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# Metabolomics and Network Biology for sensitive monitoring of how growth environment changes affect the physiology of industrial-scale perfusion cultures

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**Abstract:** Metabolomic analysis can indeed enhance the prime variable dataset for the monitoring of perfusion cultures by providing a higher resolution view of the metabolic state. Metabolic profiles can capture physiological state shifts over the course of the perfusion cultures and indicate a metabolic "signature" of the phase transitions, which is not observable from prime variable data. Notably, metabolomics provides orthogonal (to prime variables) evidence that all cultures follow this same metabolic state shift with cell age, independently of bioreactor scale. Additionally, this analysis can increase the information content of process development experiments by helping better understand the impact of changes in bioreactor operating conditions on cell physiology. In this context, metabolic profiling could be integrated into the monitoring of cell physiology in perfusion cultures.

*Keywords:* industrial cell culture engineering, GC-MS metabolomics, perfusion cultures, multivariate statistical analysis, metabolic network reconstruction, growth environmental variations

## 1. INTRODUCTION

Mammalian cell cultures have been increasingly used for the production of complex biopharmaceuticals. As these products are of significance for human health, there is a crucial need for the development of robust processes that consistently produce material of high quality. Therefore, the industry is in need of tools for sensitive, high-resolution monitoring of the cell culture physiology throughout the entire process. Conventionally, the monitoring of mammalian cell cultures has been based on a small set of prime variables (i.e. growth rate, cell density and viability, product quality, substrate consumption and lactate production) and the relevant specific rates (Goudar et. al., 2009). However, this monitoring approach has limitations more prominently apparent when changes in product quality are observed despite no evident changes in the prime variable measurements. In addition, prime variables alone cannot provide extensive information about the culture physiology to allow for better understanding and optimization of the culture process based on experiments from process development activities, such as bioreactor scale-down model qualification and design space studies.

On the other hand, metabolic profiling can inherently provide a more extensive perspective of the cellular metabolic state than the set of the prime variables (Figure 1). Therefore, the multi-compound profiles are expected to be higher sensitivity sensors of changes in the metabolic physiology of cell cultures than the prime variable dataset. Additionally, quantification of the concentration change in many intracellular metabolites between different physiological conditions could help determine the sources of the relevant metabolic variation in cell cultures. In laboratory scale cell culture engineering, metabolomics has recently been used for the characterization of Chinese hamster ovary (CHO) cells, based predominantly on the profiling of the culture medium (Chong et. al. 2011; Bradley et. al., 2010) and, lately, of intracellular metabolites (Selvarasu et. al., 2012; Dietmair et. al., 2012). Our group (Chrysanthopoulos et al., 2010) presented the first metabolic profiling analysis of industrial scale perfusion cultures of baby hamster kidney (BHK) cells, using Gas Chromatography–Mass Spectrometry (GC-MS). The study provided strong evidence metabolic profiles could sense subtle metabolic changes due to cell age.

In a recent study (Vernardis et al., 2013), an extensive overview of which will be provided in the present analysis, our group built on those results and used metabolomics and metabolic network analysis to determine characteristic metabolic patterns for the different phases of both laboratory and manufacturing scale perfusion systems. The main objective was to investigate whether comparative metabolomics across reactor scales could indeed provide information not directly obtained from the prime variable measurements as to whether the time course of the metabolic physiology is independent of reactor scale. The analysis as described below under the name Analysis 1 confirmed this hypothesis, which is sequence validates the scale-down models, which are a key component in all design space studies.

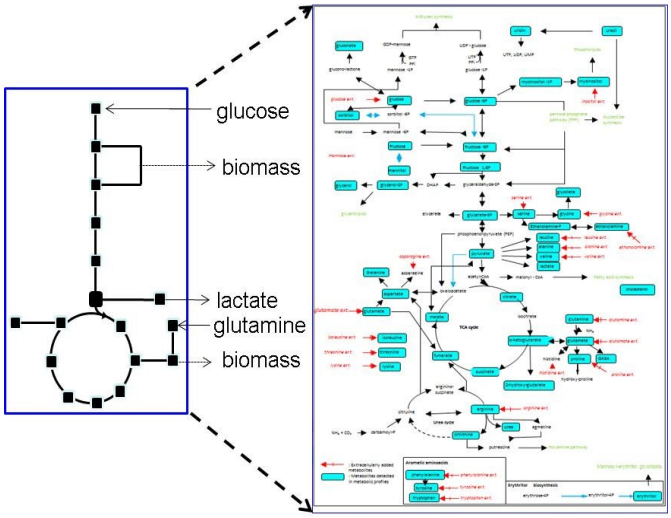


**Fig.1** The multi-compound metabolic profile provides higher resolution perspective of the cellular metabolic physiology than the prime variable measurement set. On the right-hand side network, the metabolites that are quantified using GC-MS metabolomics are shown in blue.

A subsequent design of space (DOE) experiment, which was analysed using metabolomics, has been the most extensive industrial-scale study of this kind in perfusion cultures. Specifically, in this analysis, which will be described below under the name Analysis 2, five laboratory-scale reactors derived from the same manufacturing scale were subjected to various combinations of changes in pH, dissolved oxygen (DO), temperature (T) and cell specific perfusion rate (CSPR) at various points of the culture time course. Analysing this experiment using metabolic profiling will contribute to the investigation whether changes in the metabolic physiology induced by modifications in the growth environment of the cultures may be reversible and under which conditions. This is very significant in industrial cell culture engineering, and could define metabolomics as a validation tool for the consistency of cell culture physiology and process.

## 2. EXPERIMENTAL PROCESS

### *2.1 Analysis 1*

Two experiments, experiment 1 and experiment 2 (Fig.2) were applied to study cell age effect. Extensive details can be found in Vernardis et. al.,2013. Three laboratory-scale bioreactors, L5, L6 and L7, and four manufacturing-scale bioreactors were used, connected as shown in Figure 2. Manufacturing-scale bioreactors are of an order of magnitude larger than the laboratory-scale reactors. Samples were collected in the early, middle and late phase of the cultivation processes. It has to be noted that the two manufacturing scale bioreactors M5a and M5b are derived from the same laboratory scale bioreactor L7; M5a was inoculated in the early, while M5b in the late phase of the cultures. The boundaries of the cultivation phases are currently defined in an empirical way by dividing the culture process into three equal duration parts, as there is not direct information from the prime variable measurements indicating any characteristic changes at the phase transitions. Prime variable measurements indicate a consistent process throughout the culture time course (extensive information on the prime variable time profiles could be found in Vernardis et.al, 2013).

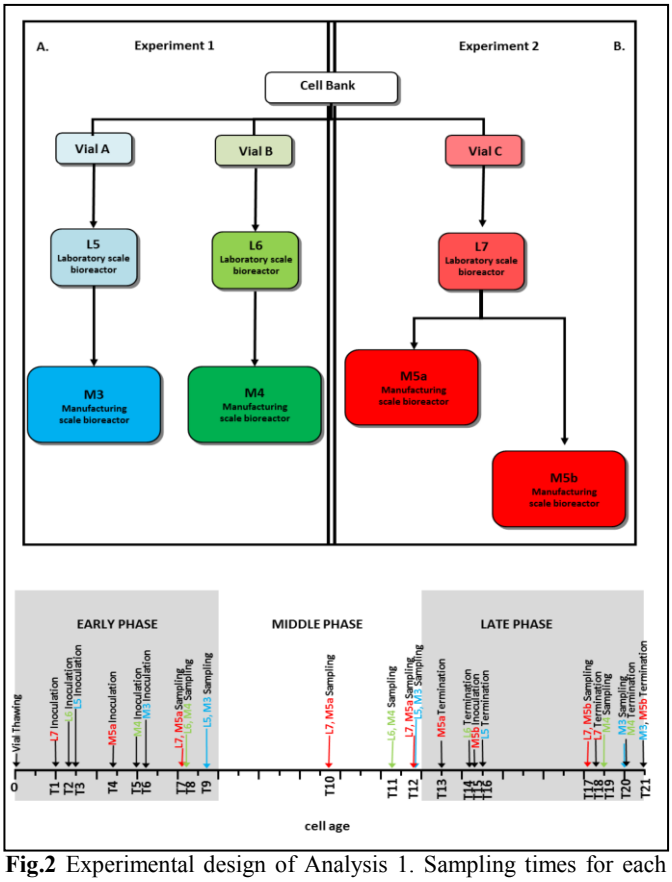


**Fig.2** Experimental design of Analysis 1. Sampling times for each reactor are shown at the bottom (the figure is identical to Figure 1 in Vernardis et. al., 2013).

### *2.2 Analysis 2*

Analysis 2 is a large-scale experiment, in which the pH, temperature, dissolved oxygen (DO) and/or perfusion rate (CSPR) in the growth environment of the cultures were varied over the course of the experiments using a Design of Experiments (DOE) strategy. The actual experimental design is shown in Figure 3. Four laboratory scale reactors were inoculated from one manufacturing scale, M6. L9-L11 were inoculated at the early phase of the cultures; L12 was inoculated from L11 at the early phase of the cultures too. L13 was inoculated at the middle phase of the cultures. pH, DO, T and/or CSPR were changed from their standard conditions at various points throughout the time course of the different cultures, as shown in Figure 3. The sampling times for laboratory scale reactor L10 are also shown. L10 metabolic profiling will be discussed in further detail below. Notably, samples were collected in the middle of the perturbation phase to allow for the cultures to physiologically adapt to the new environmental conditions. As it can be seen in Figure 3, in some cases the cultures were brought back to the standard conditions. These samples can be used to investigate whether the physiology of the cell cultures could return to the expected physiology for the particular culture age under standard conditions, even if it has been in the

meantime perturbed on a single or combination of growth environment parameters.

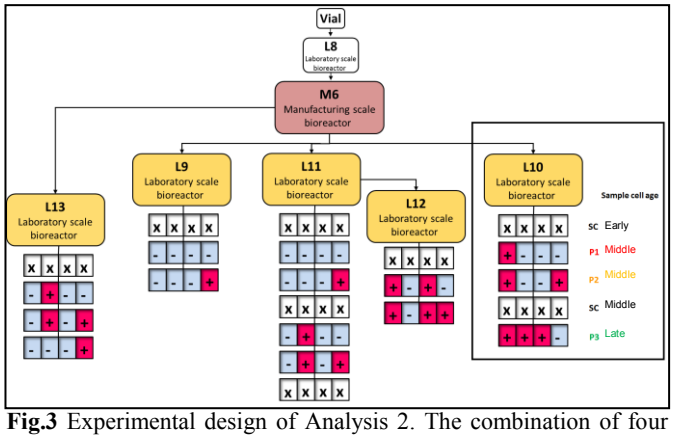


**Fig.3** Experimental design of Analysis 2. The combination of four boxes at each of the rows below the name of a laboratory-scale bioreactor corresponds to the combination of changes in the growth environment parameters in the following sequence: first box from the left: pH, second box: DO, third box: T and fourth box: CSPR. x, +, - in a box corresponds, respectively, to standard condition (white), increase (light blue) or decrease (red) with respect to the standard condition in the corresponding parameter. The sampling times of reactor L10 are also shown on the right. SC, P1, P2, P3 depict, respectively, the standard conditions and perturbations 1, 2 and 3.

### *2.2 Metabolomic data acquisition and normalization*

The dried polar metabolite extract of each sample was obtained from dried biomass samples shipped to FORTH/ICE-HT from Bayer based on a methanol/water protocol, as described in (Chrysanthopoulos et al 2010; Vernardis et. al., 2013). The extracts were derivatized to their (MeOx)TMS-derivatives followed by reaction with MSTFA and the metabolic profile of each samples was measured at least three times using a Saturn 2000 GC-(ion trap)MS (Varian, currently Agilent Inc.), as justified in (Kanani et al 2008, Kanani & Klapa 2007). The peak identification and quantification was carried out as before (Chrysanthopoulos et al 2010, Kanani et al 2008).

Metabolomic data validation, normalization and filtering were carried out as in (Chrysanthopoulos et al 2010, Spagou et al 2011). The relative areas of all identified peaks (RPAs) in each profile were estimated from their normalization with the 217 marker ion peak area of the internal standard ribitol. After normalization and filtering, the metabolic profiles used in the multivariate statistical analysis for the extraction of biologically relevant conclusions concerned approximately 70 metabolites in both analyses.

### *2.3 Metabolomic data analysis*

All applied multivariate statistical analyses were based on standardized (RPA) values. The standardized RPA of metabolite M in profile j, $stRPA_M^j$, is equal to: $stRPA_M^j = \frac{RPA_M^j - \overline{RPA}_M}{SD_{RPA_M}}$, where $RPA_M^j$, $\overline{RPA}_M$, $SD_{RPA_M}$ depict, respectively, the RPA of metabolite M in profile j, the mean RPA of metabolite M in all profiles and its standard deviation. Hierarchical clustering (HCL) (with Euclidean distance metric), principal component analysis (PCA) and analysis of differences (ratio) on the RPAs between experimental stages algorithms were used as implemented in version 4.8.1 of the open source TM4 MeV software (Saeed et al 2006, Saeed et al 2003). An extended view of the difference in the metabolic activity between two sets of samples was obtained by positioning the significant metabolites, color-coded appropriately, in the metabolic network of the BHK cells. A significantly expanded BHK metabolic network than that shown in (Chrysanthopoulos et al 2010) was reconstructed based on information from KEGG (Kanehisa et al 2012), MetaCyC (Caspi et al 2012) and EXPASY (Artimo et al 2012) databases, our metabolomic data and relevant literature.

## 3. RESULTS & DISCUSSION

### *3.1 Analysis 1: Phase transitions correspond to a characteristic shift in the metabolic profile of the cultures, which is independent of the bioreactor scale*

The time profiles of the prime variables in Analysis 1 indicate a consistent cell bank and a robust cultivation process between the two experiments comprising the analysis, throughout the time course of the culture and across bioreactor scales (Vernardis et. al., 2013). On the other hand, metabolic profiling analysis of samples at the various phases of the cultures revealed characteristic shifts in the metabolic physiology at the phase transitions, knowledge that cannot be assumed from the prime variable measurements. Figure 4 shows the heat-map of the metabolic profiles of Experiment 1 (left side of Figure 2) in Analysis 1 and the derived hierarchical tree. Interestingly, the early and late phase of the cultures, independently of the bioreactor scale, cluster together with respect to their metabolic profiles, while there is an overall decrease in the concentration of most metabolites in the middle phase. Metabolic profiling analysis of Experiment 2 (Figure 2) confirmed these findings (Vernardis et al, 2013).

This is a very important result as this metabolic shift at the phase transitions could be used as a diagnostic tool for defining the actual phase of a culture. Moreover, it shows that the time course of metabolic physiology changes in the perfusion cultures is indeed bioreactor scale independent. Finally, this is information that can be provided only from the metabolic profiles and not from the set of the prime variables, indicating that metabolic profiling could be used in extension of the prime variable dataset to monitor the cell culture physiology.

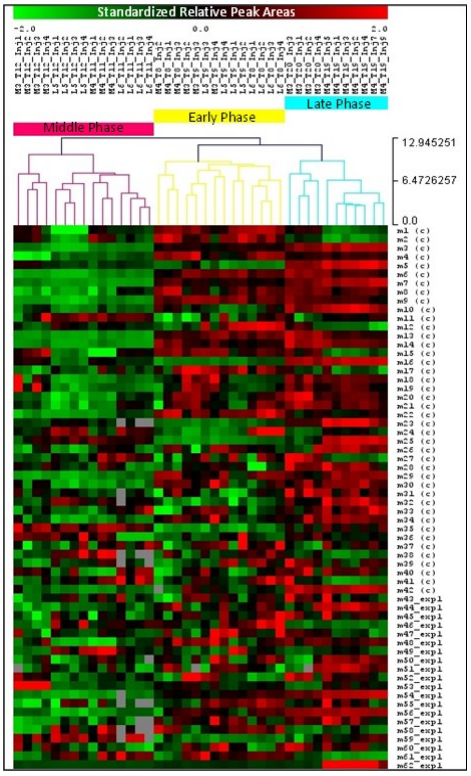


**Fig.4** Hierarchical clustering analysis of the metabolic profiles of Experiment 1 in Analysis 1 (Figure 2). There is a characteristic metabolic shift between the three phases of the perfusion cultures. Figure 4 is included as Figure 2A in Vernardis et al., 2013.

### *3.2 Metabolic Network Analysis reveals the biological basis of the metabolic shift from the early to the middle phase of the perfusion cultures*

Metabolic profiles should be visualized in the context of the metabolic network structure for correlations between metabolites to be understood and further biological conclusions to be derived. We have reconstructed an extensive metabolic network of the BHK cell line based on the mouse genome-wide metabolic network reconstruction, our metabolomic data, metabolic databases and the literature.

Figure 5 shows the results of SAM analysis of the metabolic profiles in Experiment 2 of Analysis 1 between the early and late phase in the context of the metabolic network. Specifically, the metabolites the concentration of which increased or decreased significantly in the middle compared to the early phase are shown in red and green, respectively.

Based on the fold change of the metabolite concentrations between the middle and the early phase, the metabolic shift of the cultures from the early to the middle phase is characterized by an increasing trend in citrate, malate and pyruvate (even though SAM analysis does not identify them as statistically significant). The particular physiological state that emerges in the middle compared to the early phase is characterized by (Icard & Lincet, 2012) as a "vicious cycle" between citrate, malate and pyruvate. This physiology coincided with a rather decreased rate of aerobic glycolysis and glutaminolysis required for cell survival and proliferation. These changes suggest potentially that the cell culture has been well-adjusted to the growth environment and more cell resources may be directed to lipid and protein synthesis and glycosylation. These differences are not directly apparent from the physiological data, including the production rate of the recombinant protein, further indicating the significance of metabolomics in providing this physiological information.

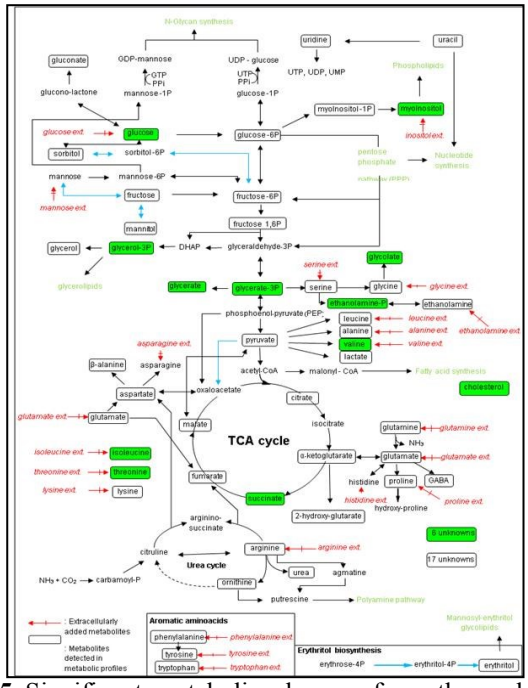


**Fig.5** Significant metabolic changes from the early to the middle phase of the Experiment 2 in Analysis 1. The metabolites the concentration of which is significantly decreased from the early to the middle phase according to SAM analysis are shown in green. The figure is included as Figure 5B in Vernardis et al., 2013.

### *3.3 Analysis 2: Can cell culture physiology return to its expected state at standard conditions even after a series of intermediate perturbations?*

Analysis 2 metabolic profiles are analysed based on the metabolic physiology shifts that were identified in Analysis 1. Knowing what the expected metabolic state would be under standard conditions in any of the phases of the perfusion cell culture, the deviations from this due to any intermediate perturbations of any growth environment parameters from their standard value could be determined. In this study, we demonstrate the results of the analysis of samples from reactor L10. All perturbations applied on L10 are characterized by high pH. The first two perturbations taking place in the middle phase of the culture combine a higher pH with a lower DO and temperature (P1 and P2). The difference between P1 and P2 is, respectively, a lower vs a higher than its standard value CSPR. L10 is returned to its standard conditions at the end of what is expected as the middle phase of the cultures under standard conditions. Subsequently, its growth environment is perturbed towards a higher than the standard pH, DO and temperature with a lower CSPR. The last perturbation is taking place in the late phase of the culture. Figure 6 shows the PCA graph of the metabolic profiles from all metabolic states of L10. According to this graph, the metabolic profiles of the culture

at standard conditions in the early and middle phase cluster together at a distance from the metabolic profiles characterizing the perturbed states. Notably, the culture under higher DO and T shows opposite with respect to PC2 profiles (P1 and P2) from the case of lower DO and T (P3). Moreover, the profiles of P1 and P2 do not differ much, indicating a lighter effect of the change in the CSPR under the particular perturbations in the other three parameters. The difference between the metabolic profiles of the standard conditions should be compared to the metabolic shift expected between the culture phases as determined in Analysis 1. In this way, we could determine whether the culture reaches the expected "well-adjusted" middle-phase physiology when returning to the standard conditions, despite the intermediate perturbations or it resembles more the "recently inoculated - early" physiological state.

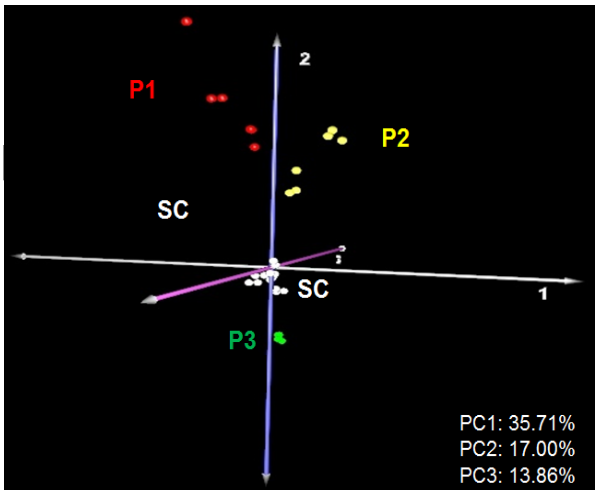


**Fig. 6** PCA graph of reactor L10 metabolic profiles (Figure 2). SC, P1, P2, P3 depict, respectively, the standard conditions, perturbation 1, 2 and 3.

Already, however, it can be concluded that the analysis of the DOE experiment using metabolomics could provide valuable information about the changes in the metabolic physiology that perturbations in the growth environment parameters could induce on the culture. Moreover, metabolic profiles could validate whether the culture "returns" to its expected physiology at standard conditions even after a single or a series of intermediate perturbations and which these perturbations are. This is very important for the industry, especially when it has to prove that the culture process is consistent despite small-scale and usually short-term changes in the growth environment.

Comparative metabolomic analysis of all reactors in Analysis 2 will provide enhanced information regarding the effect of changes in the growth environment on the metabolic physiology of the perfusion cultures.

## 4. CONCLUSIONS

Metabolic profiling can indeed enhance the prime variable dataset for the monitoring of mammalian cell perfusion cultures, by providing a higher resolution view of the metabolic phenotype. Metabolomic analysis identified the metabolic physiology shifts characterizing the phase transitions in this type of cultures, providing strong evidence that cell age related metabolic activity changes were bioreactor scale independent. Multivariate statistical analysis of the metabolic profiles indicated an overall decrease in the intracellular metabolite concentration during the middle phase compared to the early and late phases. In this context, metabolic profiling could be integrated into the monitoring of cell physiology in perfusion cultures. The acquired results are expected to extend well to the CHO and other industrial cell lines in the same type of reactors. No significant differences in the metabolic physiology are expected between two immortal mammalian cell lines from the same organism (i.e. BHK and CHO). Additionally, metabolic profiling and network analysis can increase the information content of process development experiments by helping better understand the impact of changes in bioreactor operating conditions on cell physiology. We believe that the cell culture engineering community should aim at furthering its understanding of the metabolic physiology of cancer cells, as this knowledge will have a major impact in the design and monitoring of optimal cell cultures.

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