Cell Culture Media Optimization for Increased Production of Recombinant Proteins in Insect Cells

Guenaele Raphael*, R. Logan*, and J.F. Treml*†

The insect cell/baculovirus expression system (IC-BEVS) has been at the forefront of biotechnological research, and has served as a tool to produce several human therapeutics such as Cervavix ® (preventative vaccine against the human papilloma Virus), Flublok ® (preventative vaccine against seasonal influenza virus), Provenge ® (therapeutic treatment against prostate cancer) and Glybera ® (gene therapy treatment for lipoprotein lipase deficiency). IC-BEVS is an attractive alternative to mammalian cells for biomanufacturing as it offers advantages such as easy adaptation to serum-free media, high levels of protein expression and post-translational modifications, and is appropriately scalable for manufacturing 2. Despite these advantages, the use of IC-BEVS to produce recombinant proteins can be costly and time-consuming. As the demand for new therapeutic increases, efficient, and robust methods to improve the production and screening processes of recombinant proteins in this expression system are necessary to respond to large-scale manufacturing needs. Research has shown that tailored media supplementation and optimization is an efficient, and useful strategy to reach high density cultures and increase protein production in-vitro. Therefore, this project aimed to evaluate the effects of two macromolecules (glucose in high concentration, and glutathione) as potential cell culture additives or boosters to increase the production of therapeutic proteins in the baculovirus/insect cell expression system. It was demonstrated that glutathione addition resulted in a more rapid, universal production of protein compared to minimal media, suggesting this to be a valuable addition to IC-BEVS media.

Protein-based therapeutics are highly potent medicines produced by various expression systems to prevent, treat, and cure a variety of diseases such as cancer, autoimmune diseases, and infectious diseases, as well as genetic disorders. Several currently approved human therapeutics are manufactured using IC-BEVS due to its easy adaptation to serum-free media, high levels of protein expression and post-translational modifications, and its scalability for manufacturing.[1,2] Recombinant proteins produced in this system may constitute antibodies, vaccines, growth factors, hormones, interleukins and pro-inflammatory cytokines.[3] Currently, recombinant proteins are most often produced in expression systems such as yeast, bacteria, insect cells, mammalian cells, and plant cells.[4]

Insect cell/baculovirus expression system (IC-BEVS) are well-established in the pharmaceutical and biotechnological industries to produce recombinant proteins. This expression system is particularly used due to the natural propensity of baculoviruses to infect insect cells.[2] In manufacturing, the most commonly used baculovirus is the Autographa Californica multiple-capsid nuclear polyhedrosis virus (AcMNPV) which is a double-stranded DNA virus with a genome of approximately 135 kb, which is capable of infecting multiple insect cells such as the Spodoptera frugiperda,from which the Sf9 cell line is derived.[5] Because of its large size, BEVS can accommodate larger inserts consisting of single or multiple genes; making it an ideal system to produce complex recombinant proteins.[1] Since its development in 1983, this platform has been effectively used to produce human and veterinary therapeutics.[6,7] Although scientists have developed cutting-edge tools to produce these therapeutics, there remains a great need to improve production efficiency while minimizing cost.

One major drawback of IC-BEVS is that baculovirus infection induces cellular apoptosis and lysis in insect cells, which reduces the longevity of the culture and impedes reinfection of these cells.[8,9,10] This early cell death may be due to the accumulation of reactive oxygen species (ROS) in the mitochondria of these cells upon viral infection.[8] Therefore, protein production in this platform is restricted to about three days post-infection. To remedy this issue, researchers have developed multiple strategies to increase the longevity of SF9 cultures post infection. One of the most used strategies is the use of highly specialized cell culture media. Previous research has demonstrated that the optimization of cell culture parameters, media components, and feed strategies aid in obtaining high-density cultures which result in higher yields of recombinant proteins in the baculovirus/insect cell expression system.[5]

Certain additivess such as glucose, cholesterol, and antioxidants have been shown to improve production yields and quality of recombinant proteins in the IC-BEVS system.[10] Glucose supplementation, in particular, as the main source of energy for uninfected SF9 cells, increases the longevity and supports higher density SF9 cell cultures.[11,12] Moreover, although poorly

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

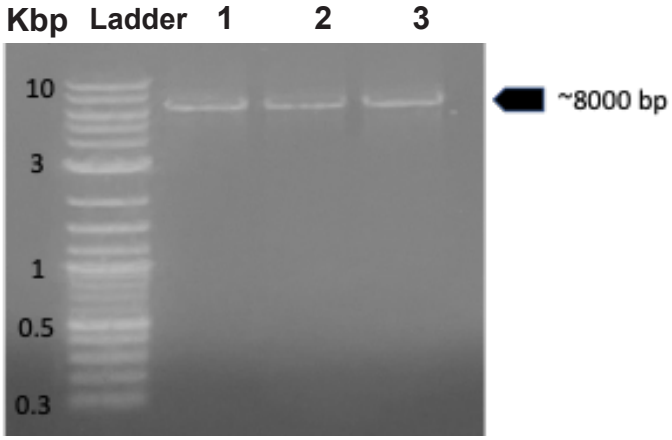

Figure 1 | The KIF1C gene is present in the newly made recombinant plasmid.
Agarose gel electrophoresis of KIF1C PCR fragment amplified from the recombinant baculovirus using flanking m13 forward and reverse primers. Electrophoresis was performed for 1 hour at 100 volts. A 1kb plus DNA ladder was used. Lanes 1, 2, and 3 contained three distinct colonies picked from a transformation plate.

studied in insect cells, the antioxidant, glutathione, acts as a Radical Oxygen Species scavenger and protects mammalian cells from premature cell death.[13] More recent studies by Monteiro et al., extend the idea that glutathione is a promising addition to insect cell culture media as well.[10]

Therefore, this research project aimed to examine the effects of the two macromolecules, glucose and glutathione, as cell culture media additives to increase the yield of recombinant proteins in the IC-BEVS system. Protein expression and production were analyzed using flow cytometry and spectrophotometry.

Materials and Methods

Cell culture and maintenance

The *Spodoptera frugiperda*-derived Sf9 cell line (ThermoFisher, Cat# 11496015) was maintained in Sf-900TM II (ThermoFisher, Cat #10902088) serum-free, protein-free insect cell culture medium in 125 mL Erlenmeyer flasks with 30 mL working volume. Cultures were kept in a 27ºC, non-humidified incubator, oscillating at 130 rpm. Cultures were split routinely, to maintain a density of 3-5x10^6 cells/mL, every 3-4 days. Cell concentration and viability were determined and evaluated by the hemocytometer and trypan blue exclusion method.

Baculoviruses and stock of recombinant bacmids were prepared using the Bac-to-Bac™ Baculovirus Expression System (ThermoFisher, Cat# 10359016). A pFastBac plasmid encoding for the KIF1C kinesin family member, fused N-terminally with a poly-histidine tag and C-terminally with GFP, pFastBac-M13-6His-KIF1C-GFP, was a gift from Anne Straube (Addgene plasmid # 130975 ; http://n2t.net/addgene:130975 ; RRID:Addgene_130975). Gene insertion was accomplished by co-transfection with these vectors and monitored using X-Gal blue-white screening. This was confirmed by PCR using recombinant bacmid as template and pUC/M13 forward and reverse primers. PCR product was run on a 0.8% agarose gel. P0 virus stock was generated by transfecting 25 mL of Sf9 suspension cells in Sf-f900TM (viability was greater than 95% and density was at 2.5x10^6 cells/mL) in a 125 ml shake culture flask. For each transfection sample, a mix of 30 µL of ExpiFectamineTM Sf Transfection Reagent (ThermoFisher, Cat# A38915), 1 mL Opti-MEMTM I Reduced Serum Medium (ThermoFisher, Cat#31985070) and 12.5 µg of undiluted recombinant bacmid was added dropwise onto the cells. Cells were incubated at 27º C for 72-96 hours in non-humidified, non-CO_2 incubator while oscillating at 130 rpm or until visible signs of virus infection. Upon reaching 50% viability, culture was harvested and centrifuged at 1700g for 10 min. The supernatant was stored at 4°C and protected from light to be used for virus titration and/or as inoculum in subsequent infections.

Baculovirus titration and total particles quantification

Virus titers were to be determined by a plaque assay. Briefly, a 6-well plate was seeded with 0.5×10^5 cells/mL per well, where serial dilutions of the viral sample was inoculated. Two replicates for each dilution were performed on the same plate Sf-f900TM insect culture media was used as negative control. Plates was screened after 7 to 10 days for plaque formation. The relative number of viral particles (titer) was to be calculated using the following equation: Titer (pfu)/mL = number of Plaques / (Dilution factor x Volume of inoculum per well).

Additives preparation

A stock of 100 g/L of glucose (Thermofisher, Cat# A16828), and a stock of 50mM of Glutathione (SigmaAldrich, Cat#G4251) were prepared in deionized water, filter sterilized, and stored according to the manufacturer's instructions. Each stock was diluted accordingly to test cytotoxicity on Sf9 cells.

Cytotoxicity of additives on SF9 cells

To determine the optimal concentration of each additive, SF9 cells were seeded in a 12-well plates, treated with different concentrations of glucose or glutathione, and incubated in a non-humidified, non-CO_2 incubator at 27ºC for three days. Samples were then collected and diluted using a dilution scheme of 10x using Sf-900TM II (ThermoFisher, Cat #10902088) serum-free, protein-free insect cell culture medium for a total volume of 1 mL of cell suspension. After dilution, each sample was treated with SYTOX AADvanced Ready Flow (Invitrogen, Cat. No. R37173) as described on ThermoFisher.com.[14] Samples were then analyzed using the Attune NxT Flow Cytometer. The relative cell density, as well as the percentage of live and dead cells were determined.

Cell growth, infection, and production of recombinant protein

Sf9 cells (ThermoFisher) were seeded with the optimal additive concentration (determined in previous Sytox viability/cytotoxicity assay) and infected at 1.0x10^6 cells/mL with an MOI of 5 pfu/cell with baculovirus-expressing GFP, using 125-mL non-baffled, vented shake flasks The samples were incubated in a non-humidified, non-CO_2 incubator at 120 rpm and 27ºC.

This study included control shake flasks also incubated at 27ºC with agitation at 120 rpm: Sf9 seeded at $1.0x10^6$ in a non-supplemented medium, Sf9 cells seeded at $1.0x10^6$ in supplemented medium, and Sf9 cells seeded and infected at $1.0x10^6$ in a non-supplemented medium with a multiplicity of infection (MOI) of 5 pfu/cell. Samples was collected daily for five consecutive days to assess protein production and viability of cells post-infection.

Viability of cells post-infection

Viability of Sf9 infected cells was determined by SYTOX™ AADvanced™ Dead Cell Stain (ThermoFisher, Cat# S10274) assay as described on ThermoFisher.com14 every day for five consecutive days. Each sample was then diluted using a dilution scheme of 10x using Sf-900TM II (ThermoFisher, Cat #10902088) serum-free, protein-free insect cell culture medium for a total volume of 1 mL of cell suspension. After dilution, each sample was treated with SYTOX AADvanced Ready Flow (Invitrogen, Cat. No. R37173) as described on ThermoFisher.com.[14] Samples were then analyzed using the Attune NxT Flow Cytometer.

Relative Quantification of Recombinant Protein

GFP fluorescence was measured two ways: GFP expression inside the cells via flow cytometry, and GFP release in the culture environment via spectrophotometry.

To detect GFP expression inside the cells, samples of cell suspension were collected and diluted using a dilution scheme of 10x using Sf-900TM II (ThermoFisher, Cat #10902088) serum-free, protein-free insect cell culture medium for a total volume of 1 mL of cell suspension. After dilution, samples were analyzed using the Attune NxT Flow Cytometer.

To detect GFP release in the cell culture media, samples of cell suspension were collected and centrifuged at 500g for 5 minutes. Supernatant were then collected and analyzed on a SpectraMax M3 using an excitation max of 488 nm and an emission max of 510 nm.

Results

KIF1C gene insertion into baculovirus construct was confirmed via PCR and gel electrophoresis.

In order to produce a baculovirus construct that can successfully infect insect cells to produce a protein, DH10Bac E.coli cells -containing bacmid construct-were chemically transformed with pfastbac vector encoding for a kinesin-like protein c-terminally linked to a GFP gene and n-terminally linked to a poly-histidine tag. The presence of the mini-attTn7 attachment site on the bacmid constructs allows proper transposition of the KIF1C genes into the bacmid construct which disrupts the expression of the LacZ peptide. When the newly transformed cells are grown on a plate, the colonies that express the LacZ gene due to the absence of the KIF1C gene appeared blue while the colonies expressing the KIF1C gene due to disruption of the LacZ gene, appeared white. Three white colonies and three blue colonies were picked from plates, then grown in liquid broth.

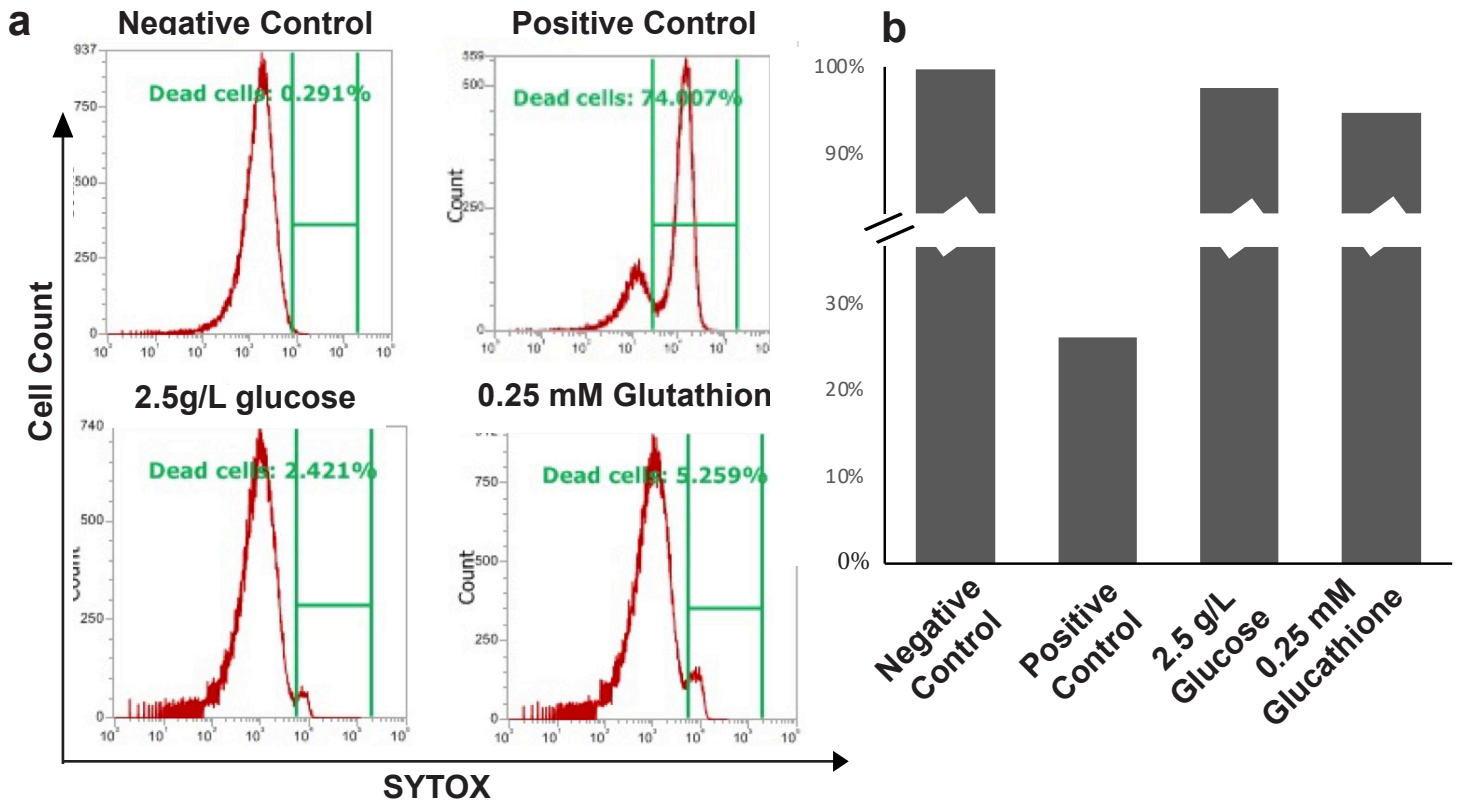

Figure 2 | Glucose and Glutathione Media Supplements maintained cell viability.
Cells were grown at 27º C for three days, agitated at 125rpm, and with a variety of concentrations of glucose or glutathione. Following incubation, cytotoxicity and cell viability were assessed using SYTOX AADvanced Ready Flow on an Attune NxT Flow Cytometer. Negative control represents a culture grown in basal (unsupplemented) media. Positive control represents a culture grown in basal media treated with 10% ethanol for three days. (**a**) Histograms of SYTOX AADvanced incubation with or without additives. (**b**) Bar graph of percent viability with basal media vs basal media + additives (as indicated).

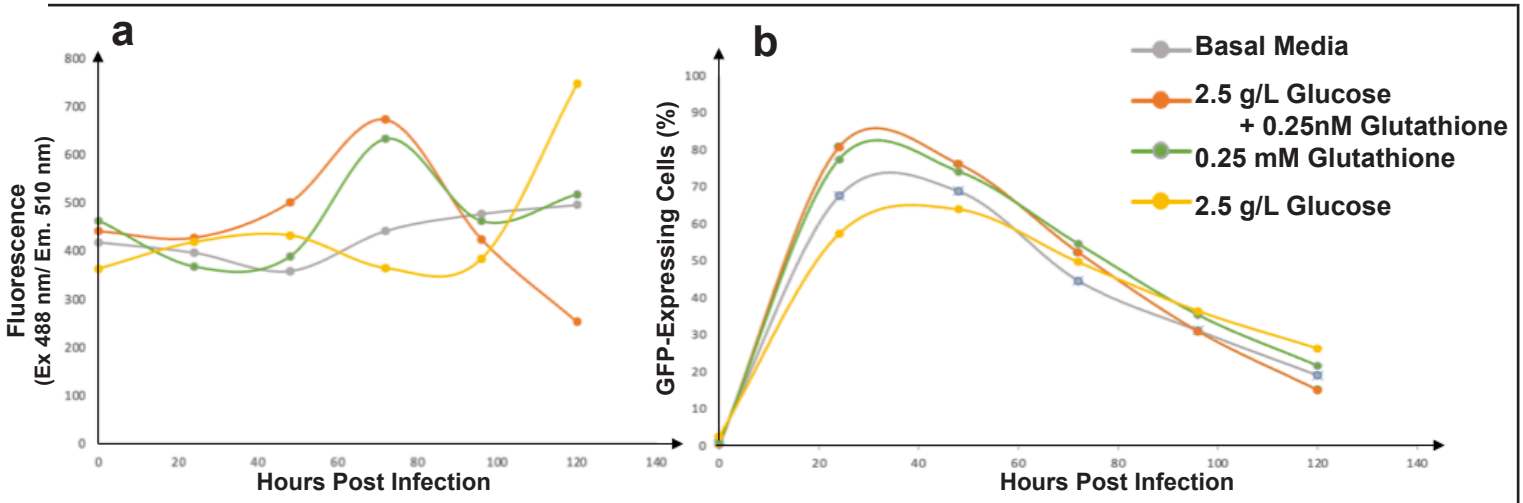

Figure 3 | Relative Quantitation of soluble, secreted- and cellular- GFP.
Sf9 cells were seeded at 1 x 10^6 cells/mL and infected at MOI 5 pfu/mL. Samples from each culture were collected daily over five days. **(a)** Relative secreted GFP fluorescence intensity was measured in spent media at the indicated times post-infection. **(b)** Relative cellular GFP fluorescence was analyzed by flow cytometry.

KF1C-expressing baculovirus were then extracted and subjected to a PCR using m13 forward and reverse primers to confirm proper transposition. A gel electrophoresis of the PCR product was performed. As expected, a single band of 8000 base pairs was seen on the gel, indicating the presence of the KIF1C gene in the recombinant bacmid (**Figure 1**).

Titration of baculovirus expressing KF1C

A plaque assay was performed to determine the viral titer of the baculovirus-expressing KIF1C stock. Contrary to what was expected, after 7 days of incubation, no plaque formation was observed. This assay was repeated twice over, with the same results obtained: No plaque formation. It was hypothesized that the method used to transfect Sf9 cells in the previous step was faulty, accounting for the lack of baculovirus produced. Due to lack of time, a baculovirus-expressing GFP was used for the remaining of this project to test if the addition of glucose and glutathione to basal media affects the production of protein the IC-BEVS system.

Addition of glucose and glutathione helped maintain high viability of uninfected cells.

Before assessing protein production, the effects of glucose and/or glutathione on cell viability were assessed using flow cytometry. SF9 cells were incubated with different concentrations of glucose (starting from 1 g/L to 30 g/L) and glutathione (starting from 0.25 mM to 15 mM), or with basal media for three days. Cells from each condition were harvested and treated with SYTOX dye. The percentage of dead cells were determined by the uptake by the SYTOX dye by dead cells. The negative control, which represents cells grown in basal media, maintained a viability of 99.5%. Cells treated with 2.5 g/L glucose only maintained the highest viability (97.6%) of all the different concentrations tested. Among the different concentration tested, cells treated with 0.25 mM of glutathione maintained a viability of 94.7% (**Figure 2b** and data not shown). These concentrations were used moving forward in this project.

Relative quantitation of GFP post-infection

To determine the effect of glucose and glutathione on production of GFP after infection with a baculovirus, the relative production of GFP produced after infection with the baculovirus stock was measured two ways: GFP expression inside the infected SF9 cells via flow cytometry, and media analysis of GFP released in the culture post-infection via spectrophotometry. Cells were seeded with optimal concentration of additive determined in prior viability assay then infected with baculovirus expressing GFP with a MOI of 5pfu/mL. **Figure 3a** illustrates the relative intensity of GFP released in the cell culture environment post infection. Glutathione seemed to favor rapid production and release of GFP. A maximum concentration seemed to occur at 72-hour post infection. Glucose on the other end seems to favor GFP release much later in the infection which occurred around day 5 post-infection, while basal media showed a steady release of secreted GFP in the media. **Figure 3b** illustrates the relative fluorescence intensity inside Sf9 cells post infection. Interestingly, the cells demonstrated a similar pattern of GFP expression, no matter the conditions they were grown and infected in. Expression of GFP seemed to peak between 24 to 48 hours post infection and then tailed off thereafter. Again, cells grown in glutathione-supplemented media seemed to favor higher expression of GFP.

Viability post-infection

To elucidate the possible cause of the early release of GFP in the media when the expressing cells were grown in glutathione, the viability of these Sf9 cells post-infection was analyzed using the SYTOX AAdvanced reagent on cells three or five days post-infection using the flow cytometer. As baculovirus is a lytic virus, we hypothesized that, contrary to what was seen in the prior experiments using uninfected cells, glutathione might not impede cell death in infected cells, resulting in the rapid release of GFP. As the highest level of expression and release of GFP was observed during day 3 post-infection and day 5 post-infection, the viability of the infected Sf9 cells was analyzed during these timepoints (**Figure 4**).

Already, at day 3 post-infection, the cells grown in glucose-supplemented media and basal media were less viable compared to the one infected in a glutathione. However, by day 5 post-infection, no matter the conditions, the cells appeared to be dead, similar to the positive control.

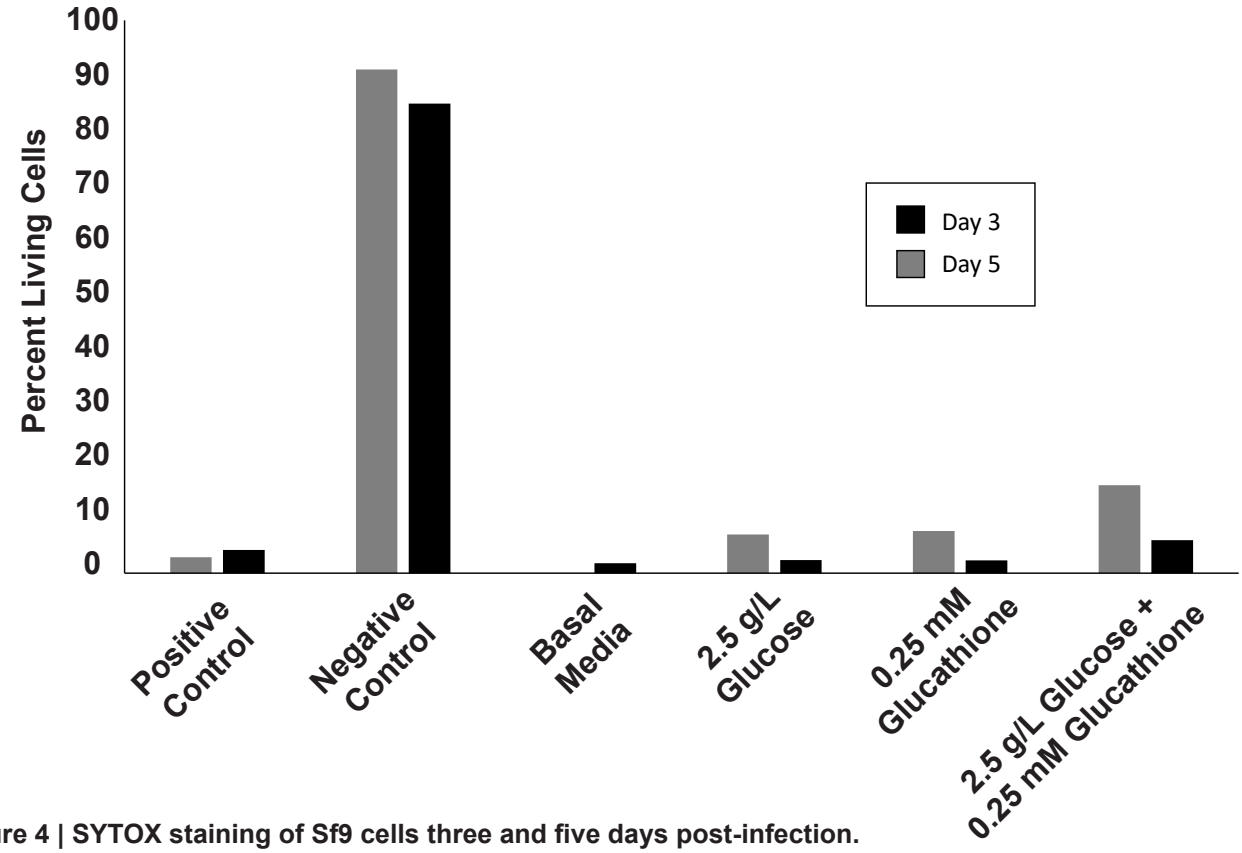

Figure 4 | SYTOX staining of Sf9 cells three and five days post-infection.
Cells were infected and grown at 27º C for three days, agitated at 125rpm, and with basal media, or basal media supplemented with 2.5 g/L glucose, 2.5mM glutathione, or both. The positive control consisted of uninfected sf9 cells grown in basal media, then treated for 10% ethanol for three days. The negative control represents a culture of non-infected sf9 cells grown in basal media. Basal media represents infected cells grown in media without supplementation. Graphs depict the percentage of SYTOX AADvanced-unstained (living) cells from the various conditions indicated at either 3 or 5 days post infection.

Discussion

The assays run during this project did not paint a full picture. The did not elucidate the reason why there was an early release of GFP in a glutathione supplemented media, and a late release in a glucose supplemented media. But nonetheless, from the data gathered, the authors observed that both glutathione and glucose seemed to aid in maintaining high viability in uninfected Sf9 cell cultures. Glutathione might also aid in the early and rapid release of recombinant proteins in-vitro as well as maintaining higher viability post-infection. However more research is required to better understand mechanism of infection, as well as the correlation between nutrients availability and protein production in the baculovirus/insect cells expression system.

Author Biography

Guenaele Raphael is a senior in Biotechnology at the University of Kansas. After graduation, she hopes to continue working in the biotechnology industry where she can gain experience in research and development of new therapeutics.

Acknowledgments

This author would like to thank Dr Jack Treml and Dr. Randall Logan for their guidance and well needed support on this project. The author would also like to thank Dr. Rollie Clem, Diane Wyatt and ThermoFisher for their generous donations; Ben Parrack, Sophie Bock, and Brandon Hamilton for their assistance with this work, as well as the University of Kansas, JCERT, and the people of Johnson County, Kansas for all their support. This work was funded in part by the KU Edwards Research and Innovation Grant with additional funding generously provided by Catalent, Boehringer Ingelheim, ICON, KCAS, and Hill's Pet Nutrition.

Author Contributions

G.A.R contributed to the experimental work, design, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

References

1. Hong, M. et al. Genetic engineering of baculovirus-insect cell system to improve protein production. Front. Bioeng. Biotechnol. 10, 994743 (2022).
2. Felberbaum, R. S. The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol. J. 10, 702–714 (2015).
3. Dimitrov, D. S. Therapeutic Proteins. Methods Mol. Biol. Clifton NJ 899, 1–26 (2012).
4. Tripathi, N. K. & Shrivastava, A. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. Front. Bioeng. Biotechnol. 7, (2019).
5. Chiou, T.-W., Hsieh, Y.-C. & Ho, C. S. High density culture of insect cells using rational medium design and feeding strategy. Bio-

process Eng. 22, 483–491 (2000).
6. Yee, C. M., Zak, A. J., Hill, B. D. & Wen, F. The Coming Age of Insect Cells for Manufacturing and Development of Protein Therapeutics. Ind. Eng. Chem. Res. 57, 10061–10070 (2018).
7. Aucoin, M. G. et al. Virus-like Particle and Viral Vector Production Using the Baculovirus Expression Vector System/Insect Cell System. in Baculovirus and Insect Cell Expression Protocols (ed. Murhammer, D. W.) 281–296 (Humana Press, 2007). doi:10.1007/978-1-59745-457-5_14.
8. Wang, Y., Oberley, L. W. & Murhammer, D. W. Evidence of oxidative stress following the viral infection of two lepidopteran insect cell lines. Free Radic. Biol. Med. 31, 1448–1455 (2001).
9. Steele, K. H. et al. Improving the baculovirus expression vector system with vankyrin-enhanced technology. Biotechnol. Prog. 33, 1496–1507 (2017).
10. Monteiro, F., Bernal, V., Chaillet, M., Berger, I. & Alves, P. M. Targeted supplementation design for improved production and quality of enveloped viral particles in insect cell-baculovirus expression system. J. Biotechnol. 233, 34–41 (2016).
11. Käßer, L., Harnischfeger, J., Salzig, D. & Czermak, P. The effect of different insect cell culture media on the efficiency of protein production by Spodoptera frugiperda cells. Electron. J. Biotechnol. 56, 54–64 (2022).
12. Bédard, C., Tom, R. & Kamen, A. Growth, Nutrient Consumption, and End-Product Accumulation in Sf-9 and BTI-EAA Insect Cell Cultures: Insights into Growth Limitation and Metabolism. Biotechnol. Prog. 9, 615–624 (1993).
13. Chevallier, V., Andersen, M. R. & Malphettes, L. Oxidative stress-alleviating strategies to improve recombinant protein production in CHO cells. Biotechnol. Bioeng. 117, 1172–1186 (2020).
14. SYTOX® AADvancedTM Dead Cell Stain Kits.