# MVDA and Transcriptomic Analyses of LDHa + PDK-1/2 Knockout CHO-S Cells in Fed-Batch Culture

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Abstract

A key bottleneck in CHO bioprocesses is inefficient glucose metabolism during log-phase, where excess lactate secretion diverts pyruvate from high-yield TCA-OxPhos pathway. This study evaluates LDHa + PDK-1/2 knockout as a strategy to increase carbon to biomass efficiency, using mass balance, MVDA, and transcriptomics to compare WT and KO cultures at both reactor and cellular metabolic scales.

## Introduction

Chinese Hamster Ovary (CHO) cell cultures undergo a shift from a lactate-production phase (characterized by aerobic glycolysis or glutaminolysis) in the exponential phase to a lactate-consumption phase (characterized by oxidative phosphorylation) in the stationary phase [1].

Such a metabolic shift is considered beneficial since lactate production is known to limit cell growth and lead to an increased osmolality when efforts are taken to maintain a constant pH of the reactor. Lactate accumulation in the media is also known to have a detrimental impact on quality attributes of the recombinant protein product, such as glycosylation; moreover, the 'lactate shift' is also correlated with high productivity in cell lines that produce a recombinant product [1].

The conversion of pyruvate to lactate is catalyzed by the Lactate Dehydrogenase (LDH) enzyme (Figure 1), which is composed of LDH-M and LDH-H subunits encoded by the LDHa and LDHb genes, respectively. While LDH-M has a higher affinity for pyruvate, LDH-H has a higher affinity for lactate. LDH isozymes with different constitution of these subunits preferentially catalyze either the forward reaction or the reverse reaction [1].

The lactate shift is primarily driven by a change in the redox state of the cell. The conversion of pyruvate to lactate leads to the consumption of NADH, and the production of NAD+, which is required for reactions of the Glycolysis Pathway. Thus, the LDH complex allows for replenishing NAD+ when it is depleted, and also facilitates the production of pyruvate, which is sensed by a decrease in the NADH levels.[1].

The distribution of pyruvate flux towards lactate or Acetyl CoA, is also regulated by the Pyruvate Dehydrogenase Kinase (PDK) enzyme, which deactivates Pyruvate Dehydrogenase by phosphorylation. The Pyruvate Dehydrogenase complex catalyzes the conversion of Pyruvate to Acetyl CoA [1].

A concomitant knock-out of LDHa and PDK genes is considered as a promising strategy due to its potential to target two important regulators that determine the fate of the carbon flux through the glycolysis pathway.

Therefore, as part of this study, fed-batch cultures of WT CHO-S cells along with those of LDHa/PDK-1 and LDHa/PDK-2 KO cell lines were subjected to multiple analyses to observe changes at the culture and cell-level with relevance to metabolic changes across different phases of culture growth.

## Dataset Description

### Carbon Balance and PCA

Spent Media Data for 8 15-day long Fed-Batch operations under 3 different conditions (WT, LDHa/PDK-1 KO and LDHa/PDK-2) were obtained.

### Flux Balance Analysis

The iCHO2441 Genome Scale Metabolic Model (GEM) was utilized to perform Flux Balance Analysis (FBA) to determine fluxes through and visualize the differences across relevant reactions.

Fed-batch operations U1 (WT), U4 (LDHa/PDK-1 KO) and U6 (LDHa/PDK-2 KO) were used as representative samples for analysis. Carbon balance, PCA and FBA were performed for the exponential (Day 2 to Day 4) and stationary (Day 6 to Day 8) phases.

### Transcriptome Analysis

A transcriptomic gene expression table quantified as TPM values from eight independent experimental runs (U1–U8), spanning CHO-S Wild Type (WT), LDHA/PDK-

1 knockout, and LDHA/PDK-2 knockout conditions, and a corresponding metadata table containing sample annotations.

Day 4, 8, and 14 were analyzed due to complete time-point coverage across all runs.

**Data Acquisition**

The transcriptome (TPM data) and spent media data were provided to us by Ms. Tejaswini, with the authorization and consent of Prof. Meiyappan Lakshmanan. Data regarding biomass composition of CHO-S cells were obtained from previously published reports [2]. The iCHO2441 GEM was obtained (in the form of a '.xml' file) from the Mendeley Data repository curated by researchers who developed the model [3].

**Data Pre-processing**

**Calculation of Specific Rates**

Integral Viable Cell (IVC) counts and Specific Metabolite Rates were calculated using the formulae given in (Equations 1 and 2). Since metabolite concentrations were not determined for some intervening days of the fed-batch operations, the missing concentrations were obtained by linearly interpolating substrate concentration as a function of time, as shown in (Equation 2).

**Flux Balance Analysis**

The transcriptome data consists of TPM data for 32895 different transcripts; however, the iCHO2441 GEM consists of 2441 genes that are involved in different metabolic pathways. Therefore, genes whose NCBI IDs matched with those included in the GEM were extracted from the transcriptome data.

**Methodology**

**Carbon Balance and PCA**

Specific Growth Rate and Specific Metabolite Rates were computed. Specific Rates could not be computed for metabolites with concentration values less than the respective Limit of Detection (LOD) value on consecutive days.

Carbon flux to biomass, extracellular media and Carbon influx were considered for mass balance calculations.

**Flux Balance Analysis**

The E-flux method was used to integrate transcriptome data with the GEM [4]. [refer to 'A note on Flux Balance Analysis']

For the exponential phase, the biomass reaction ($biomass\_cho$) was used as the objective function for the exponential phase and a linear combination of the biomass and ATP maintenance reactions ($DM\_atp[c]$), with equal weights, was used as the objective function for the stationary phase.

The Flux Balance Analysis was performed using the COBRApy toolbox in the Python programming language [5]. Expression data from the exponential and stationary phases of the WT, LDHa/PDK-1 KO and LDHa/PDK-2 KO cell lines were used to perform FBA.

Flux Distribution, flux values for reactions involving Pyruvate and reactions with fluxes above an arbitrary large value (0.1 for the exponential phase and 0.01 for the stationary phase, in $mmol \cdot gDCW^{-1} \cdot h^{-1}$ units), were visualized using bar graphs.

**Transcriptome Analysis**

Differential gene expression (DGE) analysis followed by pathway enrichment analysis was performed using transcriptome data from Day 4 (exponential phase), Day 8 (stationary phase), and Day 14 (death phase). The workflow was executed independently for each selected day.

The analysis was performed using limma, as it enables precision-weighted linear modeling on continuous TPM expression values. In contrast, methods like DESeq2 require discrete, integer raw counts to apply negative binomial modeling on sequencing read counts.

Statistical grouping of biological replicates was performed based on cell line identity (Note that the samples were not merged or pooled).

TMM normalization was used to correct sample composition bias while preserving relative expression differences. The voom transformation was applied to model the mean–variance trend and compute precision weights, converting TPM-based transcriptome abundances into $\log_2$ expression values suitable for linear modelling in limma.

Contrast comparisons were set up based on cell type, after which contrast-specific model coefficients were estimated. Empirical Bayes moderation (eBayes) was then applied to stabilize gene-wise variance and generate moderated test statistics for differential expression inference. All steps above were carried out using the built-in **default parameters** of the respective functions.

To determine differentially expressed genes for pathway enrichment analysis in DAVID, only gene lists meeting the same DEG inclusion criteria (**$|\log_2 FC| > 1$** and **adjusted p-value < 0.05**) were considered. These thresholds were selected because they are widely adopted in transcriptomics studies, a common standard in the literature.

In DAVID, official gene symbols were used as the identifier (species: *Cricetulus griseus*), and differential gene lists for each contrast were evaluated against a background gene universe derived from the same dataset to identify statistically enriched pathways [6,7].

Filtering of enriched pathways was performed to retain only statistically significant results using literature-standard thresholds (**Counts ≥ 5**, **Fisher Exact p < 0.05**). The **Benjamini–Hochberg** cutoff was relaxed to **0.1** (instead of the conventional 0.05) to account for the limited number of biological replicates (2–3 per cell line), thereby reducing the likelihood of false negatives.

## Results and Interpretations

### Carbon Balance

Exponential Phase (Day 2 - 4 of operation)

*Carbon Distribution*

While Carbon flux towards Biomass possesses similar values for all cultures, more flux is diverted towards Lactate in the WT cells as compared to the KO cells; this indicates that the LDHa/PDK-1/2 KO suppresses inefficient glucose utilization through anaerobic respiration during exponential growth.

The flux diverted towards Carbon Dioxide is relatively lower in the WT cells, in contrast to KO cells. This indicates that the KO cells move pyruvate into the mitochondrial oxidation via the TCA cycle instead of converting it into lactate.

It is intriguing to note that the LDHa/PDK-1 cells divert more flux towards biomass than the WT or LDHa/PDK-2 cells and have the highest Biomass/Total C ratio among the three cell lines. An increased flux towards Alanine is also observed in the KO cells, as compared to the WT cells, even as fluxes directed to other secreted AA remain similar across the three cell lines [Figure 2].

*Carbon Uptake*

Among the various sources of Carbon, Glucose remains the largest contributor across all cell lines. However, the WT and LDHa/PDK-2 KO cells consume more Glucose than the LDHa/PDK-1 cells. This points to a more efficient conversion of glucose to biomass in the LDHa/PDK-1 cells since they direct more flux to biomass as compared to the other cell lines (as discussed above), thus suggesting that a PDK-1 KO might be more beneficial for cell growth (and, therefore, for the production of growth-dependent products) in LDHa/PDK KO cell lines.

Furthermore, Asparagine and Glutamine contribute significantly to the Carbon Influx of all cell lines in the exponential phase. Glutamine is presumably utilized by glutaminolysis, while Asparagine can enter the TCA Cycle upon its conversion to oxaloacetate [Figure 3].

Stationary Phase (Day 6 - 8 of operation)

*Carbon Distribution*

All the three cell lines show similar behavior vis-à-vis the consumption of lactate; WT cells divert more flux towards lactate than their KO counterparts. However, an increased flux towards Carbon Dioxide in the WT cells as compared to the exponential phase is indicative of the ‘lactate shift’; i.e. a shift towards oxidative phosphorylation from anaerobic respiration.

A decreased Biomass/Total uptake C in comparison to the exponential phase indicates that more flux is diverted towards maintenance of cellular functions than cell growth in the stationary phase [Figure 4].

*Carbon Uptake*

The uptake of glucose and amino acids by all cell lines in the stationary phase is significantly smaller than their counterparts in the

exponential phase. This could be due to reduced metabolic demands of the cells in the stationary phase. A reduction in the uptake of different metabolites can be attributed to a reduction in the expression of the relevant transporter proteins [Figure 5].

**Principal Component Analysis**

Multivariate Statistical Analysis was performed using Principal Component Analysis (PCA) to gain insights about how different metabolites contribute to the differences across cell lines.

Exponential Phase

Principal Component (PC) 1 explains 35.1% of the variation between the various clones, PC2 25.7%, and PC3 15.8%. WT and KO cells are observed to form different clusters, separated along PC2. However; two clusters are observed within the KO cells, separated along PC1.

Glucose/Pyruvic Acid and Ethanol are observed to correlate negatively with each other along PC2. Moreover, Lactic Acid and Ethanol have oppositely directed loadings along PC1, indicating Ethanol and Lactic Acid fermentations to be two competing by-products of anaerobic respiration in the exponential phase. Ethanol fermentation may compete with Oxidative Phosphorylation in the KO cells, in the absence of LDHa expression.

Glutamine and Pyruvic Acid have oppositely directed loadings around PC2, indicating that glutaminolysis can be a mechanism by which Pyruvate is replenished in the cells during the exponential phase [Figures 6,7]

Stationary Phase

Principal Component (PC) 1 explains 65.1% of the variation between the various clones, PC2 15.3%, and PC3 6.67%. WT and KO cells are observed to form different clusters, separated along PC1, with the exception of a WT batch run (denoted by the label U3).

Glucose and Specific Growth Rate correlate positively with each other along PC1; both of them correlate negatively with Lactic Acid, indicating that the pattern of Lactate consumption in is the key differentiator between the WT and KO cells. This result aligns with the desired KO strategy implemented in the KO cell lines.

Pyruvic Acid and Lactic Acid correlate positively along PC1, indicating that Pyruvate consumption correlates with Lactate consumption, presumably due to the conversion of lactate into pyruvate for utilization in the TCA cycle through LDHb dominant LDH isozymes. This observation is indicative of the ‘lactate shift’ characteristic of the stationary phase. Similarly, Pyruvic Acid and Glutamine correlate positively along PC1, indicating that Glutamine consumed through glutaminolysis is utilized in the TCA cycle [Figures 8, 9].

**Flux Balance Analysis**

The specific growth rates calculated using FBA match closely with the experimentally measured values for the different cell lines in different phases of cell growth; these observations are reflective of a suitable choice of parameters during the integration of transcriptome data and the Flux Balance [refer Table 1].

A large portion of the total 6337 reactions [3] have an absolute value of flux close to or equal to zero [Figures 10, 13, 16, 19, 22, 25]. Therefore, the non-zero fluxes through reactions involving Pyruvate were visualized.

In the exponential phase of the WT cell line, a relatively large flux through reactions comprising the Glycolysis Pathway, in comparison to other reactions involving Pyruvate, indicates high glucose uptake by cells in the exponential phase due to high metabolic requirements. Moreover, a negative flux through the reaction catalyzed by L-Lactate Dehydrogenase indicates that anaerobic respiration is the primary mechanism by which ATP generation occurs [Figure 11].

A relatively large negative flux through the reaction catalyzed by D-Lactate Dehydrogenase could indicate that the conversion of pyruvate to D-Lactate could be a mechanism by which NAD+ is replenished for consumption in the Glycolysis Pathway [Figure 11].

Relatively smaller fluxes through reactions involving the export of pyruvate, cysteine transamination, etc. indicate utilization of

pyruvate in reactions that are not part of the central metabolism but are required for cellular functions (cysteine metabolism, for instance) [Figure 12].

The presence of several Glycolysis Pathway reactions and ATP Synthesis in the reactions with relatively higher flux as compared to others is reflective of the high flux directed towards glucose metabolism and ATP production [Figure 12].

The stationary phase of WT cells, however, has a relatively higher positive flux through the reactions catalyzed by LDH (that produces pyruvate) and PDH (the first step in the production of Acetyl CoA), indicating a shift towards consumption of lactate and ATP production via Oxidative Phosphorylation [Figure 14]; these results are in agreement with previous reports regarding the 'lactate shift' observed in CHO cell cultures [1].

This observation is reinforced by the fact that the transport of water and carbon dioxide outside the mitochondria seem to occur at a relatively high rate [Figure 15].

The LDHa/PDK-1 and LDHa/PDK-2 KO cell lines show similar behavior in both phases of cell growth. The changes in cellular metabolism due to the KO strategy are described as follows.

In the exponential phase of the LDHa/PDK-1/2 KO cell line the reaction catalyzed by L-LDH is turned off. However, a relatively high negative flux through the reaction catalyzed by D-LDH points to an additional mechanism in which flux is diverted away from the Oxidative Phosphorylation pathway. However, a relatively high negative flux through the reaction catalyzed by PDH indicates the success of the PDK KO strategy in activating the PDH enzyme (through the KO of PDK) and, thereby, increasing the flux towards the formation of Acetyl CoA from pyruvate [Figures 17, 23].

The exponential phase continues to be characterized by a relatively high flux through various reactions of the Glycolysis Pathway, indicating the high glucose demand of the rapidly proliferating cells [Figures 18, 24].

The stationary phase of the LDHa/PDK-1/2 cell line is characterized by relatively high positive fluxes through the reactions catalyzed by L-LDH and PDH [Figures 20, 23 ], indicating a shift to Oxidative Phosphorylation, as in the WT cells.

This observation is also supported by the evidence that the transport of water and carbon dioxide outside the mitochondria occurs at a relatively higher rate, as in the WT cells [Figures 21, 24].

Thus, a shift towards Oxidative Phosphorylation appears to occur with the onset of the stationary phase in the KO cells as well; as in the case of the WT cells, this 'lactate shift' presumably occurs through an increase in the synthesis of the LDHb subunit [1].

Moreover, relatively high fluxes through ATP maintenance and ATP Synthase reactions (in both WT and KO cells), in addition to reactions of the Glycolysis pathway, reflect a shift from the utilization of energy gained from metabolism for cell growth and proliferation (in the exponential phase) to its utilization for storing energy for cellular functions (in the stationary phase).

**Transcriptome Analysis**

Although several genes showed differential expression between the PDK-1 KO and PDK-2 KO cell lines, no metabolic pathways met the criteria for statistical significance in the KO-vs-KO comparison. In the exponential phase analysis (Day 4), a limited DEG signature (~300 genes) was observed, which was insufficient to yield significant pathway enrichment across contrasts involving WT, PDK-1 KO, and PDK-2 KO conditions. Consequently, no statistically significant enriched pathways could be identified as different between the three cell lines in this phase.

*Pathways affected across both phases*

Down-regulation of the HIF-1 signaling pathway in cells during both the stationery and death phase reflects a metabolic reprogramming towards oxidative phosphorylation, as the absence of lactate leads to a breakdown of HIF-1α in the KO cells. [Table 2,3]

Up-regulation of glutathione metabolism in both the stationery and death phases indicates enhanced engagement of cellular antioxidant defense against mitochondrial ROS in the KO

cell lines, consistent with increased reliance on oxidative phosphorylation, a metabolic state known to elevate reactive oxygen species, for which glutathione acts as a well-established redox stabilizer and ROS scavenger [Table 2, 3].

Stationary Phase *[Refer Table 2]*

Downregulation of the TNF signaling pathway indicates reduced activation of apoptosis-associated transcriptional programs in the KO cell lines. This aligns with a delayed transition toward the death phase, as indicated by a comparatively slower decline in cell-stress and apoptotic signaling signatures (as illustrated in Figures 51 - 53).

Up-regulation of cell cycle and DNA replication pathways on day 8 indicates sustained proliferative activity in the KO cell lines, extending into the stationary phase. This trend is further supported by the higher peak viable cell density (VCD) reached by the KO cell lines compared to the Wild-Type (Figure 51).

The elevated replicative stress in the KO cell lines increases the likelihood of DNA damage, which is accompanied by transcriptional activation of major DNA repair pathways, including the Fanconi anemia and homologous recombination pathways. Furthermore, the observed upregulation of glutathione metabolism suggests that the DNA damage in the KO system may arise not only from replicative pressure but also from mitochondrial ROS-driven oxidative stress.

Up-regulation of ribosomal pathways reflects an increased transcriptional and translational load in the KO cell lines, which is explained by the sustained cell division that occurs well into the stationary phase, requiring more cellular machinery.

Death Phase *[Refer Table 3]*

The KO cells at this stage exhibit downregulation of the Glycolysis/Gluconeogenesis pathway and upregulation of the Oxidative Phosphorylation pathway. Hinting that the KO cell lines might be utilizing amino acids as the raw materials for the TCA cycle, compared to Glucose.

This is supported by the fact that several metabolic pathways also appear to be upregulated in the KO cell lines compared to the Wild-Type.

The downregulation of the cell cycle pathway indicates that the KO cell lines are no longer maintaining strong proliferative transcriptional programs at the end of the run.

The overall effect of these differences is that the KO cells appear to be in a prolonged exponential and stationary phase as compared to the wild-type cell line.

**Limitations**

While this study has relied on transcriptome data, expression of mRNA transcripts need not always be directly proportional to the corresponding protein expression.

Moreover, some gene labels (outdated NCBI IDs, unannotated loci, etc.) could not always be mapped from the transcriptome dataset to the iCHO2441 GEM or the David software.

Furthermore, a lack of biological replicates made it difficult to obtain exact results for the Pathway Enrichment Analysis.

**Summary**

Residual lactate formation in the LDHA/PDK-1/2 knockout cultures is attributed to LDHb activity, while the combined LDHA+PDK KO de-represses PDH, driving greater pyruvate flux into mitochondrial acetyl-CoA and TCA oxidation. This results in enhanced oxidative phosphorylation and improved glucose-to-biomass conversion efficiency. The increased mitochondrial oxidation is accompanied by elevated ROS-associated oxidative stress, supported by the upregulation of the glutathione metabolism pathway. Excess ATP fuels sustained proliferation in the stationary phase, characterized by upregulation of cell-cycle and DNA replication/repair pathways.

**Future Directions**

Studies that map proteomics data to existing reaction networks such as GEMs can be performed to obtain a more accurate nature of the metabolic processes in different growth phases of the cell culture.

Advanced methods of integrating omics data into GEMs can be implemented to solve the optimization problem inherent to the FBA more accurately.