APPLICATION OF METABOLOMICS AND METABOLIC FLUX ANALYSIS TO IMPROVE RECOMBINANT AAV PRODUCTION IN THE SF9/BACULOVIRUS SYSTEM

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ABSTRACT

The Sf9-baculovirus expression system is widely used to produce recombinant adeno associated virus (rAAV) vectors for gene therapy applications. The manufacturing process generally involves baculovirus infection of Sf9 cells at low cell density, as infection at higher cell densities results in lower specific productivity. This “cell-density effect” is thought be a result of nutrient or metabolic deficiency; however, the exact mechanism and its resolution is not well defined yet.

In this study, Sf9 cells cultivated in an in-house medium, co-infected with baculovirus at different cell densities were examined to understand the metabolic phenomena driving the cell-density-effect. Using steady state metabolomics, we tracked metabolite levels across glycolysis, the TCA cycle, amino acid catabolism as well as nucleotide synthesis and degradation pathways, enabling the identification of potentially limiting metabolites. These novel results were matched with a detailed carbon balance flux analysis to investigate major carbon utilization pathways. Sf9 cells cultivated with C-13 labelled Glucose were infected at different cell densities and C-13 metabolic flux analysis (MFA) was performed across major metabolic pathways. These studies show that Sf9 cells experience major decrease in glycolysis and TCA cycle fluxes when infected with Baculovirus at high cell densities. We were also able to identify the major amino acids catabolized after baculovirus infection. Additionally, certain metabolites had different utilization rates between low and high cell density infections.

These in-depth studies to understand Sf9 metabolism pre and post baculovirus infection have been instrumental in designing an in-house feed supplement that boosts AAV productivity by enabling high cell density infections.

INTRODUCTION

Upstream Process utilizing Baculovirus-Sf9 System to Manufacture rAAV

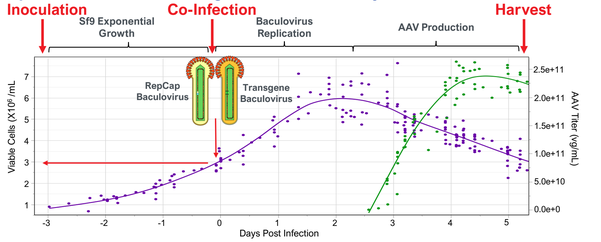


Figure 1. Viable Cell Density (purple line) and AAV titer (green line) during baculovirus infection and rAAV production

“Cell Density Effect” is a Productivity Limit in the Baculovirus System and is Partially Due to a Nutrient Deficiency

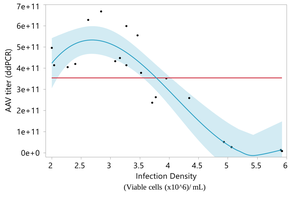


Figure 2. Sf9 Cells are co-infected at various cell densities ranging from 2 to 6 E6 vc/mL. Cells had peak productivity at around 3E6 vc/mL and this productivity decreased with increasing cell densities reaching undetectable levels after 5.5 e6 vc/mL

EXPERIMENTAL DESIGN

Investigating Cell Density Effect by Comparing Low and High Cell Density infections Utilizing 2 different Metabolomics Approaches

i) Metabolomic Snapshot

ii) C13 Glucose labelled metabolomics + Metabolic FLUX analysis

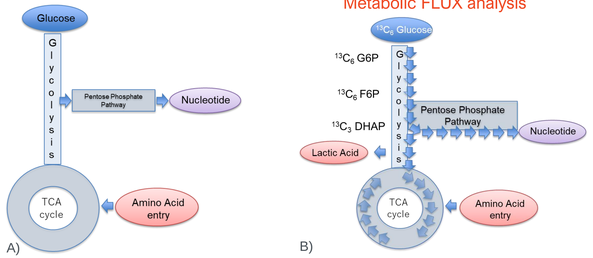


Figure 3A) Metabolomic Snapshot: Extracellular (media) and intracellular metabolites were monitored during conditions of cell growth (red), low cell density infection at 3E6 cells/mL and high cell density infection at 5-6E6 cells/mL.Quantitative analysis was performed by Human Metabolome Technologies Inc using capillary electrophoresis mass spectrometry (CE-TOFMS and CE-QqQMS)

Figure 3B) C13 Glucose labelled metabolomics + Metabolic FLUX (MFA) analysis Cells are labelled with uniformly labelled C13 Glucose at time of infection, and samples are collected every 4 hours. C13 labelled metabolites are analyzed by Human Metabolome Technologies Inc using capillary electrophoresis mass spectrometry (CE-TOFMS and CE-QqQMS) The results of these analysis along with cell growth and titer data are processed through Metalytics Inc' s CoreMFA software to calculate and visualize the flux rates, the rate at which carbon is transferred from the tracer molecule through the various metabolic pathways to end-product(s).

RESULTS

i) Metabolomic Snapshot

TCA cycle: Very different pattern in TCA cycle metabolites between LDI and HDI

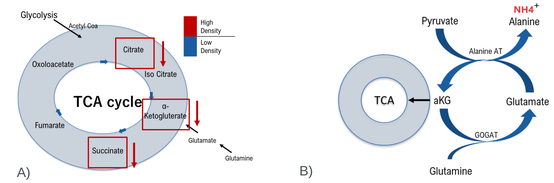


Figure 4. A) Intracellular TCA cycle metabolites following High Density Infection. **B)** Depiction of α-Ketoglutarate mechanisms in Sf9 cells. α-Ketoglutarate can enter TCA cycle, can accept NH4 from Glutamine and generate Glutamate by the aid of enzyme GOGAT, and can also play as a key metabolite in NH4 clearance by aiding generating of Alanine by Alanine AT enzyme.

- Low amounts of citrate, succinate and α-Ketoglutarate in cells infected at High Density
- A shift observed high density condition after α-KG node between High and Low cell density infections, and the later TCA cycle metabolites were lower in cells infected at High Cell Density

→ α-Ketogluterate might be key TCA cycle metabolite that has limited supply in cells infected at High Cell Densities

→ α-Ketogluterate is also an important metabolite that interplays GOGAT and AlanineAT enzymes

AminoAcids: Certain Amino Acids are depleted throughout the culture duration in both LDI and HDI

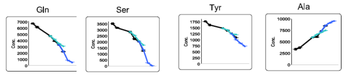
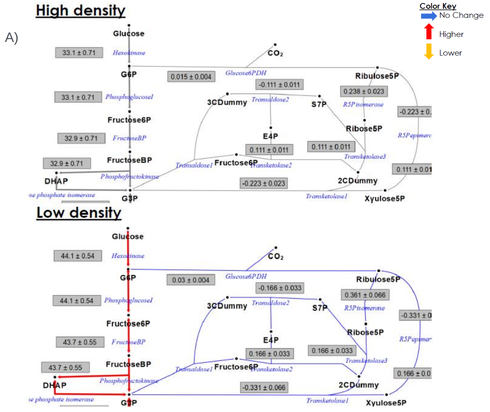


Figure 5. Extracellular Amino Acids are measured from media that are obtained from Sf9 cells during the course of Baculovirus infection

- Amino Acids such as Glutamine are Serine are completely depleted from media during infection and their replenishment is needed
- Most Amino Acids are decreased in the media during the rAAV production method
- Alanine, since it accepts NH4 and acts as by-product in Sf9 cells, accumulates throughout the culture

ii) C13 Glucose labelled Metabolomics + Metabolic FLUX analysis (MFA)

Upper Glycolysis and Pentose Phosphate Pathway:



Lower Glycolysis and TCA cycle:

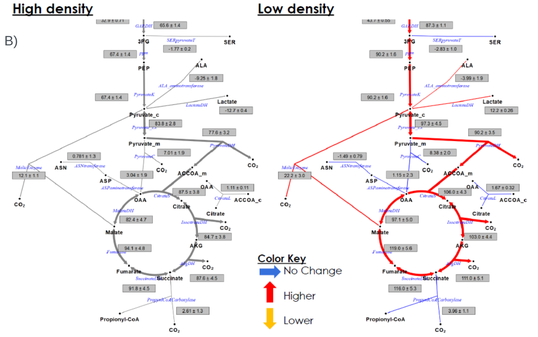


Figure 6. Metabolic FLUX analysis. Comparison of the metabolic flux rate through **A)** Upper glycolysis and PPP and **B)** Lower Glycolysis and TCA cycle in SF9 cells following exposure to uniformly labeled [U-13C6]-D-glucose in cell infected at HD (High density) and LD (Low density) . Color coding in LD is a comparison with HD. Blue represents no significant difference in flux rate between two cases, while yellow indicates a significantly lower flux rate in LD as compared to HD and red represents a significantly higher flux rate in LD. Metabolic Flux Analysis was conducted by Metalytics, Inc.

Glycolysis and TCA cycle FLUX analysis showed significant increase in glycolysis in cell infected at Low Density

- Higher metabolic activity is observed in glycolysis and TCA cycle in cells that are infected at Low Density compared to cells that are infected at High Cell Density
- Carbon flux towards the pentose phosphate pathway was negligible in both scenarios
- High glycolytic and TCA cycle fluxes suggests production in the Low Density case, which could explain the high AAV productivity

Metabolomics Data Aided Media Development to Overcome Cell Density Effect and Improve Productivity

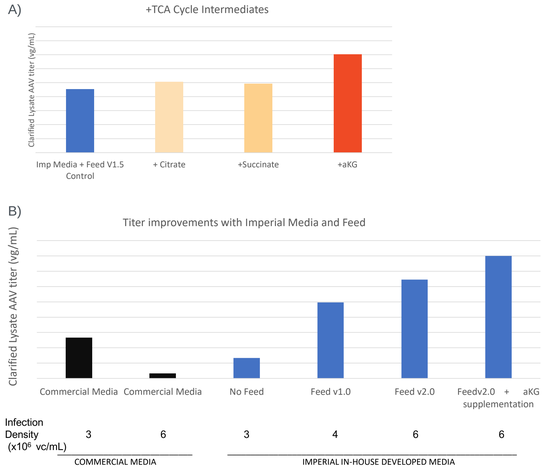


Figure 7: rAAV titer with media additions and feed supplementation. A) TCA cycle Intermediate additions. Sf9 cells are lysed and rAAV titer is measured by ddPCR analysis

B) Titer improvements with Imperial Media and Feed. Sf9 cells are infected in various Cell densities. They are either cultured in commercial or in Imperial in-house developed media. Feed versions are supplemented around the time of infection. Sf9 cells are lysed and rAAV titer is measured by ddPCR analysis.

SUMMARY AND CONCLUSIONS

- Sf9 metabolism is investigated using Metabolomics and Metabolic FLUX analysis by comparing Low and High Cell density infected cells in order to shed light into cell density effect.
- Metabolic flux analysis showed that Sf9 cells have more active carbon metabolism (both glycolysis and TCA cycle) if they are Baculovirus-infected at lower cell densities (Figure 6).
- Metabolomic analysis showed that couple of amino acids and some TCA cycle intermediates are decreased during High Cell Density infection (Figure 4 and 5).
- One TCA cycle intermediate: -ketoglutarate has a dual role in TCA cycle replenishment and NH4 clearance (Figure 5B). It might be a key limiting metabolite in baculovirus infections. Supplementation of a-ketoglutarate resulted in increased rAAV titers (Figure 7A).
- Cell density effect and nutrient limitations can be overcome with specific feed design and metabolite additions (Figure 7B).