Insect Cell Expression System: A Review

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ABSTRACT

Vaccines are widely used as a preventive measure against influenza virus infection. However, these vaccines gain concerns regarding their safety during the production process as well as the poor production yield and efficacy. A breakthrough that uses insect cells as a production substrate to produce protein rapidly, especially viral antigens for the potential avian influenza outbreak, is being extensively researched. Insect cells infected by baculovirus (BV) are utilized to express proteins known as virus-like proteins (VLP). This review aims to assess the production of the avian influenza vaccine (i.e., H5N1 and H7N9 strains) made from VLP by utilizing a baculovirus-insect cell (BV-IC) expression system. A narrative review was conducted by screening international indexed journals about the topic from the last 10 years. Previous studies have indicated that VLP vaccine development using BV-IC expression is more cost-effective and produces a higher yield compared to the live attenuated and inactivated vaccines. The upstream process consists of the IC infection by the BV and BV-IC cell cultivation inside the bioreactor. The downstream process consists of the purification of the VLP product until it becomes a functioning vaccine. The VLP vaccines have improved immunogenic quality, enabling a more specific immune response than other influenza vaccines. However, the downstream processing (DSP) of VLP production had not been well-elaborated in available studies. Therefore, further studies are required to improve the current VLP vaccine production processes.

Keywords: *bioprocessing; virus-like particles; baculovirus-insect cell expression system; avian influenza; vaccine*

HIGHLIGHTS

- ❖ The baculovirus-insect cell (BV-IC) expression system is a cheaper, faster, and safer method to produce a virus-like protein (VLP) for commercialized avian influenza vaccines.
- ❖ The upstream process consists of the IC infection by the BV and BV-IC cell cultivation inside the bioreactor.
- ❖ The downstream process consists of the purification of the VLP product to become a vaccine.

INTRODUCTION

Influenza virus infections have been an issue since 1918 when the first influenza pandemic started (CDC, 2019). It causes human respiratory problems and becomes a concerning public health issue due to its unpredictable occurrence timing, whether seasonal, endemic or even a pandemic. One of the preventive measures as the first line of defense upon the potential outbreak in the population is vaccination (Thompson

et al., 2015). There are several common types of vaccines that utilize different types of formulations, such as the split antigen, attenuated virus, and inactivated whole-virion (Lai et al., 2019). Several types of vaccines have been discovered, approved by the FDA, and mass-produced by several companies, such as Afluria, Flucelvax, and FluMist (Chen et al., 2019). Common influenza live attenuated vaccines generally have a relatively poor production yield. Meanwhile, influenza inactivated vaccine induced a relatively poor level of mucosal immunity. More importantly, these vaccines gain many concerns regarding the safety issues during their production process. Efforts to increase the safety level in live attenuated vaccines likely decrease the efficacy of the vaccine (Jang & Seong, 2012).

The usage of insect cells to produce protein rapidly has been a breakthrough in the medical field, especially in producing the viral antigens for the potential avian influenza outbreak. Insect cells infected by baculovirus (BV) utilize the expression of proteins known as virus-like proteins (VLP). VLP is essential for vaccine creation during the timeframe where a potential pandemic might occur. It has been found that insect cells produce a higher VLP yield compared to mammalian cells, specifically in the attempt to manufacture the avian (H7N9) influenza vaccine (Thompson et al., 2015). This review aims to assess the production of the avian influenza vaccine (i.e., H5N1 and H7N9 strains) in the form of VLP by utilizing a baculovirus-insect cell (BV-IC) expression system. Currently, there are no review papers discussing this particular topic; while comparing techniques used in the BV-IC expression system for VLP production.

After covering baculovirus and its role in VLP production, this paper reviews the bioreactors used in the production, modes and process parameters of cultivation, followed by the technology used in downstream processing. Lastly, this paper will discuss the advantages and disadvantages and the possible methods to overcome the disadvantages within this paper. The reviewed studies were selected based on the keywords of bioprocess, avian influenza vaccine, insect cells, BV, and VLP.

BACULOVIRUS

Baculovirus (BV) is an enveloped, rod-shaped nucleocapsid virus with a genome length of 8 to 180 kbp and a budded virus's phenotype to infect the insect cell. The BV infection occurs when the BV is attached to the insect cell membrane, which leads to the fusion or penetration of the infected cell membrane caused by a viral glycoprotein that interacts with the cell's receptor (Pidre et al., 2013). The infection process consists of four phases: immediate-early, delayed-early, late, and very late. In the immediate-early phase, the genes encoding for transcription factors are transcribed by the host's RNA-polymerase II, which causes the activation of delayed-early and late gene promoters. The expression of the genes responsible for replicating the BV and manipulating the host's system occurs during the delayed-early phase. The transition from delayed-early phase to late phase is indicated through DNA replication. In the late phase, the DNA is replicated, viral proteins are synthesized, and nucleocapsids are assembled, leading to the production of the budded virus VLP. While a high BV concentration is produced at the early infection process, a high concentration of occluded virions, namely the polyhedrin and p10 proteins, are produced at the very late phase. However, the polyhedrin and p10 proteins are not needed to form the budded virus and occlusion bodies (OB). Thus, genes encoding the polyhedrin and p10 proteins can be replaced by genes of interest to produce target proteins. In this case, the *polh* and *p10* promoters are used to express high amounts of foreign proteins in the insect cell only during the very late phase while limiting the formation of OB in the insect cell culture (Contreras-Gómez et al., 2014; Pidre et al., 2013; Saxena et al., 2018). BV as insect pathogens is known for infecting a specific host, thus, limiting the ability of BV to infect humans and minimizing the safety concerns in vaccines' manufacturing (Cox, 2012; Pidre et al., 2013; Pushko & Tretyakova, 2020).

VIRUS-LIKE PROTEINS

The BV-IC expression system is capable of producing a virus-like protein (VLP) for commercialized vaccines. VLP is composed of viral proteins constituted through self-assembly mechanisms, forming similar conformation of the mature virus, yet lacking in the viral genetic material. VLP derived from the BV-insect cell technique is considered a subunit vaccine (World Health Organization, n.d.). The ability of VLP to trigger strong immune responses and their rapid production is considered advantageous during a pandemic when large scaling and fast production are needed.

The manufacture of VLP for influenza vaccines has become a new trend since less investment cost is required while achieving a high yield. Adjustment in the cell culture strategy can give a high VLP yield, which corresponds to the significant reduction in the production cost, namely growth media enhancement and molecular biology approach (gene manipulation); for example, gene manipulation within the insect cell line that affects the expression of BV. The decrease of vaccine buyers causes a decline in vaccine supply sustainability, causing many vaccine manufacturers to be overwhelmed. With the usage of the BV-IC expression system, the flexibility of making a broad range of vaccines is possible, thus maintaining the sustainability of vaccine supply that corresponds to the demand for a specific area and time (Cox, 2012).

Novavax is the first to establish an avian influenza vaccine using the BV-IC expression system. Their nanoparticle vaccine technology takes advantage of the BV-IC expression system to produce subunit vaccines. The lack of viral genetic materials in the VLP will not raise safety concerns, unlike the usage of live attenuated vaccines. The final composition of the vaccine is composed of hemagglutinins (HA) protein, neuraminidase (NA), and matrix 1 protein (M1). HA and NA proteins are the two significant glycoproteins that distinguish the structure found in influenza viruses. HA protein is a significant protective antigen on the surface of the avian influenza virus, thus, having a significant role in the correct vaccine production to be marketed. The vaccine was delivered with the aid of an adjuvant with a cage-like form to increase the vaccine's antibody response (Fries et al., 2013; Ge et al., 2016).

UPSTREAM BIOPROCESSES

A. Cell culture

Bioreactor Design. The standard bioreactor design for insect cell culture in large-scale productions is suspension-based bioreactors (Unger & Peleg, 2012). Insect cells-infected BV grows best in suspension culture, resulting in high cell density due to the ease of accessibility of BV to infect the insect cells. Furthermore, no limit in surface areas resulted in a higher production rate during the infection phase (Drugmand et al., 2012).

The bioreactor design for insect cells is similar to mammalian cells. Nevertheless, some bioreactor components need adjustment to enhance and optimize the production using BV and insect cells. Insect cell damage is often caused by shear stress or energy dissipation rates from sparging. Insect cells are found to be shear sensitive at more than 0.59 N/m^2 shear and $2.25 \times 10^4 \text{ W/m}^3$, although this range is higher than values generated in usual stirred-tank reactors (Drugmand et al., 2012).

A wave bioreactor can be used on a smaller scale, such as the inoculation stage. Novavax reported the use of wave bioreactor up to 200 L capacity (Hahn et al., 2013). The use of wave bioreactor avoids shear stress due to impellers. The stirred-tank and air-lift bioreactors are common in insect cell cultivation among many suspension systems. The air-lift bioreactors have a simple design and construction, which is more beneficial economically. However, stirred-tank is common in applications because of its capability for industrial-scale production, compared to air-lift (Contreras-Gómez et al., 2014).

Cultivation Mode of the Bioreactor. Batch and fed-batch bioreactors offer simplicity and flexibility in usage, as it is highly suitable for BV during the infection cycle. However, it is reported that the production in the batch system is lower than the fed-batch and limited due to nutrient depletion and waste accumulation (Contreras-Gómez et al., 2014). Target-protein production is reduced at high cell density infection in a “cell density effect” phenomenon, which is not thoroughly studied but suspected to be caused by nutrient depletion and waste accumulation. However, a study shows that the cause might be metabolism-based inhibition at high-cell densities (Carinhas et al., 2010). The proposed solution to this particular problem is to replenish the culture medium or selective nutrient feeding. However, total replenishment of culture medium might be costly, so partial medium replenishment is recommended (Beas-Catena et al., 2013).

The fed-batch system is an alternative to the medium replenishment system. The fed-batch system is similar to the batch system but with the feature of fresh medium addition. This system is easier and cheaper than continuous and perfusion systems while also producing high cell density and productivity. Moreover, the fed-batch is safer for insect cells since it avoids the damage caused by cell retention systems. However, this system requires knowledge of cell physiological requirements and optimal medium supplementation (Drugmand et al., 2012).

Continuous bioreactor systems are rarely used in insect cells. The withdrawal of the medium causes cell and product dilution. Moreover, the two-stage process, cell infection and cultivation, is challenging to be implemented into the continuous system (Drugmand et al., 2012).

Perfusion systems were also tested on insect cells. In perfusion systems, the cells are continuously fed, and the product is harvested continuously; and equipped with cell retention mechanisms and continuous cell monitoring. This system is advantageous as it produces higher cell productivity than the fed-batch system while also removing toxic by-products. However, the perfusion system is unsuitable for productions involving BV, as high and medium withdrawal rates can dilute the cells and products that often occur when using the continuous system. Retention systems are usually membrane-based devices used to collect the product to be harvested while also retaining the cells to be used again. Unfortunately, the retention system can damage the cells that have been infected with the BV system (Drugmand et al., 2012). Furthermore, retention systems are also expensive and difficult to apply.

Currently, the popular alternative for fed-batch and batch stirred-tank suspension-based reactors is the single-use or disposable bioreactor. This reactor consists of single-use sterile bags equipped with a wave or stirred-induced mixing, making it low-maintenance. In addition, it has a decreased likelihood of cross-contamination or regular contamination since the bag is only for single use. This type of bioreactor is also suitable when vaccines need to be produced in a pandemic situation due to time constraints. An example of a large-scale production using disposable bioreactors is done by Novavax, precisely on the avian (H7N9) influenza vaccine production. Their production uses a 50 L WAVE bioreactor, a 200 L WAVE bioreactor, a single-use cell culture bioreactor, a 1000 L Xcellerex production bioreactor, and a single-use stirred tank bioreactor (Hahn et al., 2013).

In summary, batch and fed-batch are preferred over all cultivation modes that have been tested on insect cells. Both bioreactors are suited for production using BV. The single-use or disposable bioreactors are the perfect alternatives, especially in pandemic situations where vaccines are needed fast.

Cleaning and Heat Exchange Units. Cleaning plays an important role in the post-fermentation process in bioreactors. According to the cGMP provisions, it is essential to prevent product contamination in different batches by thoroughly cleansing the equipment to achieve a high standard of cleanliness, sanitization, and reproducibility. Cleaning also helps preserve and maintain the bioreactor surface to prevent scale build-up or corrosion, decreasing efficiency of operation. Different types of cleaning are required depending on the type of bioreactors (Koller, 2020; Flickinger, 2010). In this review paper, the cleaning process for stainless-steel-based bioreactors and other alternatives will be discussed.

The cleaning-in-place (CIP) method is the most commonly utilized method to clean an industrial-scale bioreactor is the cleaning-in-place (CIP) method. CIP thoroughly cleans the bioreactor used and all linings linked to the bioreactor to ensure thorough cleaning. It utilizes four main circulating cleaning agents within the process, which are the spray balls, sterilizing and cleaning chemicals, high-pressure pumps, and steam (Chisti & Moo-Young, 1994). Various equipment where the CIP's adequate flow is insufficient to clean and generate dead zones may also be detached and cleaned-out-of-place (COP). The cleaning efficiency depends on various factors such as the cleaning procedures, type of bioreactor system used, material of the bioreactor, piping and ancillary process equipment design, cleaning reagent used, and others. The complete requirements and procedures for CIP are available online. In summary, equipment with a cleanable smooth surface and seamless tubing is preferred with the piping design, ensuring direct contact with cleaning reagents. The correct reagent capable of dissolving organic residues is also preferred to ensure no remnants are left (Flickinger, 2010). However, the CIP method takes a long time (up to 10 hours) depending on the repetitions, cycles, and procedures done to ensure cleanliness. Continuous and repeated cleaning processes also usually cost a large sum of money due to the continuous supply and usage of chemicals and utilities used for cleaning. Furthermore, sterilization of the equipment is still needed by utilizing high-heat pressurized steam or autoclave when available (Kirkland, 2015; Hebel, 2014). The usage of the single-use bioreactor (SUB) and wave bioreactor has been emerging for the BV-IC expression system because they do not need to be cleaned. Compared to traditional stainless equipment, single-used bioreactors provide ease for modification, reconfiguration, modulation, and transportation. The SUB can also immediately be discarded after usage, which omits the need for cleaning. Therefore, it reduces the processing time significantly in several ways, such as eliminating the time needed for developing service and design for the cleaning and steaming procedure, the cost required in building the cleaning system, and others (Whitford, 2015; Vicente et al., 2011, Hahn et al., 2013). Wave bioreactor is also disposable, but it can only hold up to 25L for each process due to its small size. This reusable bioreactor costs lower; it has a longer life cycle and flexibility in its accessory configuration and handling due to its rigid shape (Flickinger, 2010).

Heat exchanger units are majorly used to control the temperature of the bioreactor to maintain the temperature adequate for the fermentation process. Various heat exchanger units are commonly utilized, such as the cooling jacket, internal or external coils, and an external heat exchange unit with their advantages and disadvantages. However, this heat exchanger unit's usage is mainly used in a lab-scale bioreactor since it is easier for control and observation. A large-scale bioreactor has a lower heat transfer area per volume, which causes the heating from units, such as the cooling jacket, insufficient to control the temperature. Therefore, internal or external coils are usually used for a bigger-scale bioreactor (Flickinger, 2010). The heating unit used for the BV-IC expression system is generally attached together with the bioreactor, such as the one used in lab-scale experimentation, the Advanced Microscale Bioreactor (AMBR24c), a high-throughput microbioreactor that utilized additional cooling elements (Monteiro et al., 2016). Other bioreactors, such as the BioFlo® 310, utilize recirculated cooling water jackets (Lai et al., 2019).

B. Parameters and Controls

Medium. Before discovering a convenient Sf9 (*Spodoptera frugiperda*) culture medium, Grace's medium and TnMFH insect medium was used with fetal bovine serum (FBS). The TnMFH medium is the mixture of Grace's medium with lactalbumin and yeastolate (Palomares et al., 2015). López-Vidal et al. (2015) added 10% heat-inactivated fetal bovine serum and gentamicin 50 µg/ml alongside TnMFH medium, all produced from PAN Biotech GmbH, Germany. Nowadays, the Sf9 cell culture often uses Sf 900 II SFM and Sf-900 III SFM (Hattori et al., 2016; Thompson et al., 2015; Unger & Peleg, 2012). All of the studied Sf9 cell cultures are maintained and incubated at 27°C without CO₂. The acceptable temperature range for the growth of the Sf9 cells is 25-30°C.

Small-scale cultivation methods (e.g., shake flask and suspension culture) lack real-time monitoring. Therefore, a recent robust benchtop μ -bioreactor system called BioLector[®] was developed, allowing for a high-throughput lab-scale fermentation. It is assembled with continuous online monitoring of key parameters, such as temperature, pH, DO, and biomass concentration, via non-invasive probes. Strobl et al. (2020) conducted a study in VLP production using BEVS in Sf9 cells using BioLector[®] with a 1.7 mL working volume. They described BioLector[®] as a cost-effective, time-saving, and attractive alternative to the conventional shaker flask. Similarly, a study by Monteiro et al. (2016) also used a high-throughput microbioreactor, namely the Ambr model AMBR24c. It allows continuous online monitoring at a 10-15 mL volume. The installed cooling elements and additional air supply with a flow rate up to 0.9 mL/min keep the temperature at 27°C and maintain the DO percentage to not drop below 70%. Additionally, the high-throughput μ -bioreactors allow online monitoring of the cell density by backscattering and fluorescence detection.

Infection & Post-infection. Five main parameters that must be considered in Sf9 infection are incubation condition, dissolved oxygen, harvest time, the multiplicity of infection (MOI), and cell concentration at infection (CCI). These parameters are within the scope of Sf9 infection with BV to promote optimal conditions for high productivity and quality of the desired protein.

The infected Sf9 are incubated and maintained under 27°C without CO₂. The acceptable temperature range for the production of proteins is 25-27°C. Temperature plays a role in the BV titer, protein production, and glycosylation. The pH in the medium during the infection process was reported to be slightly higher than 6.5 to ease BV entry. However, no significant changes were reported on the pH until the end of production. It was also mentioned that Sf9 cells are less sensitive to pH change than HighFive insect cells (Contreras-Gómez et al., 2013; Drugmand et al., 2012; Palomares et al., 2015). Small-scale cultivation (e.g., shake flask and suspension culture) lacks online monitoring; hence the control of parameters relies on the incubator and offline measurement usage. The pH of suspension culture is maintained by the buffers included in the media and pH indicator (e.g., phenol red) typically added to monitor medium acidity. More quantitative measurements could be obtained from the phenol red absorbance spectrum using a standard curve as described by Michl, Park, & Swietach (2019).

It was observed that the oxygen uptake rate (OUR) increased after the infection of BV for the insect cell to generate energy via respiration, which led to the increase of dissolved carbon dioxide (dCO₂). A high amount of dCO₂ will lead to the slow production of the target protein due to the limited insect cell growth (Contreras-Gómez et al., 2013; Palomares et al., 2015). Moreover, the dissolved oxygen (DO) concentration during the cells' infection has become a crucial parameter to consider. It was reported that the cells were sensitive to DO concentrations, thus, resulting in an increase in the consumption rate of oxygen or OUR (Contreras-Gómez et al., 2013; Palomares et al., 2015). DO is often monitored offline by sample withdrawal and measured by a meter or probe. An additional oxygen supply will compensate for any decrease in the DO. The Sf9 cell culture may also be treated with DO ranging from 30% to 100%. It was reported as well by Sequeira et al. (2018) that insect cell maintenance does not rely on the amount of DO.

MOI is the number of infectious particles, the BV, involved in the VLP production. The MOI value indicates the amount of BV that will infect a single cell. A low MOI value indicates a lower BV concentration introduced to the initial phase of infection; the BV would then replicate and infect the insect cell rapidly and efficiently. The latter causes secondary infection since the replication of the introduced BV is used for insect cell transfection. A lower value of MOI will reduce the production of defective interfering particles (DIP) in the culture. Meanwhile, a high MOI limits the infection of BV since the total BV is higher than the total insect cells. (Palomares et al., 2015). The most-reported MOI values for the infection of Sf9 cells are 0.01, 0.1, and 1 (Hattori et al., 2016; Smith et al., 2015; Thompson et al., 2015; Unger & Peleg, 2012).

Several harvest durations are reported, with 72 hours as the most common applied time of harvest for BV-insect cell culture post-infection. The range set to harvest the culture is 48-120 hours, where

harvesting the culture 48 hours post-infection aims to recover the BV. The long hours of cell harvest aim to maximize the production of proteins, which correlates with the insect cell's mechanism known as the very late infection phase (Hattori et al., 2016; López-Vidal et al., 2015; Smith et al., 2015; Unger & Peleg, 2012).

CCI is one of the parameters that must be considered in maximizing the desired target proteins. The CCI indicates the initial cell concentration for infection to take place. Therefore, it is important to consider the correct value of both CCI and MOI during the vaccine production to prevent nutrient limitation and toxic production (Palomares et al., 2015). The most-reported CCI of Sf9 cells is 2106 cells/ml, considering the range between 1.8-3106 cells/ml (López-Vidal et al., 2015; Smith et al., 2015; Thompson et al., 2015; Unger & Peleg, 2012).

In summary, growth mediums, cell lines, parameters, and methodologies will give varying results. All variables must be considered, such as choosing the proper cell line to produce the target protein. Different cell lines have other capabilities; thus, cell lines will contribute significantly to the desired outcome. After choosing the cell lines, planning strategies for the parameters and growth mediums should optimize its growth and production. The findings found in common are that the incubation of Sf9 cells for growth before infection should be under 27°C and pH 6; limitation on the dCO₂ inside the medium since it will limit the cell's growth; and CCI 2,106 cells/ml. In contrast, the findings notice different reports on harvest time, MOI, and dissolved oxygen value.

As for the control, bioreactor scale cultivation requires automated, online monitoring to increase process efficiency, reduce operator-dependent errors, and allow real-time data analysis. Bioreactors are equipped with invasive temperature, pH, DO, and gas sensors with appropriate actuators. For example, a study by Lai et al. (2019) used a 5-L BioFlo® 310. The BioFlo® 310 is a bioreactor equipped with a thermowell for resistance temperature detector (RTD), a gel-filled pH sensor, and a polarographic DO probe. The temperature, pH, DO, and dCO₂ were controlled using a recirculated cooling water jacket and proportional & integral (P & I) controllers. P & I controllers arrange acid and base addition through two peristaltic pumps. In contrast, P & I controllers change the impeller speed, the flow rate of oxygen and carbon dioxide supply through the sparger. A similar control system for baculovirus-mediated expression for VLP production was also reported in a 2-L Biostat® B-DCU (Sequeira et al., 2018).

C. Product Yield, Product Titer, and Volumetric Productivity

The result of bioreactors can be measured, analyzed, and compared using performance assays. The assays used for bioreactor performance are product yield, product titer, and volumetric productivity.

Product yield measures how much production occurs for the amount of substrate used. This assay is essential when the substrates are prominent in the product's final cost. Some factors influence product yield and quality during the upstream process (Eibl et al., 2013). The factors include the following: (i) the recombinant BV itself, (ii) the passage number should be a maximum of five times for contamination prevention from defective interfering particles, (iii) maintaining genetic stability and limit variations by the sub-cultivation number is limited to 30-50 times, (iv) the insert cell line must also be monitored, (v) the medium's selection and additives must not hinder the virus-host interaction and (vi) the bioreactor with its bioengineering and production parameters. The most critical point of the production is the first generation of VLP after the infection, which usually requires more O₂ and is more susceptible to shear stress.

The product titer and volumetric productivity measure its concentration and production over time, respectively. The product titer is essential for the product purity and purification costs, while volumetric productivity shows the bioreactor's efficiency. Volumetric productivity is also necessary for production optimization and improvement in specific scales or possible future scale-up processes. Durous et al. (2019) suggest feeding strategies to increase VLP productivity, as adding antioxidants and cholesterol results in 6-fold productivity. In contrast, protein, vitamin, and lipid supplements after 72 hours result in a 4-fold increase of HA titer. Unfortunately, the suitability of these methods in insect cells is unknown and yet to be tested.

The data of these production performance assays are limited in availability, specifically in avian influenza VLP vaccine production. Novavax, as the only known avian influenza VLP vaccine producer, did not reveal their production data. Production data are available through the bioreactor data sheets used by Novavax, even though it is not specifically for the Novavax vaccine. Other found data are mostly small-scale and not specific to the avian influenza VLP vaccine. The maximum bioreactors concentration compares the single-use bioreactors' production with the batch, fed-batch, even the perfusion bioreactors. It can be seen in Table 1., and the comparison suggests that product titers of the single-use bioreactors barely equal the batch bioreactors specifically. It can also be seen that the perfusion bioreactors have the highest product titer. However, this particular bioreactor is not suitable for production using BV.

The comparison cannot describe the overall avian influenza VLP vaccine production with a BV-insect cell expression system due to the limited data. However, this comparison shows that single-use bioreactors can be the alternative to batch and fed-batch bioreactors.

Table 1. The Comparison of Product Yield, Product Titer, and Volumetric Productivity in Bioreactors

	Insect cells, vector, and product	Bioreactor description	Product Yield	Product Titer	Volumetric Productivity	Reference
Datasheet of the bioreactor used by Novavax	insect cell baculovirus no product	WAVE single-use bioreactor	Not available	Over 9×10^6 cells/mL - 1×10^7 cells/mL	Not available	GE Healthcare, 2011
Possible Scale-up Study	Hi5 cells baculovirus influenza VLP vaccine	5L benchtop bioreactor	Not available	Before infection: 2.4×10^6 cells/mL After infection: $\pm 1.7 - 2 \times 10^6$ cells/mL	Before infection: 3.3×10^5 cells/mL/h After infection: $\pm 2.4 - 2.8 \times 10^5$ cells/mL/h Time: 72 hours	Lai et al., 2019
Fast single-use VLP Production	Sf9 cells, baculovirus, influenza VLP vaccine	BIOSTAT CultiBag RM (single-use)	Not available	2×10^6 cells/mL	2.3×10^5 cells/mL/h Time: 88 hours	Eibl et al., 2013

Bioreactor Maximum Concentration (Review)	insect cells, baculovirus, protein	Batch, fed- batch, perfusion bioreactors	Not available	Batch: 2-8 x 10 ⁶ cfu. mL ⁻¹		
				Fed-batch: 50 x 10 ⁶ cfu. mL ⁻¹	Not found	Contreras- Gómez et al., 2013
				Perfusion: 55 x 10 ⁶ cfu. mL ⁻¹		

DOWNSTREAM BIOPROCESS

Downstream processing (DSP) is the recovery and purification process of fermentation products, which is essential for the commercialization process. The process starts by clarifying the fermentation product from a wide range of contaminants from the cell culture process, including cell debris, vesicles, baculovirus, host cell nucleic acids, and host-cell protein. Several critical points are located in the separation of exosomes and cell microvesicles, which have similar sizing to the VLP itself (Ladd Effio et al., 2015). Afterward, the separation process' outcome will be further concentrated through VLP primary isolation and removal of water. Lastly, purification of the concentrated VLP from contaminating chemicals is performed (Shuler, Kargi & DeLisa, 2017). The major challenges in DSP are the co-production of baculovirus, which has a similar size to VLP, causing the separation to be more complicated to be done. Aside from that, VLP purification has a low binding capacity, needs long processing time, low production yield with high heterogeneity, and low stability (Carvalho et al., 2018; Ladd Effio et al., 2015). Therefore, the DSP process design should cover the best balance between purity, cost, and process to obtain the wanted product. The processes should also be produced on an industrial scale with high quality and yield while keeping the cost most effective (Vicente et al., 2011). In the DSP of VLP by Novavax, several vital processes are repeated to obtain the highest quality VLP (Hahn et al., 2013). The same processes were also adapted by Ladd Effio et al. (2015), where several processes are repeated but with different purposes (Figure 1.). This paper will review different types of processes and available techniques to purify VLP based influenza vaccines to achieve the required quality for the vaccine. The methods include cell lysis, clarification, capturing and purification, polishing, and final result quality.

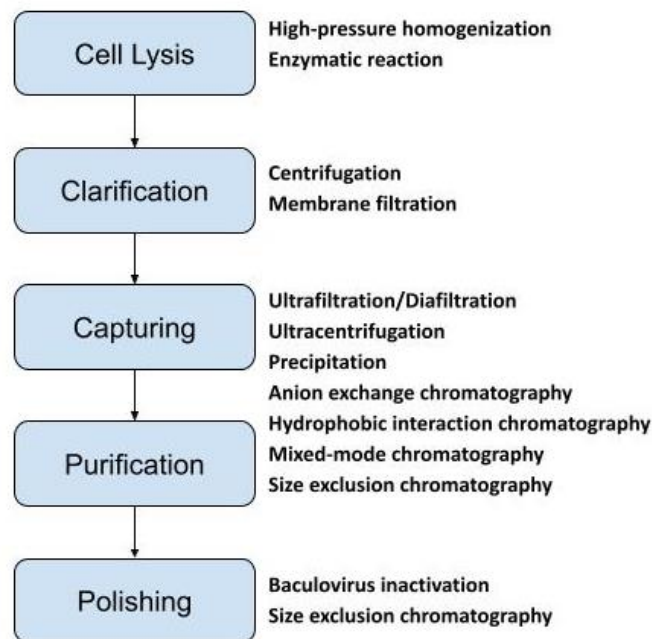


Figure 1. Downstream Processing Overview Adapted from Effio et al. (2015) & Hahn et al. (2013).

A. Cell Lysis

Cell disruption or cell lysis is necessary when the VLPs are less in the extracellular medium, resulting in the low VLP content. Large-scale cell lysis can be done by high-pressure homogenization or the addition of an endonuclease, Benzonase®, to digest the nucleic acids of the insect cells (Besnard et al., 2016; Durous et al., 2019; Effio & Hubbuch, 2015). It is also found by Besnard et al. (2016) that the addition of Benzonase® helps to solve the difficulty in the filtration step, which previously causes a loss of 30% of VLP. However, Meyer & Schmidhalter (2014) specify that the VLPs used for influenza vaccines do not require the cell lysis process. The natural budding and self-assembly of the VLPs processes that occur within the insect cell membrane eliminate the cell lysis process. At the same time, the nucleic acids will be removed in the clarification process. It can also be seen within the production line of the avian influenza VLP vaccine by Novavax that no cell lysis should be conducted (Hahn et al., 2013).

B. Clarification

The clarification process focuses on the separation of the solid-liquid particles within the cell culture broth, which resulted in the removal of unwanted biological materials, such as cells, cell debris, and large aggregates (Besnard et al., 2016; Durous et al., 2019; Effio & Hubbuch, 2015). In choosing the suitable clarification technique, there are criteria such as the following: (i) ease scalability, (ii) manufacture-friendly, and (iii) low cost of goods sold (Besnard et al., 2016; Carvalho et al., 2019b; Cherradi et al., 2018; Durous et al. 2019; Meyer & Schmidhalter, 2014). Specifically on the clarification process of viral vaccines, choosing the technique with high retention capacity and robustness is favorable. The high retention capacity will enable the separation of VLPs from the high nucleic content, while the technique's robustness helps to maintain the process performance against high feed variability (Cherradi et al., 2018).

Centrifugation. The most common method for clarification is centrifugation. Centrifugation has been used for decades due to its ability to perform for samples with high cell density (Besnard et al., 2016). The separation by centrifugation is based on size and density properties (Zhou et al., 2020). A high separation resolution and its straightforward process have become the advantage of centrifuge usage in industries. The centrifugation process allows the separation of the products and contaminants, resulting in cell pellets and supernatant (Mannon, 2012). The supernatant containing VLP will be further processed (Effio, 2016; Hahn et

al., 2013). However, in terms of scalability for large-scale production, as well as the trend of incorporating disposable technologies (i.e., cleaning free, single-use technologies), membrane filtration has become an alternative for centrifugation (Besnard et al., 2016; Durous et al., 2019; Effio & Hubbuch, 2015). Though its popularity within the industrial-scale clarification process, centrifugation has a significant disadvantage of causing physical shear stress (Durous et al., 2019; Effio, 2016).

Membrane filtration. The two most common membrane filtrations used in the clarification process are depth filtration utilizing the dead-end filtration (NFF) mode of action and tangential flow filtration (TFF). The separation by membrane filtration is solely based on particles' size (Zhou et al., 2020). For example, Novavax uses depth filtration for their influenza (H7N9) vaccine production. Depth filtration uses a porous filter medium that consists of multiple layers with descending pore size to entrap the unwanted biological materials. The usage of depth filtration in the industries is advantageous; it increases the efficiency of the sieving and particulate retention process due to the depth-dependent size separation and enables the removal of residual nucleic acids (Vedvick et al., 2013; Vicente et al., 2011). Additionally, depth filtration has the advantage of being easy to be scaled-up, low cost, and has a high capacity (Besnard et al., 2016; Meyer & Schmidhalter, 2014).

Occasionally, when centrifugation and depth filtration is used together in the clarification of viral vaccines, centrifugation is used to harvest the cell by separating the VLP and the broth medium, while the depth filtration process focuses on the removal of cell debris and contaminants (Hahn et al., 2013; Kis et al., 2019; Le Merdy, 2015). The most commonly used depth filtrations in the production of influenza VLP are Millistak+[®], Opticap[®], Polysep™ II, and Milligard[®] (Carvalho et al., 2018; Carvalho et al., 2019b; Cherradi et al., 2018; Le Merdy, 2015). The study by Carvalho et al. (2019b) suggests the usage of Millistak+[®] eases implementation in the clarification of influenza VLP. Le Merdy (2015) explains that the clarification by NFF, performed by Millistak+[®], works by adsorption mechanism, where it relies on the interactions, namely electrostatic attraction, hydrophobic interaction, and Van der Waals forces, between the filtration media and the particles. The adsorption mechanism is advantageous to remove small particles. Conversely, in VLP clarification, the adsorptive depth-filtration usage can cause a major yield loss (Besnard et al., 2016; Le Merdy, 2015; Merck Millipore, 2016). To overcome this problem, Novavax pre-treated the feed with the addition of salt, NaCl, to prevent VLP aggregations (Durous et al., 2019; Smith et al., 2013), as also suggested by Le Merdy (2015).

TFF is another alternative that provides high recovery yield and scalability (Durous et al., 2019). TFF has several advantages, such as low energy consumption, high membrane packing density, high solid loading, robustness, and is available in both single-use and reusable TFF (Besnard et al., 2016). TFF can be used as a membrane filtration with the combination of low-speed centrifugation to avoid the VLPs being captivated in the filter (Carvalho et al., 2019b). The TFF membrane ranges between 0.1–0.65 μm . Specifically, on the filtration of influenza VLP, the pore sizes of 0.2 μm or 0.45 μm are proven to be effective (Besnard et al., 2016). The parameters when operating clarification processes using TFF should be carefully planned since it inflicts high shear stress and results in VLP damage. Lastly, TFF is considered costly due to its expensive membrane modules and pumping equipment (Besnard et al., 2016; Carvalho et al., 2019b). Many reports suggest using open-channel TFF devices, flat sheet or hollow fibers, which reduce the shear stress (Besnard et al., 2016, Merck Millipore, 2016; Negrete et al., 2014; Schmidt & Wieschalka, 2017; Smith et al., 2013).

C. Capturing and Purification

The capturing and purification process is done to obtain concentrated VLP products and reduce a significant volume in the DSP while also removing most of the bulk impurities. The goal of capturing is to reduce the amount of material needed for further purification steps and save a significant investment required for the DSP equipment (Vicente et al., 2011). The purification goal is to remove all of the impurities and contaminants, such as baculovirus, nucleic acids, endotoxins, and proteins, from the VLP product before

it goes to the polishing step (Merck Millipore, 2016). Several ways to capture and purify VLP are ultrafiltration, ultracentrifugation, precipitation, and chromatography (Hahn et al., 2013; Wickramasinghe et al., 2010; Durous et al., 2019).

Ultrafiltration/Diafiltration. Ultrafiltration/Diafiltration (UF/DF) is the separation of low molecular weight solutes (permeates) such as salt, sugar, host cell proteins, and insoluble precipitates with the retained product, VLP. The permeates' loss volume is replaced with DI water to ensure constant volume (Shuler, Kargi & DeLisa, 2017; Hillebrandt et al., 2020). The UF/DF system could work in batches or continuously with a wash buffer that completely displaces leftover permeates and retentates. Influenza VLP usually utilizes cross-flow filtration (CFF) UF/DF devices with membrane pore size ranging from 300–1000 kDa MWCO membranes, with pore size varying from 0.1 to 0.2 μm , 0.1 m^2 effective filtration area and applied transmembrane pressure up to 1.1 bar on the retentate side valve (Merck Millipore, 2016; Eibl et al., 2013). The extraction process occurs when the desired concentration is achieved, followed by draining the CFF loop and recovery of the VLP (Moleirinho, 2015). Currently, developments on integrated processes such as CFF and ion-exchange chromatography for VLP capture and purification are highly researched. This process integration's main advantages are the product loss reduction, less precipitate re-dissolution time needed due to less compacted precipitates, and the increase of the yield's purification level. While the CFF method of operation is also preferable due to the high recovery yield in VLP capturing. It ensures turbulent flow along the membrane surface, which reduces fouling and concentration polarization while increasing its recovery rate (Hillebrandt et al., 2020). The most common ultrafiltration membranes used by various industries are the hollow fiber membrane, made from polyethersulfone (PES) or stabilized cellulose. The open-flow path membrane helps handle the virus particle gently, which keeps the integrity of the virus structure safe (Morenweiser, 2005). Research by Michalsky et al. (2010) shows that the PES membrane's utilization successfully attained 20 times concentration with 10 times reduction of liquid volume in the membrane with MWCO varying from 100 - 500 kDa. While another research utilizing hydrosart membrane with 100 kDa MWCO only increases the concentration level by 6 times. This shows that the polyethersulfone membrane can perform better and yield a higher VLP concentration than the hydrosart membrane (Aucoin, Mena & Kamen, 2010).

The advantages of utilizing UF/DF separation are that the process's operation is cheaper than centrifugation on an industrial scale, safe for the product integrity, easy to be operated, and energy-efficient. It also operates under mild conditions that do not require additional requirements for the industry to be done. While it has various advantages, membrane fouling remains a problem. In addition, since separation is based on size, larger MW transduction inhibitors become concentrated with the viral particles, which reduces the viral transduction and infectivity efficiencies due to shear force inflicted on the virus envelope (Morenweiser, 2005).

Ultracentrifugation. Ultracentrifugation is the well-studied standard for influenza virus capture and purification since the production of egg-based vaccines. Utilizing various reagents such as sucrose, iodixanol, and cesium chloride (CsCl) allows the culture to be concentrated and separated based on the density gradient. However, research shows that the utilization of CsCl causes heterogeneity in VLP size due to broken particles. This may cause impurities and complications in the DSP due to storage aggregation and functionality reduction. While so, utilizing sucrose to separate the product is tedious and inefficient as it needs different layering and concentration levels of sucrose, and analysis must be done repeatedly. It also needs further DSP to remove the sucrose through dialysis (Merck Millipore, 2016). Therefore, a good VLP volumetric concentration factor could be achieved. However, several side impacts were questioned, including the scalability and variability issues, physical degradation caused by excessive mechanical and osmotic stress, and low yield (Durous et al., 2019; Vicente et al., 2011).

Precipitation. This process is commonly coupled with bind-and-elute chromatography, which aims to reduce the bulk volume of the product. There are two types of common precipitants for high MW products:

polyethylene glycol (PEG) and ammonium sulfate. Precipitation utilizing PEG precipitant has been reported to obtain a 1.6 purification factor for VLP with a product recovery of 90%. The efficiency and high selectivity rate for VLP are associated with the hydrodynamic radius of the PEG with VLP itself. However, drawbacks from utilizing precipitation include the co-precipitation of other large biomolecules in the product, for example, baculoviruses and nucleic acid. Batch processes for precipitation could also risk variety results, irreversible aggregation, and product alterations between every batch. Precipitation also increases the product's viscosity, impairing the subsequent processing technique (Hillebrandt, 2020; Hutchins, 2000).

Chromatography. Chromatography is a process of purification based on size or affinity characteristics. It is a scalable purification method to separate VLP from bulk contaminants. The stationary phase or adsorbents of a chromatography system can be monolith-, membrane- or bead-based. For large and fragile particles like VLP, both monolith and membrane adsorbents were proven to exhibit lower pressure and better dynamic binding capacity (DBC) than traditional bead-based adsorbents (Durous et al., 2019). However, scaling-up could be difficult due to their highly exothermic polymerization process, resulting in an inhomogeneous structure (Merck Millipore, 2016).

The most common chromatography used for influenza VLP is the ion-exchange chromatography, specifically anion-exchange chromatography (AEC) (Effio & Hubbuch, 2015; Durous et al., 2019; Merck Millipore, 2016). AEC is a widespread purification method for influenza VLP. However, the AEC system has a problem dealing with larger particles since it will reduce mass diffusion in column loading. This problem can reduce efficiency and limit the column's dynamic binding capacity (Merck Millipore, 2016). AEC has been implemented in both flow-through and bind-and-elute methods. The flow-through method setup works by the adsorbents binding to the BV, nucleic acids, or other contaminants while the VLP in the mobile phase flows through the column (Hahn et al., 2013). The VLPs are recovered at a high ionic strength in the flow-through after the chromatography process, while contaminants in the stationary phase are removed. Novavax also uses this method with the utilization of Capto Q as the stationary phase. The advantage of the flow-through method is the suitability for the lipid-enveloped and less stable products such as VLP, where there is less risk towards product alteration. However, this method requires additional concentration steps, for example, precipitation, when dealing with large volumes (Effio & Hubbuch, 2015). This method recovers VLP up to 40% and 70% for pilot and laboratory-scale, respectively (Effio & Hubbuch, 2015; Merck Millipore, 2016).

The bind-and-elute method setup works by binding the VLPs and washing the column from impurities before eluting the VLPs. This method is usually set up in a two-way process with precipitation using PEG (Effio & Hubbuch, 2015). Some studies report that the purity level is above 95% without any recovery value mentioned. (Koho et al., 2012; Koho et al., 2014). However, the disadvantage of the bind-and-elute method is that it has low dynamic binding capacities and diffusion limitations (Hillebrandt et al., 2020).

The standard stationary phases used in AEC are Fractogel TMAE, Fractogel DMAE, and Eshmuno Q, which shows a significant reduction in DNA and endotoxin levels (Merck Millipore, 2016). The elution process is done with the addition of buffer solutions to the VLP product as the mobile phase, for example, phosphate, Tris-HCl, MOPS, HEPES, and sucrose (Merck Millipore, 2016).

Other chromatography types that can be used to purify VLPs are hydrophobic interaction chromatography (HIC), mixed-mode chromatography (MMC), and size exclusion chromatography (SEC). HIC is based on hydrophobicity differences, which work by bind-and-elute mode. HIC process requires an ideal salt concentration during its binding stage with the target to enhance the interaction between them, but it must have low adsorption towards the impurities (Li et al., 2018). HIC is usually done after the purification using ion-exchange chromatography, as it will bind to the leftover contaminants (Effio & Hubbuch, 2015). However, the addition of the HIC process could prove to add more cost for the purification method. MM chromatography method utilizes both size exclusion and separation using the flow-through binding mode. MM chromatography prevents VLPs, as the larger molecule, from entering and interacting with the beads. In

contrast, the smaller contaminants flow through and are trapped in an adsorptive core (Merck Millipore, 2016). Currently, there are limited reports regarding the usage of MM chromatography for influenza VLP. Lastly, SEC is used as an alternative when the VLP and its impurities have similarities in the electrostatic phase but significant differences in size (Vicente et al., 2011). However, SEC is more commonly used in the polishing steps of the VLP (Pincus et al., 2010).

D. Polishing

Polishing aims to further remove impurities, including the host cell DNA and BV recombinants formed during the infection process to obtain clinical-grade VLP. Polishing could be done by inactivating the BV, increasing the purity level of the product through SEC and sterile filtration.,

BV Inactivation. BV can induce adverse synergistic immunological reactions if not removed (Vicente et al., 2011). There are approximately 10^{10-12} BV particles present in a typical BEVS system. Therefore, BV has to be inactivated prior to the removal by using formalin or β -Propiolactone (BPL) followed by selective precipitation and chromatography (either bind-elute or flow-through chromatography). The treatment involves incubation with 0.1-0.2% BPL for 3 hours on ice to eliminate residual live BV. The BPL will then be removed through incubation at 36-38°C for 1-2 hours (Pushko et al., 2018). For the chromatography process, a study by Silva et al. (2020) has demonstrated that flow through chromatography (FTC) provides a higher recovery yield (66%) compared to bind-elute chromatography (37%).

Size-exclusion chromatography. When recombinant baculoviruses and VLP have sizes with a different order of magnitude, SEC can be used as a preparative approach for VLP polishing. While conventional SEC has been widely used in downstream processing for protein purification, a novel technology for multimodal SEC (mmSEC), Capto™ Core 400/700, has been developed for significant impurities reduction. Capto™ Core 400/700 comes with core bead technology and multimodal ligand, which allows separation based on size and binding at the same time. As a result, mmSEC provides the higher recovered mass and yield, purity, and low A260/A280, together with high productivity (Hillebrandt, Vormittag, Bluthardt, Dietrich, & Hubbuch, 2020).

Sterile filtration. After VLPs final formulation is achieved, sterile filtration is required before the vialing process. Sterile filtration of VLPs typically uses a 0.22 μ m filter. Therefore, sterile products that ensure patient safety can be obtained. Although, in general, the VLPs particle size is lower than 0.22 μ m, the selection of the membrane material can affect the result of VLP recovery. For example, when PES and polyvinylidene fluoride (PVDF) were compared in a sterile filtration experiment, both exhibited complete recovery and had similar total protein removal (PES: 6.8% and PVDF: 8.7%). However, PES showed a baculovirus log reduction value higher than 2, which makes it superior to the PVDF (Carvalho et al., 2019a). Moreover, the selection of low adsorption or inert membrane should be considered to minimize product losses due to non-specific binding (Vicente et al., 2011).

E. End Result Quality

VLP vaccines are known to elicit a more specific immune response than whole inactivated vaccines due to the role of genetic engineering in the VLPs development for improved immunogenicity (Zepeda-Cervantes, Ramírez-Jarquín & Vaca, 2020). The final product's activity and particle integrity are essential in obtaining purified influenza-VLP, which determines the vaccine's safety, efficacy, and potency. Based on the requirement given by USDA and WHO, the purity of human VLP-based vaccines is required to have protein purities above 95% with its DNA concentrations below 10 ng per dose (Ladd Effio et al., 2015).

In the large-scale production of avian influenza VLP by Novavax, the drug substance is stored at 2–8°C after the sterile filtration process. After the HA content is measured, the drug substance is diluted to the target dosage. Dilution is typically done using a mixture of different antigens, pharmaceutical excipients (e.g., buffer solutions, stabilizers, preservatives, and detergent), and adjuvant. The product is further processed by

mixing in a closed-system mixer. Finally, the resulting solutions should be aseptically transferred into glass vials to produce the end-product. Novavax had successfully implemented a GMP in its avian influenza VLP manufacturing, which expresses appropriate levels of HA, NA, and M1. In addition, certificates of analysis and quality control (QC) testing are necessary to ensure that the product displays the adjuvant immune response in a non-infectious form (Hahn et al., 2013).

FURTHER DIRECTION AND GAP ANALYSIS

Currently, there are no review papers that explicitly compare techniques commonly used by different industries in conducting BV-IC expression systems for VLP production. This review paper compares various types of medium, bioreactors, control, and parameters in the upstream processes and different techniques utilized for downstream processes.

It is highly suggested that improvements are to be made specifically in the downstream processing, where the purification method is still lacking in yield, stability, and quality. This is due to the limited reports for a detailed bioprocessing system for the BEVS-IC-based VLP production method, including its production amount data and specifically on the downstream processes. In addition, there are also numerous varieties in the DSP, both at the laboratory and industrial scale, which are not very well-described. Therefore, more studies and research for the VLP production DSP are needed to establish a satisfactory bioprocessing system with the desired yield and quality for commercialization.

CONCLUSION

The upstream and downstream processing of the avian influenza VLP vaccine utilizing the BV-insect cell expression system were thoroughly investigated. Utilizing VLP as an alternative method for influenza vaccine provides better cost-effectiveness and faster and safer methods compared to the conventional ones. The upstream processing is found to be most effective when the insect cells are grown at a single-use suspension-based bioreactor while also applying an optimized feeding strategy to improve the volumetric productivity and titer. While for the downstream processing, several processes, including clarification, centrifugation, and membrane filtration, could be done to clarify the product. The process is followed by capturing and concentrating through ultrafiltration/diafiltration, ultracentrifugation, precipitation, or chromatography. Lastly, the polishing step, including BV inactivation, size-exclusion chromatography, and sterile filtration, are done. The VLP vaccine is studied to have improved immunogenic quality, enabling a more specific immune response than other vaccines. Future studies on the efficacy and immunogenicity of the avian influenza vaccine expressed by the BV-IC expression system could be done to see its potential for further development.

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APPENDIX

Appendix 1. List of abbreviations

Abbreviation	Meaning
AEC	anion-exchange chromatography
BPL	β -Propiolactone
BV	baculovirus
BV-IC	baculovirus-insect cell
CCI	cell concentration at infection
CFF	cross-flow filtration
CIP	cleaning-in-place
COP	cleaned-out-of-place
DBC	dynamic binding capacity
dCO ₂	dissolved carbon dioxide
DIP	defective interfering particles
DO	dissolved oxygen
DSP	downstream processing
FBS	fetal bovine serum
FTC	flow through chromatography
HA	hemagglutinins
HIC	hydrophobic interaction chromatography
M1	matrix 1 protein
MMC	mixed-mode chromatography
mmSEC	multimodal size exclusion chromatography
MOI	multiplicity of infection
NA	neuraminidase
NFF	dead-end filtration/ normal flow filtration
OB	occlusion bodies

OUR	oxygen uptake rate
P&I	proportional & integral
PES	polyethersulfone
PVDF	polyvinylidene fluoride
QC	quality control
RTD	resistance temperature detector
SEC	size exclusion chromatography
SUB	single-use bioreactor
TFF	tangential flow filtration
UF/DF	ultrafiltration/diafiltration
VLP	virus-like protein
