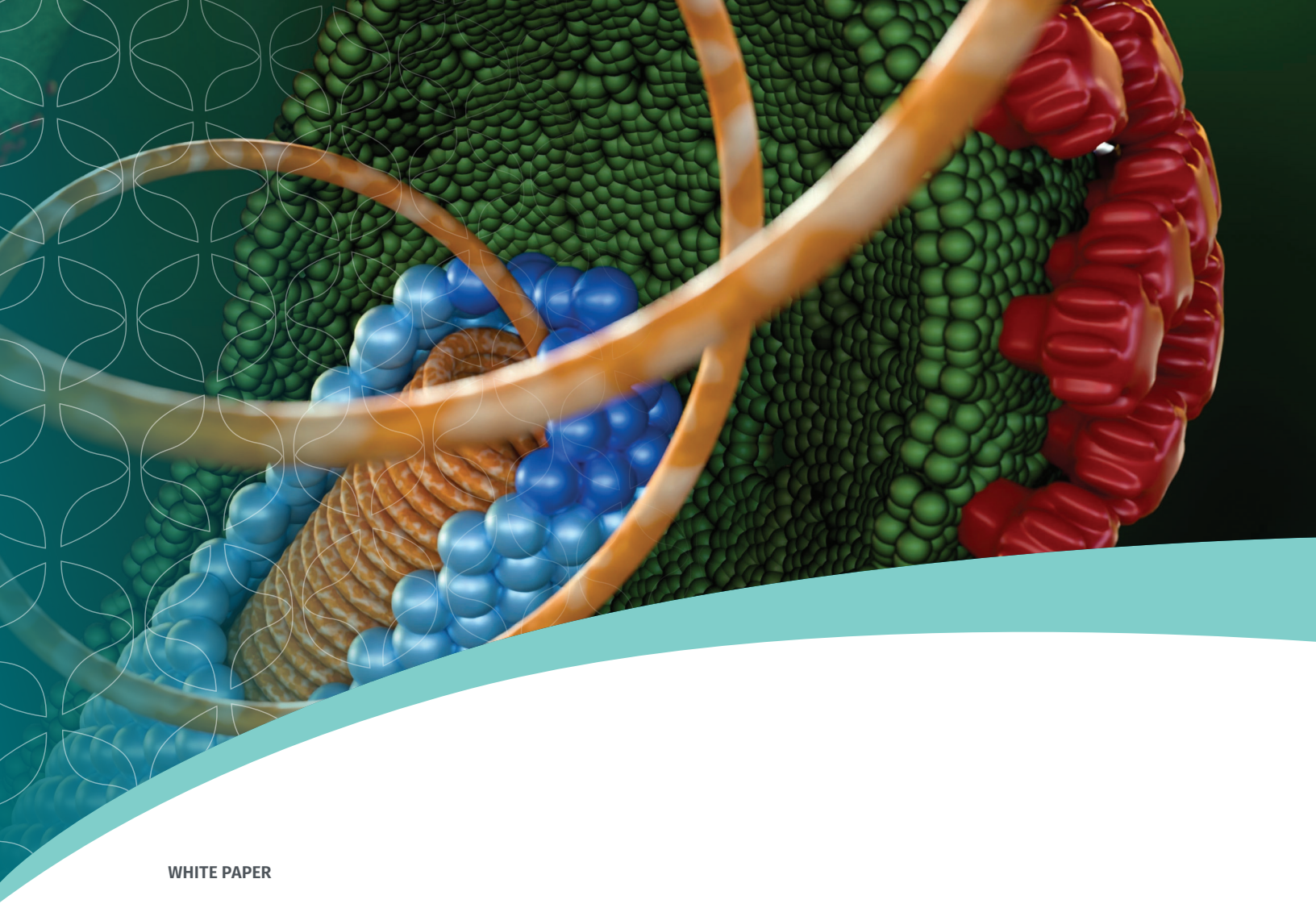

WHITE PAPER

Improved Cell Specific and Volumetric Product Titers in Insect Cells Using ESF AdvanCD™ Medium

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Abstract: ESF AdvanCD™ chemically defined insect cell culture medium from Expression Systems has proven to be highly effective to increase the production of baculovirus vectors, model proteins, and model virus-like particles. In this work, we compared traditional / recommended workflows in both Gibco™ Sf-900™ III SFM and ESF AdvanCD media. ESF AdvanCD media allowed greater per cell productivity and higher volumetric cell densities resulting in significant increases in total volumetric productivity. Notably, these improvements in yield were seen by following standard protocols rather than by extensive process optimization.

Keywords: Spodoptera frugiperda (Sf) 9 cells; cell growth; baculovirus expression vector system (BEVS); infection; high cell density; green fluorescent protein (GFP); mAzamiGreen (mAG) fluorescent protein; mKusabira-Orange-kappa (mKOκ) fluorescent protein; Gag; virus-like particle (VLP)

1. Introduction

This work investigated the utilization of two cell line and growth medium combinations: (i) *Spodoptera frugiperda* clonal isolate 9 (Sf9) (ATCC CRL-1711) cultured in the serum-free medium Gibco™ Sf-900™ III SFM (Thermo Fisher Scientific, Waltham, MA, USA) and Sf9 insect cells obtained from Expression Systems and cultured in the chemically defined medium ESF AdvanCD (Expression Systems, Davis, CA, USA). These two conditions will be referred to as (i) Sf9 in Sf-900 III SFM and (ii) ES-Sf9 in AdvanCD, respectively. Three model baculovirus vectors were used to evaluate expression capacity, two fluorescent proteins - one under a conventional p10 promoter, the other using the p6.9 promoter (Bruder and Aucoin, 2022); and one virus-like particle (Bruder and Aucoin, 2023).

2. Experimental

2.1 Cell Growth and Viability

Cell growth and viability were assessed for ES-Sf9 in AdvanCD and Sf9 in Sf-900 III SFM. Duplicate (biological replicates) 125 ml glass Erlenmeyer flasks were seeded with exponentially growing cells at a density of 0.5 x 10^6 cells/ml (working volume 35 ml). Flasks were shaken on an orbital shaker set to 130 rpm and incubated at 27°C. Cells were sampled daily and density and viability were assessed via the Trypan blue exclusion method with a Countess™ II Automated Cell Counter (Life Technologies; Thermo Fisher Scientific). Duplicate (technical replicates) cell counts and viability measurements were performed for each sample and averaged. Figure 1 shows the growth and viability curves.

The exponential growth rate for ES-Sf9 cells grown in AdvanCD was calculated as 0.0343 h^{-1} which corresponds to a doubling time of approximately 20.2 h. This is considerably faster growth as compared to Sf9 in Sf-900 III which exhibited an exponential growth rate of 0.0244 h^{-1}, corresponding to a doubling time of approximately 28.4 h. Similarly, the maximum viable cell density of ES-Sf9 in AdvanCD under these experimental conditions was found to be approximately 3.39 x 10^7 cells/ml while the maximum viable cell density of Sf9 in Sf-900 III SFM was approximately 1.42 x 10^7 cells/ml. Cell viability for both conditions was high (>97%) throughout exponential growth. Viability drop occurred earlier for ES-Sf9 in AdvanCD, with significant viability reduction observed after 250 h of culture, although this time is outside the range of normal batch culture utilization. ES-Sf9 in AdvanCD show robust and sustained exponential growth and are able to reach a higher maximum cell density compared to Sf9 cells grown in Sf-900 III SFM.

Adaptation of Sf9 (ATCC CRL-1711) to AdvanCD and ES-Sf9 to Sf-900 III SFM Media. A typical protocol was used to transition each cell line to the other medium in the study i.e., ES-Sf9 transition to Sf-900 III SFM and Sf9 transition to AdvanCD. In a first passage the amount of 'new' medium was added at 25% v/v, followed by 50, 75 and then 100% in subsequent passages. For passaging, cells were seeded between 0.4-0.6 x 10^6 cells/ml and allowed to grow to 2-4 x 10^6 cells/ml over 72-96 h. Although both sets of cells adapted well to the new media, the growth rate of both combinations converged. The new doubling time for both cell-medium combinations was ~25 hours.

2.2 Baculovirus Vector Stock Amplification

Autographa californica Multiple Nucleopolyhedrovirus (AcMNPV) baculovirus vector stock solutions were generated by infecting Sf9 cells in Sf-900 III and ES-Sf9 cells in AdvanCD at a multiplicity of infection (MOI) of 0.1.

Exponentially growing cells were seeded at either 1 x 10^6 or 2.5 x 10^6 cells/ml in 125 ml glass Erlenmeyer flasks (35 ml working volume) shaken at 130 rpm and incubated overnight at 27°C prior to infection. For the purpose of this section, flasks seeded at 1 x 10^6 and 2.5 x 10^6 cells/ml will be referred to as low and high density, respectively. Prior to infection, cells were counted and the proper volume of virus stock to add was determined. Cells were infected with passage 2 (p2) recombinant baculovirus expressing the fluorescent protein mAzamiGreen (mAG) under the control of the *Ac*MNPV p6.9 promoter (Bruder et al., 2021). The expression of mAG allows for easy infectious viral titer quantification via an end-point dilution assay (EPDA). The average cell densities at the time of infection were 1.4 x 10^6 and 3.8 x 10^6 cells/ml

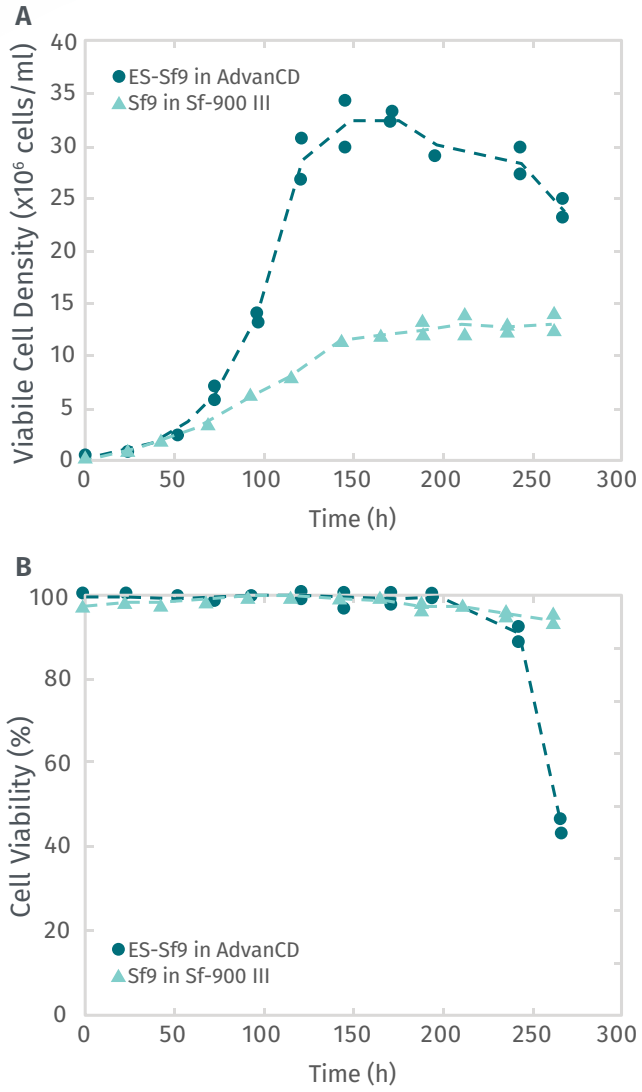

Fig. 1. Cell growth (A) and viability (B) over time for ES-Sf9 cells grown in AdvanCD medium and Sf9 cells grown in Sf-900 III. N=2; all points shown on plot. Dashed lines represent average at each time point.

for low and high seeding density of Sf9 in Sf-900 III, respectively, and 2.3 x 10^6 and 5.5 x 10^6 cells/ml for low and high seeding density of ES-Sf9 in AdvanCD, respectively. Infected cultures were harvested 72 hours post-infection (hpi). Culture supernatant was centrifuged at 800 x *g* for 10 min at 4°C to remove cells and debris. Clarified supernatant was then filtered utilizing a 0.22 µm polyethersulfone (PES) membrane filter and stored at 4°C until analysis. Infectious virus titer (IVT) was measured utilizing an EPDA protocol. Briefly, Sf9 cells in Sf-900 III SFM were grown adherently in 96-well plates and were inoculated with serial ten-fold dilutions of baculovirus stock samples. After a 7-day incubation in a humidified box at 27°C, wells were scored as either positive or negative for baculovirus based on the presence or absence of green fluorescence as determined by examination of each well under a fluorescent microscope. Total baculovirus particles (i.e., infectious and defective particles) were quantified utilizing SYBR green DNA staining analyzed via flow cytometry (Shen et al., 2002) using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, Mississauga, ON, Canada). Figure 2 shows IVT and total baculovirus particle concentrations for baculovirus stocks produced in Sf9 cells in Sf-900 III SFM and ES-Sf9 cells in AdvanCD at the infection densities studied.

For the conditions studied, ES-Sf9 cells seeded at high density in AdvanCD led to the highest IVT and total viral particle concentrations. All conditions were harvested 72 h after infection. Average cell viability at the time of harvest was approximately 73% and 80% for low and high seeding density Sf9 in Sf-900 III SFM, respectively, and 90% and 93% for low and high seeding

density ES-Sf9 in AdvanCD, respectively. To account for differences in cell density between the conditions, IVT per total cells (i.e., viable plus non-viable) at the time of harvest was calculated. Average IVT/cell was approximately 88±20 and 123±50 for low and high seeding density Sf9 in Sf-900 III SFM, respectively. Average IVT/cell was approximately 102±50 and 426±223 for low and high seeding density ES-Sf9 in AdvanCD, respectively. ES-Sf9 in AdvanCD cultures seeded at 2.5 x 10^6 cells/ml and infected after overnight culture showed the highest IVT and per cell productivity of all the conditions studied.

2.3 Fluorescent Protein Production

Protein production in Sf9 cells in Sf-900 III SFM and ES-Sf9 cells in AdvanCD was compared over time utilizing two recombinant baculovirus vectors (rBV) expressing fluorescent reporter proteins. Exponentially growing cells at a density of 2 x 10^6 cells/ml were infected at an MOI of 5 and incubated at 27°C in flasks on an orbital shaker set to 130 rpm. Cells were infected with p2 stocks of either: (i) rBV expressing mKusabira-Orange-kappa (mKOκ) under the control of the very late p10 promoter (Walji et al., 2021) or (ii) rBV expressing mAzamiGreen (mAG) under the control of the late p6.9 promoter (Bruder et al., 2021). Flasks were sampled daily, and cell

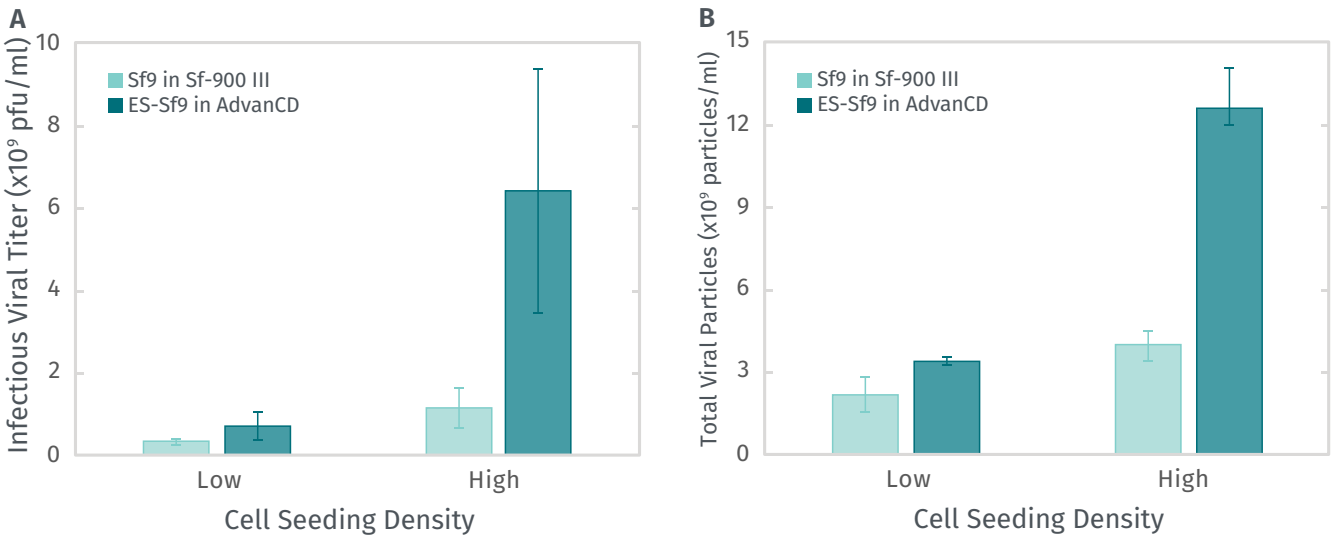

Fig. 2. (A) Infectious baculovirus titers in plaque forming units (pfu) per ml for recombinant baculovirus stocks produced under the studied conditions. Infectious baculovirus titers were quantified based on the production of mAG in infected cells and determined via EPDA. (B) Total baculovirus particle concentration in particles per ml for recombinant baculovirus stocks produced under the studied conditions. Total baculovirus particle concentrations were determined based on SYBR green staining of viral genomes quantified via flow cytometry. All conditions were tested in triplicate (biological replicates) and error bars represent +/- standard deviation. Low and High seeding density refers to cells seeded at 1 x 10^6 and 2.5 x 10^6 cells/ml, respectively. Cells were infected after overnight incubation.

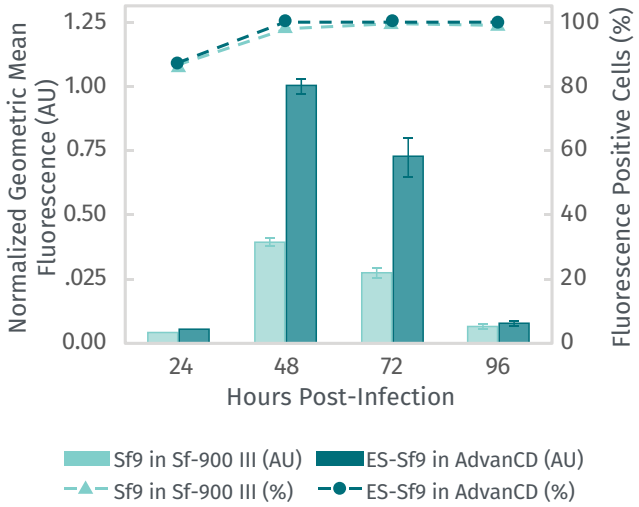

Fig. 3. Normalized geometric mean fluorescence intensity in arbitrary units (AU) and percentage of fluorescent-positive cells for Sf9 cells in Sf-900 III SFM and ES-Sf9 cells in AdvanCD infected with a rBV expressing mKOκ under the control of the p10 viral promoter over time. Each condition was run in triplicate (biological replicates) and error bars represent +/- standard deviation.

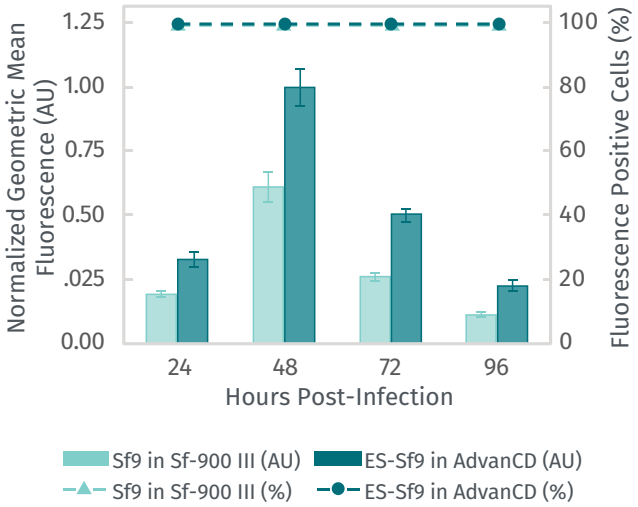

Fig. 4. Normalized geometric mean fluorescence intensity in arbitrary units (AU) and percentage of fluorescent positive cells for Sf9 cells in Sf-900 III SFM and ES-Sf9 cells in AdvanCD infected with a rBV expressing mAG under the control of the p6.9 viral promoter over time. Each condition was run in triplicate (biological replicates) and error bars represent +/- standard deviation.

viability, density, and fluorescence intensity were assessed. Cell fluorescence was quantified via flow cytometry using an Accuri™ C6 plus flow cytometer (BD Biosciences). Fluorescence of cells infected with the mKOκ - expressing rBV was measured utilizing a 533/30 nm filter and fluorescence of cells infected with the mAG - expressing rBV was measured utilizing a 510/15 nm filter. Figures 3 and 4 show the relative fluorescence intensity of both cell lines infected with both rBVs over time.

ES-Sf9 in AdvanCD produced more fluorescence per cell than Sf9 in Sf-900 III SFM in both the mKOκ and mAG reporter systems. In the case of both reporter proteins, peak fluorescence occurred at 48 hpi. Throughout the time course, the percentage of fluorescent-positive cells was highly similar between Sf9 in Sf-900 III and ES-Sf9 in AdvanCD.

2.4 Virus-Like Particle Production

Virus-like particles (VLPs) were produced by infecting either Sf9 in Sf-900 III SFM or ES-Sf9 in AdvanCD with a recombinant baculovirus expressing the HIV-1 Gag polyprotein fused to enhanced green fluorescent protein (eGFP) (Hermida-Matsumoto and Resh, 2000) under the late baculovirus p6.9

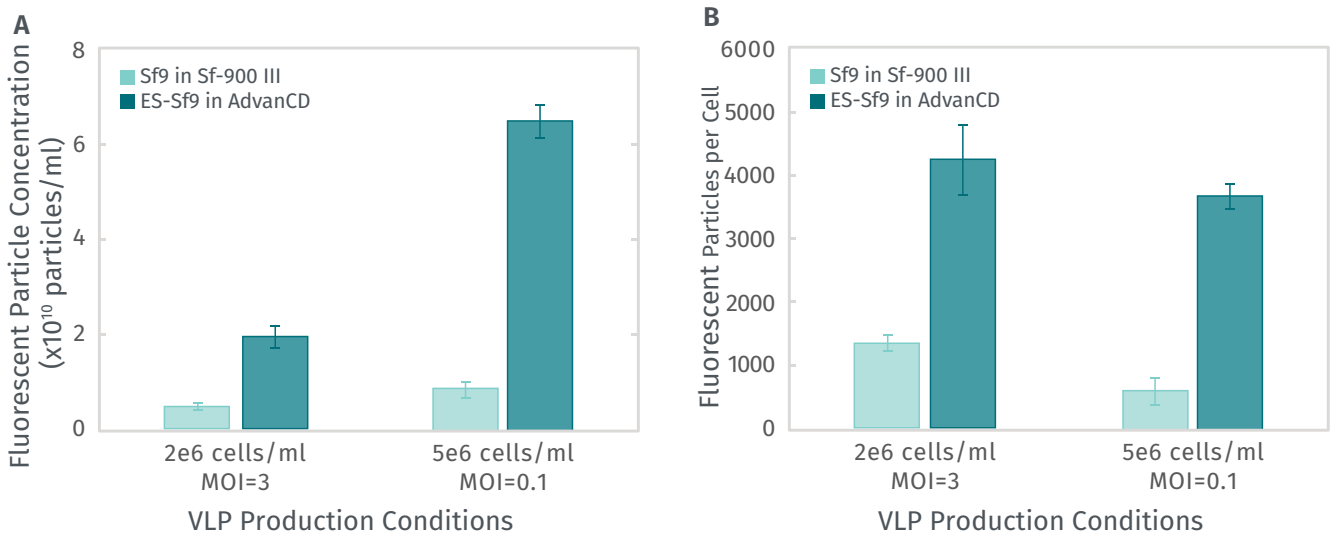

Fig. 5. A) Comparison of Gag-eGFP VLP production between Sf9 in Sf-900 III SFM and ES-Sf9 in AdvanCD under two production regimes. Regime (i) corresponds to cells at a density of 2 x 10^6 cells/ml infected at an MOI of 3 and regime (ii) corresponds to cells at a density of 5 x 10^6 cells/ml infected at an MOI of 0.1. Error bars represent +/- standard deviation. B) Comparison of per cell productivity of Gag-eGFP VLPs between Sf9 in Sf-900 III SFM and ES Sf9 in AdvanCD under the same production regimes. Fluorescent particles/cell represents the ratio of Gag-eGFP VLPs quantified via flow cytometry to the total number of cells at the time of harvest.

promoter (Bruder and Aucoin, 2023). Exponentially growing cells were seeded into glass Erlenmeyer shake flasks and incubated at 27°C overnight on an orbital shaker set to 130 rpm.

To explore a cross-section of VLP production conditions while minimizing the number of experiments, cells were either infected at a density of 2 x 10^6 cells/ml at an MOI of 3 or at a density of 5 x 10^6 cells/ml at an MOI of 0.1. The former infection conditions are representative of a typical shaker flask protocol while the latter aimed to investigate VLP production at high cell density. After infection, cell viability was monitored, and the cultures were harvested when cell viability reached 75-85%. This harvest

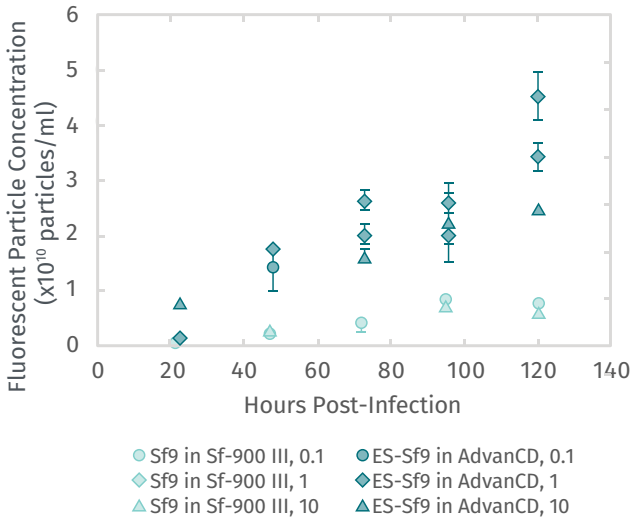

Fig. 6. Gag-eGFP VLP concentration over time for Sf9 in Sf-900 III and ES-Sf9 in AdvanCD cells infected at a cell density of 2 x 10^6 cells/ml and MOI 0.1, 1, and 10. N=2; error bars represent the range of the data.

point corresponded to 72 hpi for cells infected at an MOI of 3 and 92 hpi for cells infected at an MOI of 0.1. VLP solutions were harvested by centrifugation of culture supernatant at 800 x *g* for 10 min at 4°C, followed by filtration with 0.22 µm PES filters. All VLP solutions were stored at 4°C until analysis. VLP concentration was subsequently quantified via flow cytometry where the presence of eGFP allowed for discrimination between VLPs and other particles present (e.g., baculovirus). Gating of flow cytometric data was performed utilizing a non-fluorescent negative control (Gag VLP solution produced utilizing an rBV expressing the HIV-1 Gag polyprotein under the p6.9 promoter (Bruder and Aucoin, 2023) with conditions mirroring those studied here) and quantification was performed utilizing Flow Set Fluorospheres (Beckman Coulter Life Sciences, Mississauga, ON, Canada) run under the same conditions at known concentrations. Gag-eGFP VLP concentration and Gag-eGFP VLPs per cell are shown in Figure 5.

Gag-eGFP VLP production is significantly higher in ES-Sf9 in AdvanCD cells for all conditions. Per cell productivity is also significantly higher. Furthermore, at high cell density, the productivity relative to low cell density for ES-Sf9 in AdvanCD cells is 86%, while the productivity relative to low cell density of Sf9 in Sf-900 III cells drops significantly to 45%, suggesting a lesser cell density effect for ES-Sf9 in AdvanCD.

To further explore the production of VLPs, a time course experiment was performed where Sf9 in Sf-900 III SFM and ES- Sf9 in AdvanCD were infected at a density of 2 x 10^6 cells/ml at MOI 0.1, 1, and 10. Gag-eGFP concentration was monitored via flow cytometry as described above (Figure 6). At 72 hpi, the concentration of VLPs produced in ES-Sf9 in AdvanCD when infected at 2 x 10^6 cells/ml with an MOI of 0.1 (Figure 6) is just under half of what was produced at 72 hpi when 5 x 10^6 cells/ml were infected with an MOI of 0.1 (Figure 5). Finally, it can be seen that at 120 hpi, the ES-Sf9 in AdvanCD culture infected at 2 x 10^6 cells/ml with an MOI of 0.1 (Figure 6) was approaching the concentration of VLPs produced in 72 hpi when the culture was infected at 5 x 10^6 cells/ml with the same MOI (Figure 5).

3. Discussion

The number of permutations that can be used to produce recombinant products from a baculovirus expression vector - insect cell system is extremely large; however, the goal should always be to make the most high-quality product as possible. While MOI and time of infection are key factors, much of the thought process around these variables is how to ensure that the cells will have enough nutrients throughout the production process. Here we investigated two media formulations, Sf-900 III SFM and AdvanCD. In all conditions examined, expression in AdvanCD yielded significantly higher levels of product – whether it be baculovirus vectors, model fluorescent proteins, or a model VLP.

It should be noted, however, that increases in production often come at a cost. This is especially true for virus systems where a cell density effect is often observed: a significant decrease in cell specific production as the cell density in the culture increases. In this work, the per cell productivity of ES-Sf9 in AdvanCD remained remarkably high at high cell density (86% of the productivity of the low-density culture) together with the ability to achieve higher cells densities, the ES-Sf9 in AdvanCD system significantly increased overall production.

In this work – simply done in shake flasks – the possibility of pushing the ES-Sf9 in AdvanCD even further in a controlled bioreactor using additional fed-batch or perfusion feeding schemes, has not been explored; however, it can be expected that the benefits be easily translated. Although model products were studied, the two fluorescent proteins can be taken as representative intracellular products while the VLP can be taken as a representative secreted product. One class of product that has not been studied in this work is that of membrane-associated proteins, an example of which is the influenza hemagglutinin (HA) proteins; however, all evidence points to increased production with the ES-Sf9 AdvanCD system.

Acknowledgements

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Notes

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