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Determination of Best Nutritional Conditions for a Monoclonal Antibody-Producing Cell Line based on a Multivariate Data Analysis Approach

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Abstract

The design of mammalian cell culture processes as technological platform for monoclonal antibody (mAb) production is a complex task mainly due to partial knowledge of culture media composition impact on process outcomes. Faced with this problem, the present work aimed to characterize the metabolic profile during the early culture at lab-scale of a specific cell line transfected to obtain a monoclonal antibody (mAb) of therapeutic interest in the treatment of cancer, seeking most favorable nutritional conditions. The experimental design, based on the use of four different media in a two-liter scale culture, provided data on the content of 19 metabolites, cell concentration, and mAb concentration over the course of batches, where in the first case measurements were performed with liquid chromatography-mass spectrometry (LC-MS) as an advanced laboratory analytical support. The corresponding data-driven models, as a result of integrating Principal Component Analysis (PCA), Soft Independent Modeling of Class Analogies (SIMCA) and Partial Least Square Regression (PLSR) methods, revealed the actual difference among media regarding cell culture metabolic progression, and allowed to estimate cell growth behavior and mAb generation relative to biomass metabolites composition. Consequently, such an approach facilitated defining the metabolites that benefit the aforementioned cell culture process and those with a negative effect, as well as the choice of media that ensure the best nutritional conditions under technological and economic bases, thereby providing the essential elements for further media optimization.

Index terms— mammalian cell culture, metabolic profile, data-driven modeling, principal component analysis.

1 I. Introduction

he use of mammalian cells to produce monoclonal antibodies (mAbs) has become a widespread practice in the biotechnology domain because of its ability to largely achieve posttranslational modifications and protein folding. However, from an engineering point of view, the greatest obstacle in designing culture processes including these cells is their high complexity, as currently there is partial knowledge of laws governing such phenomena. On one hand, it shall be taken into consideration the significant amount and intricate sequence of biochemical reactions at the intra and extracellular level, while on the other hand cell environment operational conditions have also their impact on culture process performance regarding product-required quality [1].

A key issue to consider at first is the influence of media metabolites content on cellular growth and mAb generation along the process. As a sound strategy, focusing on culture metabolic profile could start at small scale, leaving the inclusion of cell environment operational variables for further studies at gradually larger scales, where fluctuations of these are better detectable and meaningful to establish culture process state [2,3].

In such research, the design of experimental plans combined with the use of multivariate data analysis (MVDA) tools has shown its advantages, by facilitating the development of data-driven models that integrates input and output variables in all its interrelation complexity, hence providing comprehensive process variability characterization and prediction [4,5].

There is a wide range of MVDA applications that has been described in the biotechnological domain, for instance: cell culture process scales comparability [6,7]; determine the relationship between process parameters and critical quality attributes [8,9]; feeding strategies for metabolic control and improving process robustness [10,11], among others. In addition, MVDA is currently recognized as a useful mean to analyze genomic and proteomic data, as it provides the tools for a significant complexity reduction in data processing [12][13][14]. Yet, regarding the implementation of MVDA in metabolic studies and media selection, there are still a discrete number of published references that manage to analyze a significant spectrum of metabolites [15][16][17].

Such praxis is in full correspondence with Quality by Design current paradigm as appointed by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Year 2023 in the Q8, Q9, and Q10 guidelines, where process understanding acquires a key role for assuring an effective scaling-up exercise toward a successful technology transfer [18].

The present work exposes a MVDA approach for characterizing metabolic profile during the early labscale culture of a specific CHO-K1 cell line transfected for obtaining a mAb under study in a two-liter bioreactor, thus determining the most advantageous culture nutritional conditions. Results derived from this research provide a valuable knowledge that shall contribute to subsequent studies as a continuity for achieving culture media optimization and further process scale-up.

2 II. Materials and Methods

3 a) Cell Culture Experiment

A transfected mammalian cell line CHO-K1 developed for expressing the mAb of interest was cultured in a two-liter volume APPLIKON bioreactor equipped with an automatic process control system, using four different protein-free and serum-free media identified as M1, M2, M3 and M4. Main chemical composition of each medium is summarized in Table 1: The operation of each run was carried out in batch mode, starting from an inoculum with a concentration greater or equal to 0.4 x 10 6 cells/ml in the medium previously loaded in the bioreactor, and then the process was allowed to carry on until viability was less than 50%. Cell environment culture conditions were set as follows: temperature at 37 ± 1°C, dissolved oxygen at 40 ± 10% and pH at 7.2 ± 0.2, as well as an agitation impeller tip speed kept at 1 m/s and aeration rate between 0.005 -0.0075 vessel volumes per minute (vvm).

4 b) Analytical Support

Several measurements were obtained off line from culture supernatant samples taken over the course of each batch:

? The concentration of metabolites was measured through a Liquid Chromatography-Mass Spectrometry (LC-MS) analytical method. The equipment configuration was composed of Heater Electro Spray Ionization source, ORBITRAP detector (AGILENT, USA) and ZIC-pHILIC column (MERCK MILLIPORE, Germany). All standard reagents used for quantification of the 19 metabolites involved in the process were from SIGMA ALDRICH (MERCK MILLIPORE, Germany).

? The mAb concentration (IgG) was determined by an own-developed ELISA sandwich method. In summary, 96-microwell plates were previously coated with human PD-1 and kept overnight at 4°C. Next, the samples and the standard were added to the plates and incubated at 37°C for one hour. Subsequently, an anti-Human IgG antibody conjugated with alkaline phosphatase was added to the plate and incubated at 37°C for one hour. Then, p-Nitrophenyl phosphate substrate was added to the plate and after 30 minutes, the plates were read by means of a spectrophotometer (JASCO, Japan) at 405 nm. ? Concentration of cells (X) was obtained from visual counting through optical microscopy, using the trypan blue dye exclusion method in a Neubauer chamber (MARIENFELD, Germany).

5 c) Data Preparation

All batch measurements collected over time were organized in a single two-way data matrix of 756 elements in a Variable-Wise Unfolded (VWU) array, Year 2023 where each column is a single variable, and each row contains measurements for the variables at a specific time point in correspondence to batches [19,20]. Notation of scored samples (S) represents first number as the specific culture medium in the batch, and second number as the time instant (T) of sampling (for example, S21 score is the first sample taken at time T1 of batch run using M2). The work matrix can be seen here.

6 d) Data Processing

MVDA was applied with the following sequence [21]:

? Use of descriptive statistic and run chart graphics for a preliminary look to the dataset in order to identify variable fluctuations, tendencies and potential correlations between them. ? Data auto-scaling standardization (ratio of centered mean and the standard deviation) in order to avoid prevalence of variables due to their magnitude. ? Use of Principal Component Analysis (PCA) for dimensionality reduction in a few independent latent variables or Principal Components (PC) in order to differentiate input variables according to their real impact on process variability and probable correlation between each other, as well as identification of score's trends.

7 ? Use of Soft Independent Modeling of Class

Analogies (SIMCA) method [22,23] to confirm differences among nutritional media according to batches progress. ? Use of Partial Least Square Regression (PLSR) to find the potential interrelation between cell and IgG concentration as output variables vs. supernatant metabolites content as input variables, focusing on data from the exponential phase of batch cell growth.

It should be noted that given the limitations to replicate experiments in this early stage of development, an internal full cross validation (leaves out only one sample at a time) procedure was applied to appraise PLSR model ability of estimation rather than prediction, which is admissible for the purposes of the present work [21,24].

An available UNSCRAMBLER X version 10.4 software (CAMO Software AS) was used to run the above MVDA methods, which does not mean a preference among other applications.

8 III. Results

A first look at batch culture metabolic dataset by applying descriptive statistics and run charts showed that all measured metabolites could be relevant for the study, as they exhibit a substantial concentration variability that can potentially impact cell culture performance, also noticing certain degree of correlation between metabolites, which in some cases is considerable. Fluctuations detected in those univariate graphs also contributed to multivariate analysis subsequently.

Additionally, it is also detected a difference of magnitude among metabolites concentration, more significant in the case of Glucose and Lactate (see a summary of univariate statistic graphs here). Since other metabolites can have a greater influence even at lower proportion in the culture as known elsewhere [25], data were standardized via auto-scaling in order to assure a proper balance among variables.

9 a) Characterization of Process Metabolic Progression through PCA

A model expressed in three principal components points out as a proper choice, covering around 90% of data variance in calibration and about 86% in validation, as shown in Figure 1a. In addition, no outliers were detected, as can be noticed in Figure 1b. Hence, such PCA model can be considered as representative of culture metabolic variability and adequate for further analysis. From Figure 2a it can be appreciated that those metabolites consumed throughout the batches, such as Asn, Gln, Leu, Lys, Met, Ser, Thr, Val, Gluc, Pyr, and produced as Gly, can be grouped in PC-1, having correlation loadings outside the margin of ± 0.7, which in regular practice is indicative of the greatest contribution to process variability. Furthermore, consumed metabolites show a strong correlation among each other. On the other side, it was found that PC-2, being the second major contributor to process variability, includes other metabolites such as Phe, Pro, Arg, Ile and Asp, having a notable disparity in initial concentration due to media differences in composition, as can be seen in Figure 2b. Moreover, in the same figure is observed that Lactate and Ala metabolites, which are first produced and later consumed during the batch course, are gathered in PC-3, with a less important relative impact on cell culture variability.

As a complement to the above, the score plot in Figure 3a shows a well-defined trajectory of the batches from right to left along PC-1 axis, with no remarkable differences in metabolites consumption or production patterns among nutritional media.

Concurrently, looking in the direction of PC-2 axis, batches using culture media M1 and M2 are very similar in tendency, as well as those using M3 and M4, both trends being distinguishable between each other. In addition, Figure 3b A simultaneous view of both, scores and correlation loadings graphs, combined as a bi-plot in Figure 4a and Figure 4b, shows that culture samples are rich in those metabolites consumed since batches start, while Gly as the one produced, reach its higher concentration at the end of the culture.

Looking through PC-2 axis it is more evident that M1 and M2 have a significant initial content of Phe and Pro, as well as M3 and M4 in Arg, Ile and Asp.

Further, Lactate and Ala come to their highest concentration in the middle of culture batches, being corroborated its production at first and consumption later on. Metabolite concentrations relationship with cell and IgG concentrations was analyzed via PLSR using data from the exponential phase of batch cell growth, given its relevance in cell culture process [26]. Consequently, a logarithmic transformation of cell growth data was applied looking for an approximation to a linear behavior.

Following PLSR procedure, a Martens uncertainty test together with full cross validation was applied in order to find input variables with more significant impact on model's response [27,28]. In this regard, Figure **??**a and Figure **??**b show in striped-shaded Through the Coomans plot shown in Figure 5, it is confirmed that media M1 and M2 are segregated into different classes and at the same time are quite distinct to media M3 and M4, while the latter are rather similar. Such result is consistent with the fact that media M1 and M2 share 30% of their initial composition, whereas M4 is the same medium M3 modified with some additives. The correlation loadings X -Y plots shown in Figure 8a and Figure 8b, reassert that incidence pattern of metabolites correlated with the output variables is comparable with the obtained from PCA model as well. It is also noticed that cell growth is closely interrelated to mAb generation, as evidence of a substantial interaction between them.

Complementarily, Figure **??** summarize those key metabolites influence on cell culture performance, based on Martens uncertainty test likewise. From the analysis integrating both Figures 8a -8b and Figure **??** it is inferred that metabolites linked to first factor, Lys, Leu, Ser and Gln contribute to cell growth and mAb generation as they are consumed, while Val only contributes to cell growth. In the case of those associated to second factor and related to initial concentration in media, Phe has a positive effect on cell growth, whereas Arg and Ile have a reverse effect on both cell growth and mAb production. In view of these findings, extra experiments shall be done to consolidate knowledge on the actual influence of their initial concentrations in the culture. Concerning Lactate metabolite linked to third factor, it does not show a significant incidence on cell growth, but on mAb concentration in a negative way, which shall be discussed later.

10 IV. Discussion

The above results, derived from early lab experimental work, depict the first insights into this particular cell culture system, in correspondence with its inherent metabolic complexity.

It was found that Lys, Leu, Val, Gln, and Ser metabolites have a major impact onto this cell culture process. In the case of Lys, Leu and Val, they are consistent with their role of being essential amino acids, in conformity with current knowledge so far [25]. Therefore, depletion of this substances could take place during the course of cell culture batches, and given the cells inability to produce them, the culture could end the exponential growth phase prematurely. Likewise, Gln is widely known as a key metabolite in mammalian cell culture due to its important function as a source of carbon and nitrogen, in addition to the influence it exerts in delaying cell death [29]. Concerning Ser metabolite, is also known to be relevant for cell metabolism. A deficiency on this metabolite in the culture can trigger several negative scenarios causing an imbalance in the tetrahydrofolate cycle, which is detrimental to cell growth [30]. Moreover, absence of this metabolite can bring on phosphatidylserine formation, a component involved in signaling and detection of cell death by apoptosis.

On the other hand, it was also found Arg, Ile and Phe metabolites as the second major contributors to cell culture behavior regarding their initial content in culture media. In fact, culture performance depends on cells capability to sense somehow nutrients availability at batch start, thereby stimulating the metabolic interactions that lead to primary growth and mAb generation concurrently. Hence, in the specific case of Arg and Ile it should be elucidated if their concentration at start exceeds the limit that leads to culture inhibition in further studies, which shall also include Phe in search of media optimization.

In regard to Lactate, it is well known that glucose/glutamine metabolism leads to formation and accumulation of this metabolite, which is more accentuated in cell culture batch mode [31]. Although in this cell culture system has a minor effect on cell growth, it shows a significant negative impact on mAb generation. This could be due to the potential effect of Lactate to divert cells specific metabolic pathways that subsequently lead to a decrease in its specific productivity [32].

Comparing these results with those obtained in other references, the substantial diversity and variability in CHO cell culture process is corroborated [33][34][35]. In some cases, a specific amino acid is relevant in a positive way, while in others is quite the opposite, or does not impact the process at all in certain cases. It may even be the case that most favorable cell culture nutritional conditions to ensure maximum cell growth may not necessarily be the best for cell productivity and product quality [36]. Thence the importance of proper culture media optimization based on cell specific nutritional profile understanding.

Given the difference among culture media derived from the above results, it can be deduced for this cell culture process that M1 provides most favorable nutritional conditions in terms of the content of those amino acids found as key contributors, followed by M4 in order. Paradoxically, from the economical point of view M1 have the highest unit cost, while M4 have the lowest. Hence, the alternative of using M4 becomes attractive if supplemented with Leu, Lys, Val and Phe in similar proportions as in M1, because it already has similar contents of Ser and Gln.

It is recognized that predictability of the MVDA models used for analysis is limited given the lack of additional data for performing an external validation. Nevertheless, the internal validation carried out on such models with the available dataset evidenced they have an adequate estimation ability to provide, in this early research, valuable insights on the cell culture system in question, thus assuring the necessary groundwork for further studies.

11 V. Conclusion

The applied MVDA approach showed its potential by providing advanced data processing tools for achieving this study. It facilitated the understanding of metabolic variability in this particular cell culture process in batch mode at an early experimental stage, as well as disclosing the difference among culture media according to their nutritional effect on batches. In addition, the PLSR model derived from the available dataset contributed to identify those key metabolites that benefit cell growth and mAb production and those with a negative incidence, thus giving a rationale for the proper choice of culture media with most advantageous nutritional conditions. Finally, these outcomes offer the essentials needed for subsequent media optimization, which shall consolidate future scale-up studies.

12 Funding

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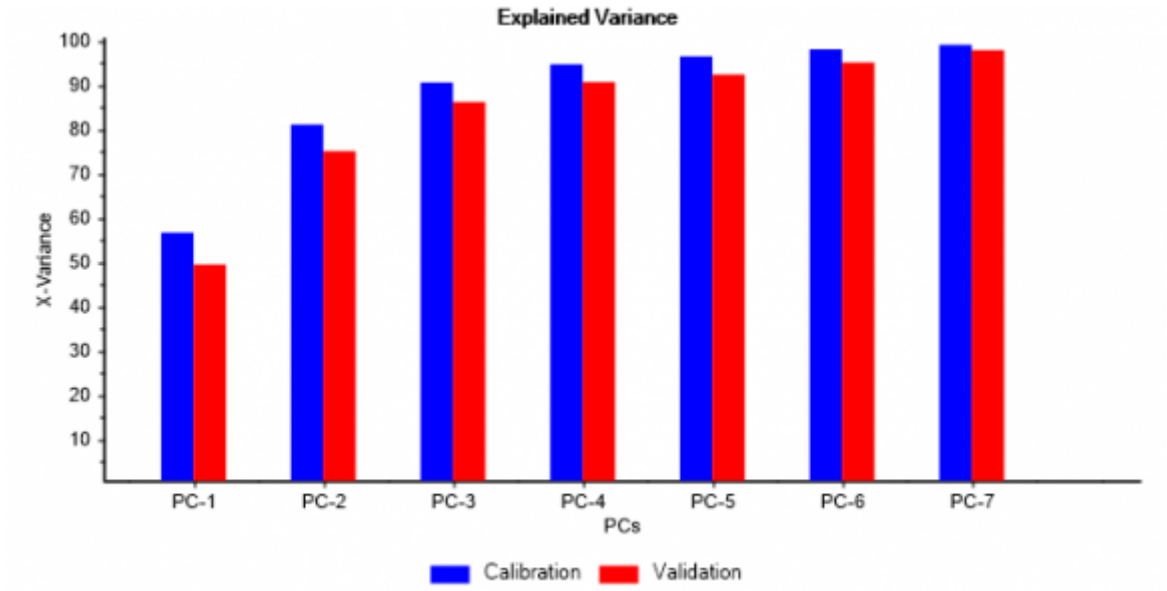

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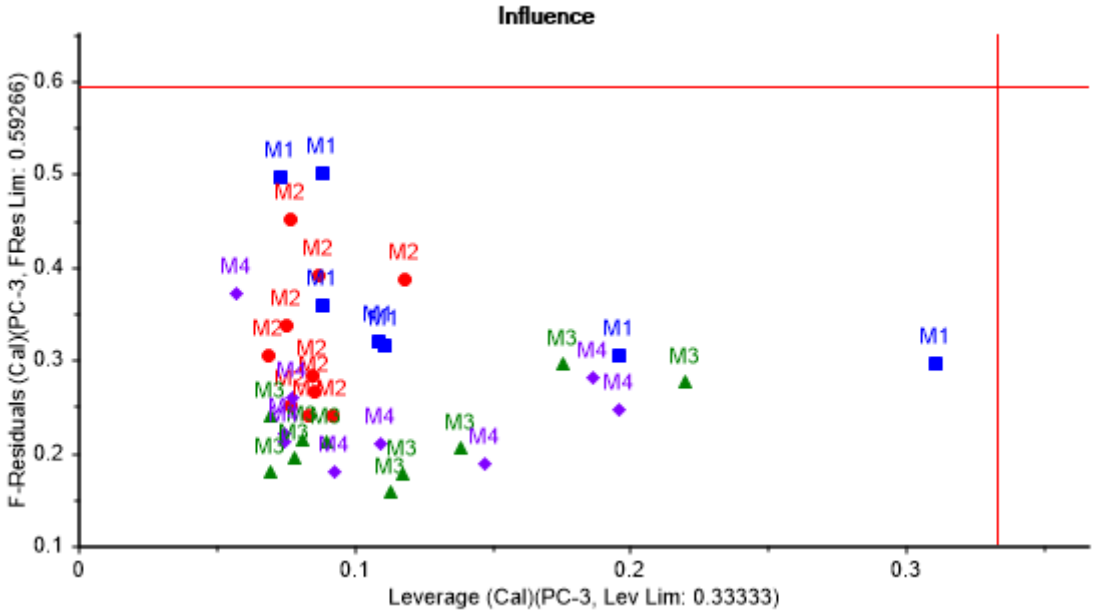

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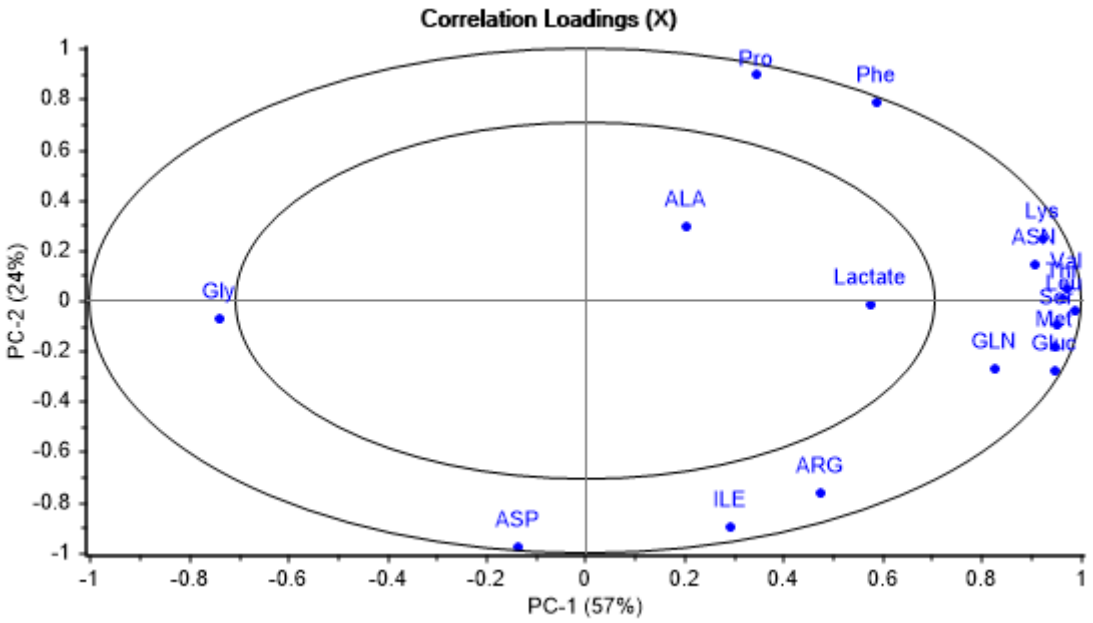

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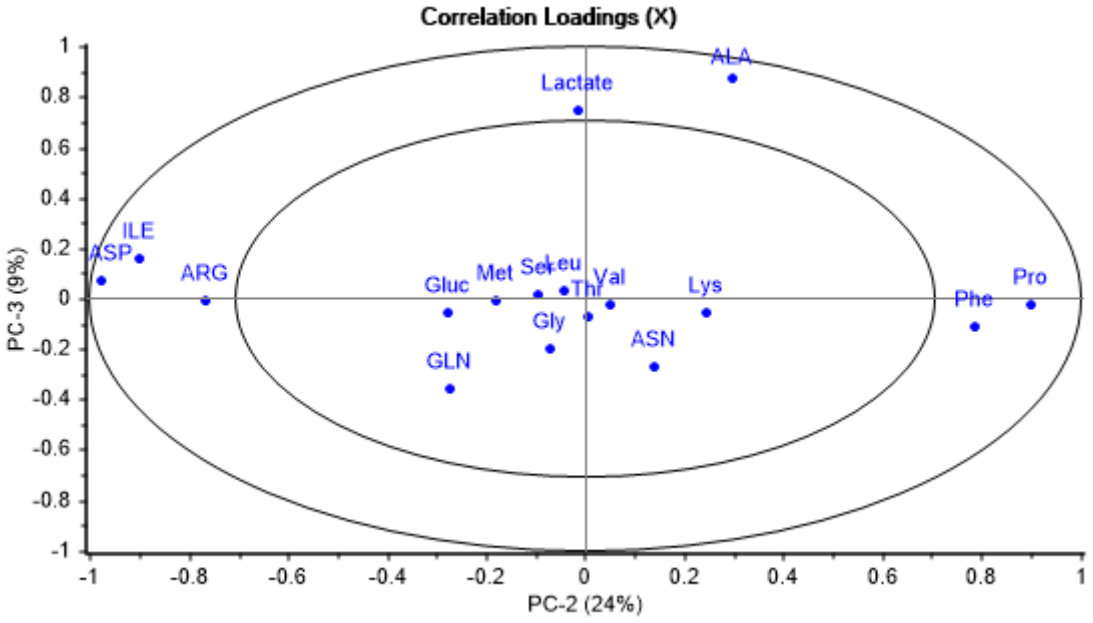

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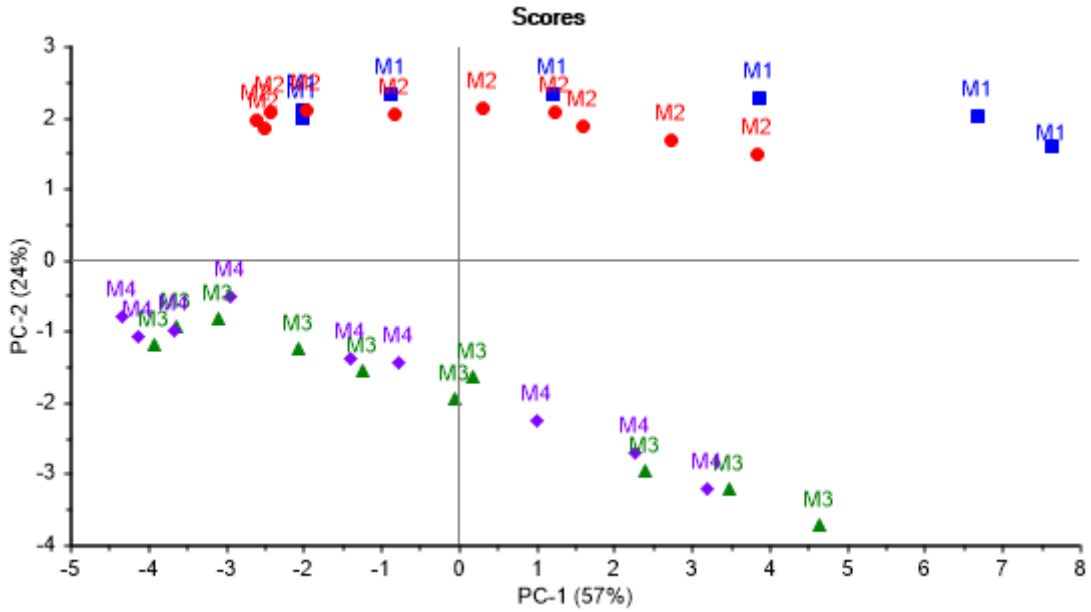

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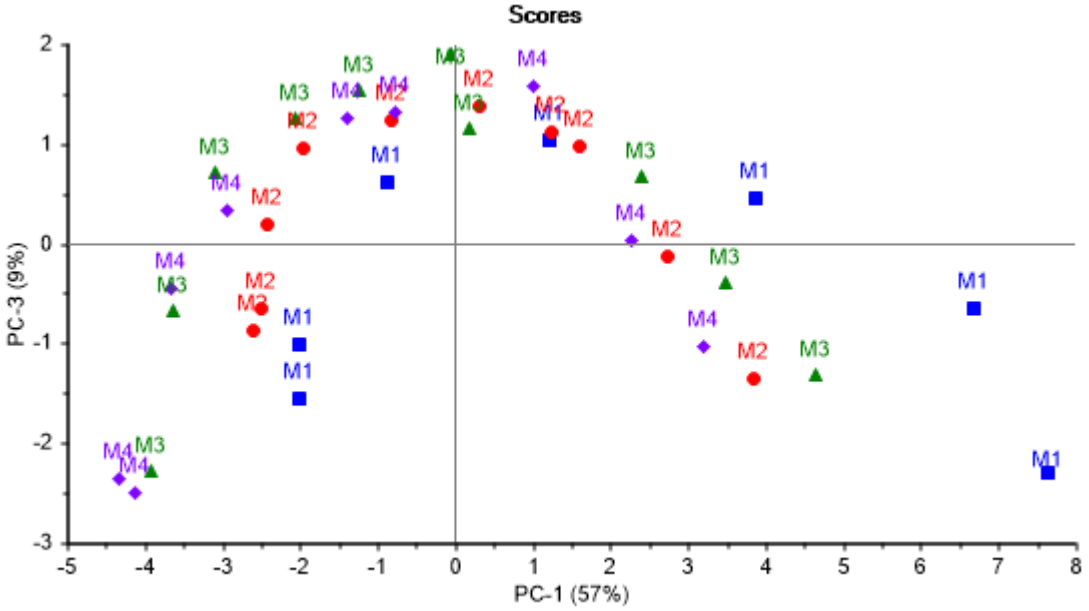
Figure 5: Figure 5 :



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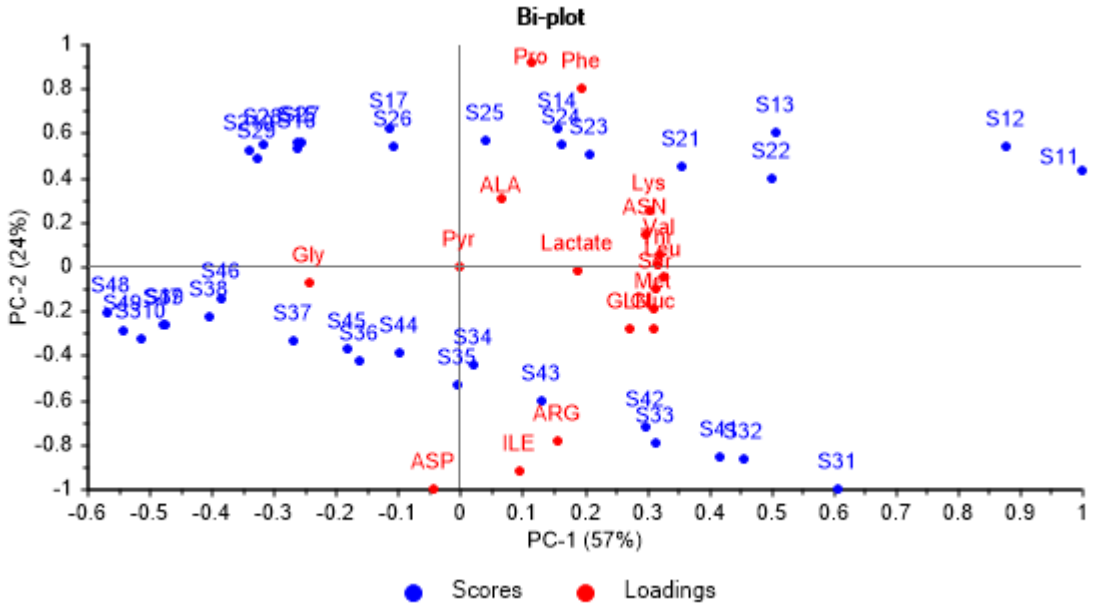

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Figure 7: Figure 6 :Figure 7 :

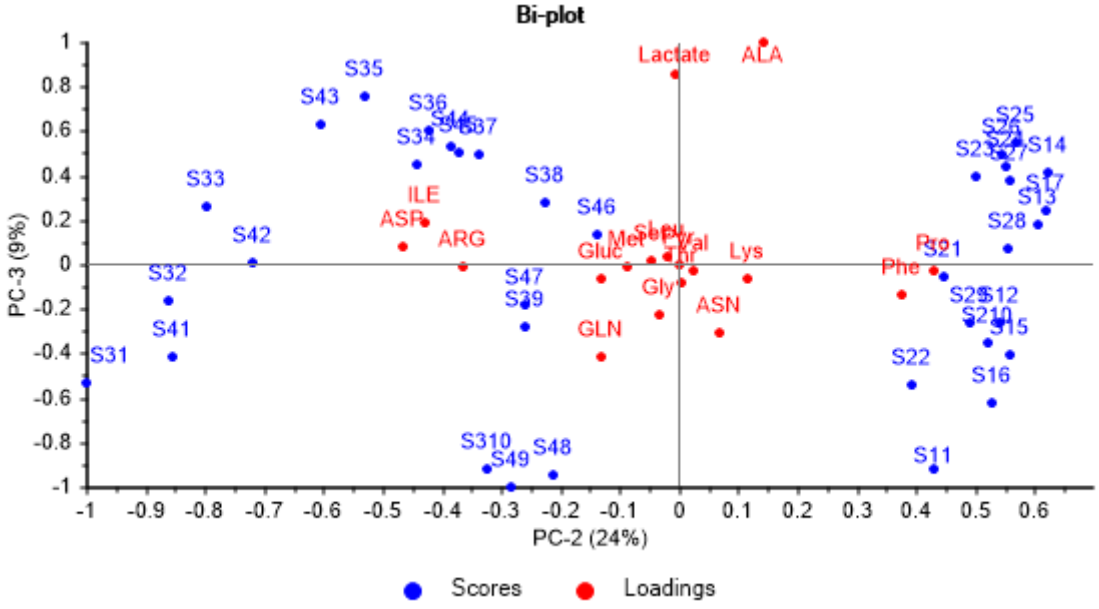

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Figure 8: Figure 8 :

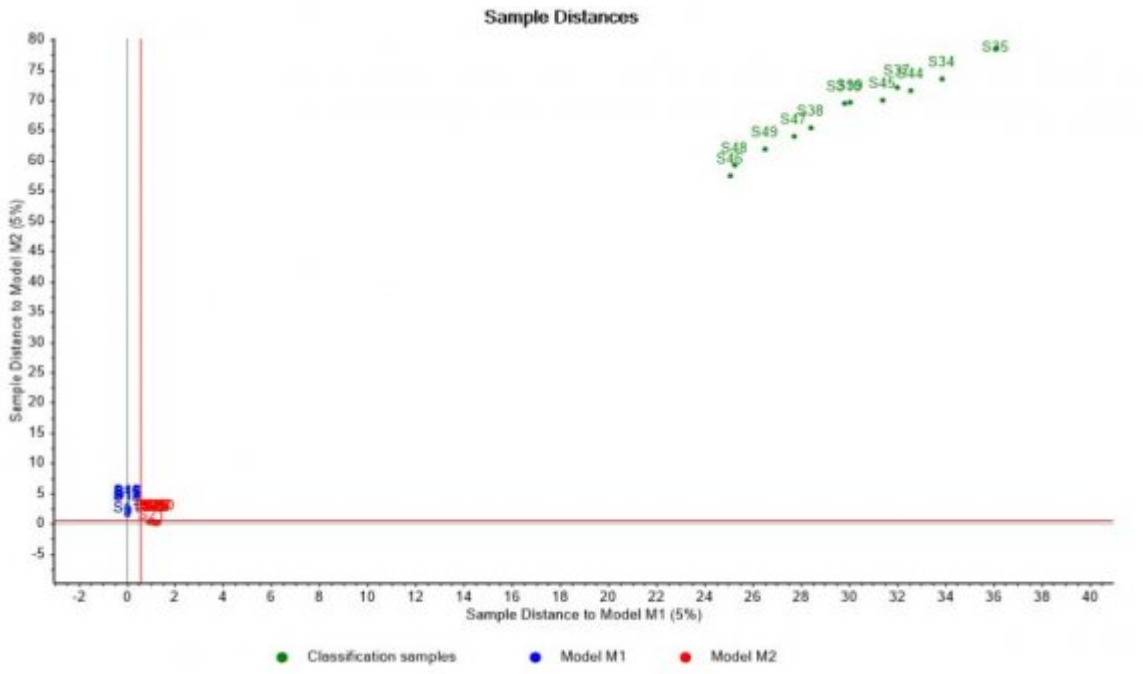

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Figure 9: Figure 9 :Figure 10 :

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Components	Medium M1	Medium M2	Medium M3	Medium M4
Ala	1.052	1.311	0.952	0.895
Arg	1.562	1.356	1.792	1.640
Asn	3.727	1.371	1.512	1.363
Asp	0.028	0.020	0.702	0.687
Gln	4.236	2.507	5.133	3.745
Glu	0.396	0.514	0.548	0.525
Gly	0.261	0.411	0.549	0.605
Ile	0.104	0.054	1.690	1.539
Leu	2.304	1.359	1.836	1.573
Lys	1.806	1.209	1.367	1.172
Met	0.471	0.298	0.439	0.374
Phe	0.775	0.431	0.051	0.044
Pro	1.207	1.210	0.084	0.089
Ser	1.510	1.373	1.449	1.324
Thr	1.184	0.572	0.835	0.713
Val	1.786	0.913	1.278	1.038
Pyr	1.543	1.044	0.973	0.666
Gluc	24.182	10.887	20.138	17.348

Figure 10: Table 1 :

Figure 11:

.1 Acknowledgments

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.2 Conflicts of Interest

The authors have no conflict of interest to declare regarding this research. ? Complexities derived from the analysis of such a volume of experimental data are managed effectively by applying a multivariate data analysis approach to attain data-driven models, which lead to key findings that contribute to understand the metabolic behavior of this particular cell culture process. ? Best nutritional conditions determined at this stage provided the necessary groundwork for subsequent culture media composition optimization. In addition, such practice can be generalized to deal with similar high complex research of this kind in the biotechnological domain.

.3 Abbreviations

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