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Original Research Article

Revealing metabolic performance of chinese hamster ovary cell capable of producing erythropoietin by *in silico* analysis

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ABSTRACT

Erythropoietin (EPO) is one of the most important hormones in the human body, due to its effective pharmaceutical performance, recombinant human EPO (rhEPO) is often produced by Chinese Hamster Ovary (CHO) cells as host cells through recombinant DNA technology on an industrial scale. In this study, the reported genome-scale metabolic network of CHO cells was upgraded to integrate EPO production pathways using the INIT algorithm in the RAVEN Toolbox. After quality analysis for the reconstructed model, performance of the model was examined under two different culture conditions provided within the literature. Such analysis were implemented through Flux Balance Analysis (FBA) and Multi-objective Analysis techniques and the results highlighted the effectiveness of these culture conditions. To enhance the efficiency of rhEPO production, analysis of essential genes and reactions, sensitivity of essential amino acid supplementation and flexibility of amino acid uptakes was also performed through a series of standard *in silico* techniques in constraint-based analysis.

***Keywords*:** EPO production, genome-scale metabolic model, constraint-based analysis

INTRODUCTION

Erythropoietin (EPO) is a hemopoietin hormone which is secreted by liver in fetus or by kidney in adults, in response to a low hematocrit or hypoxia in the bloodstream. EPO is used as a therapeutic agent in the treatment of conditions in which the normal secretion of the hormone is limited because

of kidney damage [1]. It plays an important role in producing Red Blood Cells (RBCs) and is a secretory glycoprotein belonging to a large family of the growth factor proteins [1,2]. When an adult suffers from cellular hypoxia, EPO is secreted by kidney cells as a stimulant to increase the production of RBCs in the bone marrow until the oxygen concentration in the target cells or tissues return to normal level by the circulatory system [1]. Due to the key role of EPO in response to hypoxia, since 1989, the Food and Drug Administration (FDA) has licensed the clinical use of recombinant human EPO (rhEPO) to treat the anemia in Chronic Kidney Disease (CKD) [1,3,4]. Nowadays, rhEPO is known as a performance-enhancing drug [5] because it is beneficial not only in the treatment of CKD but also for other diseases, including anemia in myelodysplasia [6] and anemia in cancer chemotherapy and radiation [7]. In recent decades, the advances in recombinant DNA technology have provided to produce exogenous rhEPO, called Erythropoiesis-Stimulating Agents (ESAs), in cell cultures [8].

Selection and preparation of a right host for appropriate cell culture is the first and most important step to achieve high quality and efficient yields of ESAs. Human genes related to recombinant therapeutic proteins are commonly duplicated and replicated into cell cultures created by mammalian cells due to their high productivity and capability for producing biopharmaceutical products [9]. Mammalian cell lines have been extensively used for the production of complex therapeutic proteins and monoclonal antibodies due to their ability to properly glycosylated recombinant proteins of human or mammalian origin. Chinese Hamster Ovary (CHO) cells are one of the achievable mammalian cell lines that are frequently used as the commercial host cells for significant production of ESAs on an industrial scale [2,8,9,10]. Epoetin alfa and Epoetin beta are two anti-anemic agents obtained from CHO cell-derived rhEPO that are often used to treat anemia arising from CKD and cancer chemotherapy and radiation [8].

Meanwhile, CHO cell growth has met severe problems in bioreactors due to the non-optimal growth conditions of the cells in the bioreactor. A mixture of essential growth factors has to be added to the cell cultures to ensure survival and growth of these cells as well while enhancing efficiency of rhEPO biosynthesis [11]. Accordingly, careful adjustment of exogenous growth factors in bioreactors is

a significant problem for scientists to acquire optimal growth conditions [12]. CHO cell growth depends on the concentrations of nutrients added to the culture medium [11,13]. Among main nutrients, amino acids play a fundamental role in synthesizing peptides, proteins, DNAs, and RNAs within the cell [14]. Generally there are twelve amino acids which can be regarded as essential: Arginine (Arg), Cysteine (Cys), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Histidine (His), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr), and Valine (Val), which all are L-amino acids [14,15]. However, CHO cells need the nine amino acids His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val as nutrients, because they cannot be synthesized by these cells [14]. In addition to the effect of specific amino acids on CHO cell growth, their amounts also affect the quantity and quality of EPO production [11].

It is well known that *in silico* modeling methods are reliable tools to predict and design the required conditions for optimal cell growth [16]. Nowadays, the development of mathematical methods has been facilitated to simulate the genome-scale metabolic networks of cellular metabolisms. Metabolic Flux Analysis (MFA) [17,18,19] and Flux Balance Analysis (FBA) [20,21] are two of these mathematical methods which are widely used for modeling metabolic networks containing metabolites and enzymatic reactions.

The first genome-scale metabolic network of CHO cells, comprising 1302 metabolites and 1540 reactions, was reconstructed [22] by adapting the existing metabolic model for mammalian cells [23]. Since CHO cell lines are also the host cells to produce the recombinant monoclonal antibody (IgG) [24], cell biomass and IgG manufacture equations were included within this CHO metabolic model as well [22]. Additionally, the mathematical model of rhEPO biosynthesis reaction was developed by including EPO production equation into the CHO metabolic network model by applying the MFA method [25].

To simulate human cellular metabolisms, a wide range of computational methods have been developed to reconstruct the tissue-specific metabolic networks for different types of human cells [26]. Integrative Network Inference for Tissues (INIT) is known as a powerful approach to reconstruct the Genome-scale Metabolic

models (GEMs) by analyzing the cell-type-specific data obtained from the Human Proteome Atlas (HPA) as the main source of evidence [27]. The INIT algorithm is an implementation of a Mixed Integer-Linear Programming (MILP) problem which use the Human Metabolic Reaction (HMR) database to construct template human metabolic model as well as applies available protein sequences in the HPA database to generate template human enzyme model as its input data. In a high quality model derived from this algorithm, all reactions can carry a flux and the synthesis of most metabolites becomes possible [27]. The RAVEN (Reconstruction, Analysis, and Visualization of Metabolic Networks) Toolbox is usually used to reconstruct the tissue-specific metabolic models in the framework of the INIT algorithm [28]. The RAVEN Toolbox is a complete software suite to generate, analyze, simulate, visualize, and run the GEMs within MATLAB, Mathworks. In order to apply biochemical constraints to the mathematical scaffold of the GEMs, Constraint-Based Reconstruction and Analysis (COBRA) methods are developed as a Toolbox within MATLAB [29]. FBA is the oldest COBRA method that is widely utilized for modeling cellular metabolisms [30]. However, there is an ever-growing trend in development and improvement of these methods [31].

In this study, the primary objective is to upgrade the genome-scale metabolic model of CHO cells with the capability of EPO production. After sufficient analysis of the quality of the reconstructed model, the performance was evaluated under two already mentioned culture conditions. Besides, the reactions that may raise or limit the EPO synthesis were identified and the performance of CHO cells in the trade-off between cell growth and EPO production was also evaluated. Then, the effect of the type and amount of amino acids on CHO cells expressing EPO will be investigated by a series of *in silico* modeling to investigate the physiology of CHO cells producing EPO.

MATERIALS AND METHODS

Upgrading CHO GEM

A generic metabolic network of CHO cells has been previously reconstructed and represented. The first version of the high quality model was released in 2012 by adapting a reconstruction of the metabolic network of CHO based on genomic and literature data which the model contains

1065 genes, 1545 metabolic reactions, and 1218 unique metabolites [22]. This model characterizes the CHO cell lines expressing recombinant monoclonal antibody (IgG). Therefore, in addition to overall metabolic reactions, it includes two balance equations representing biomass and IgG synthesis which both of these reactions mainly composed of the biosynthetic precursors and relevant cofactors with appropriate coefficients that are experimentally measured or obtained elsewhere for mammalian cells [22]. Meanwhile, Fernández and Chico, 2012, performed MFA to provide an explicit stoichiometric reaction for production of EPO [25]. The equation of EPO biosynthesis in the model is represented in follow in Eq. 1:

0.045ALA + 0.029ARG + 0.014ASN + 0.014ASP + 0.010CYS + 0.088GLN + 0.021GLY + 0.005HIS + 0.012ILE + 0.055LEU + 0.019LYS + 0.002MET + 0.010PHE + 0.019PRO + 0.024SER + 0.026THR+ 0.007TRP + 0.010TYR + 0.026VAL + 0.143F6P + 0.052G6P + 0.071AcCoA + 0.124UTP + 0.026CTP + 0.026HO + 0.071GTP + 1.480ATP + 0.026PEP → EPO + 0.030ADP + 0.452P2 + 0.043MAN + 0.021GLC+ 0.116UDP + 0.071GDP + 0.026CMP + 0.007UMP + 0.071CoA + 0.043GLUi Eq. (1)

Where: F6P: fructose-6-phosphate; G6P: glucose-6-phosphate; GLC: glucose; MAN: mannose; PEP: phosphoenolpyruvate; UMP, UDP and UTP: uridine mono-, di- and triphosphate, respectively. Standard three-letter codes are used for amino acids. The important precursors for production of EPO can be seen in Eq. (1). In addition, Table 1 demonstrates the stoichiometry of precursors (amino acids, glucose and oxygen) to produce 1 mmol biomass and 1(mmol) EPO. According to the table, 1(mmol) biomass requires higher supplements rather than 1(mmol) EPO synthesis.

The INIT algorithm [27] is used to reconstruct the genome scale metabolic networks. The INIT algorithm requires a connected template human metabolic model as input and protein sequences for the template models and for the model of the organism of interest. This template model was generated from the Human Metabolic Reaction database (HMR), containing the two existing genome-scale metabolic models, Recon1 and EHMN, as well as incorporating information from HumanCyc and KEGG [32]. The HMR database has a hierarchical structure in which the genes are at the top and are linked to information about their tissue specific expression profiles reported via BioGPS [32]. The protein sequences for the normal

liver tissue are provided from Human Protein Atlas (HPA) [27].

In this research, the EPO producing reaction is added to the previous CHO model using the INIT algorithm to characterize the EPO production rate. Accordingly, the demonstrative IgG synthesis equation is replaced with the balance equation of rh-EPO production. Then, the consistency of the model was analyzed by available tools within the RAVEN Toolbox [28].

Evaluation of Performance of the Model

The reconstructed model was simulated under two different culture conditions by the FBA method within COBRA Toolbox, MATLAB. The upper bound of target fluxes was adjusted according to the default values in the initial model [22,25] which provides two different conditions that are presented in Table 2. The further multi-objective analysis of the model to investigate the behavior of the EPO integrated reconstructed model was proceeded using ORCA Toolbox within MATLAB, 2013 [33].

Determination of Essential Genes and Reactions

The gene deletion and reaction deletion analysis was performed to realize if changing the objective affected the essentiality of any of the reactions or genes in the model. To model the deletion of the reaction *r* (or deletion of the gene associated with reaction *r*), we added another constraint, n*r* = 0, to the previous set of constraints. In three different investigations, to calculate the maximum growth, maximum EPO-production and relative contribution of biomass/EPO production (Biomass + 0.98 EPO=>), constraints for reaction fluxes mentioned in 1^{th} simulation condition are set on the exchange reactions in the FBA model. Then, the essentiality of each gene and reaction was examined. In our study, modeling gene and reaction deletion were performed by *singleGeneDeletion* and *singleRxnDeletion* functions in the COBRA Toolbox [35].

Investigation of Essential Amino Acid

Next, in order to investigate robustness of growth and EPO biosynthesis against perturbations in uptake of essential amino acids in culture media, the exchange flux of each essential amino acids is varied and the optimal objective value is calculated as a function of this exchange flux. This analysis shows how sensitive the objective is to a particular exchange reaction. Therefore, other exchange reactions are fixed according to constraints of the 1^{st}

simulation in Table 2 and in two separate analyses, the objective function was regarded as growth and EPO production, respectively. This analysis was performed by *Robustnessanalysis* scripts in COBRA toolbox in MATLAB [35].

Flux Variability Analysis for Amino Acid Reactions

Moreover, in order to determine the optimal window for uptake rate of all amino acids, a series of Flux Variability Analysis (FVA) was performed. FVA is a mathematical tool to find the optimal range of each reaction flux while the objective has its maximum value [34]. All amino acid uptake fluxes were maximized and minimized, subjected to the stoichiometric, capacity and exchange rate constraints (according to conditions of 1st Simulation in Table 2) while keeping the objective function of previously solved multi-objective FBA problems at its optimal value. The purpose of such analysis is to detect amino acid exchange reactions that represent higher activities in EPO production phenotype. This analysis was performed by *fluxVariability* scripts in COBRA toolbox in MATLAB [35].

RESULTS

Automated reconstruction of the GEM of EPO-producing CHO cells based on protein homology

The automated reconstruction can lead to some loss of control compared to a stricter manual, bottom-up approach. It is therefore important to identify and fill gaps in the model to ensure that the network is functioning as required. In a high quality model all reactions should be able to have a flux if all uptake and excretion reactions are allowed and net synthesis of most metabolites should be possible (the exception would normally be some co-factors). Gap filling traditionally centers on adding reactions in order to able production of all precursors needed for biomass production. To compare the quality of the automatically generated model to a manually curated one, some kind of reference was needed [32,36]. As all models contain errors it would not be very relevant to simply compare the similarity between the RAVEN Toolbox generated model and a previously published model. Table 3 presents the number of genes, reactions and metabolites for the published model in literature (iHepatocytes 2322) [32] and the reconstructed model by INIT algorithm in RAVEN toolbox. The models

are also compared with respect to identical genes, reactions and involved metabolites which the percent of similarity between the two models are 62, 54 and 50, respectively.

Two models are tested by simulating 256 different biologically defined metabolic functions [36] (for example, the synthesis of FAs, amino acids, cholesterol and bile acids) that is known to occur in hepatocytes using the RAVEN Toolbox. The representative results of the simulated biological tasks was provided. The results revealed that the generated model by us is successful in performing biological functions as well as iHepatocytes2322 model.

In second part of analysis of the reconstructed model, reactions present in model are classified using Parsimonious enzyme usage Flux Balance Analysis method by *pFBA* function in COBRA toolbox [37]. It optimizes the growth and then minimizes the flux through the model and subsequently classifies each reaction by how it contributes to the optimal solution on 6 classes: Essential ones, pFBA optima, Enzymatically Less Efficient (ELE), Metabolically Less Efficient (MLE), pFBA no flux and Blocked ones. The pie plot shown in Figure 1, demonstrates the percent of distribution of genes reactions in each class.

Results of Evaluation of Growth and EPO Production

In order to investigate the amino acids effects on EPO production by CHO cells, Constraint-Based Modeling (CBM) [38] had been used as the main framework of this study. As described in pervious section, the genome scale metabolic network of CHO cell is upgraded to synthesize EPO as secondary metabolite. To simulate the growth or any other objective under different culture conditions, constraints for reaction flux are set on the exchange reactions, while constrains for flux of other reactions remained in default values which demonstrate the thermodynamic and capacity constraint of the reactions [38]. In this study, growth and EPO production are simulated regarding to two different culture conditions in previously published researches [22,25] which are already shown in Table 2. Thus, upper bounds for uptake rates of the supplied nutrient (amino acids, glucose and oxygen) are constrained based on the mentioned conditions.

In both simulations, the optimal value of growth rate is the same (0.0257 mmol $gDW^{-1}h^{-1}$), whereas the EPO synthesis fluxes are different. The computed Flux

distribution intervals of EPO are shown in Figure 2 for each simulation. Red and blue solid lines in this figure show EPO production interval computed on the basis of the underlying conditions for 1th and 2nd simulations of Table 2 when the sole objective is maximizing the growth rate. Therefore, the solid blue line confirms that more flexible EPO synthesis conditions occur by application of the first simulation conditions. We hence concluded that the maximum ability of EPO production provided by the conditions applied within the first simulation, is almost ten times more than that of the second simulation. This ability can be interpreted by the proper balance in supplying Glucose (GLC), O_2, and amino acids nutrients.

Meanwhile, Biotechnological optimization of natural product biosynthesis often suffers from pathway competition with fluxes leading to the biosynthesis of biomass components [39,40]. In order to assess competition between secondary metabolite biosynthesis and biomass production for selected key compounds and metabolites, Multi-objective analysis has been used to calculate Pareto fronts between the biomass objective and the EPO production objective ([41], [42]). Here, this analysis is used to identify theoretical EPO production capacity in CHO cells and differences in the extent to which biomass production competes with EPO biosynthesis. Multi-objective analysis results in a trade-off between the two objective reactions (biomass production and EPO production reactions). The coefficient lambda (λ) with values between 0 and 1, is a coefficient assigned for each individual objective to adjust the relative contribution of each objective in a multi-objective optimization. Extreme values of λ (λ = 0) represents the metabolic state where the target metabolite cannot be produced and the growth is the sole objective, while λ = 1 demonstrate that all metabolic resources are devoted to supporting the production of a target metabolite. Fixing λ to a series of values and maximizing the compound objective can produce the points of the curve that describe the relationships among different objectives. The multi-objective optimization was performed for the two mentioned culture conditions depicted in Table 2. Figure 3 displays the results of 1th and 2nd simulations. Blue and red lines are related to trade-offs between growth and EPO formation for 1th and 2nd simulations, respectively.

In two cases maximum growth rates were the same (0.0257 mmol $gDW^{-1}h^{-1}$). To

estimate the transition from a biomass to EPO-only phenotype, the enforced growth rate was stepwise decreased from 100 to 0 % of the maximum rate, where the EPO secretion rate go up to 1.429 (mmol gDW^{-1} h^{-1}) in 1^{th} simulation and 0.285 (mmol $gDW^{-1}h^{-1}$) in 2^{nd} simulation when the EPO production was the sole objective (100 % contribution to the objective function). It is observed that the majority of fluxes decrease in a transition from the multi-product synthesis of sole biomass toward the sole objective of EPO synthesis. Specifically, we observe a decreasing flux for 331 reactions (of 1545 total reactions in the network, and of 917 minimally required reactions for the synthesis of biomass), of which 250 reactions attain a zero flux in the EPO production-only state. Thus, 1^{th} simulation is again identified such that the growth and EPO production are in much better conditions rather than 2^{nd} simulation. The difference between the two environments conditions is in glucose and amino acids nutrients, these values are increased in first simulation and thus, it can be concluded that the increase in glucose and amino acid supplements has positive effect on EPO production.

Essential reactions and genes

Table 4 presents the amount of essential genes and reactions corresponding to different assumed objectives. Among these three objectives, the lowest number of essential genes and reactions is related to the case in which sole objective of the cell was production of EPO which these reactions are responsible for synthesizing amino acids required for biosynthesis of EPO. Furthermore, when "Biomass + 0.98 EPO=>" was the objective, the number of essential genes and reactions are increased compared to wherein the biomass as the objective is maximized.

Table 1: The computed contribution (stoichiometry, mmol) of amino acids in 1(mmol) biomass and EPO synthesis

Material	biomass reaction	EPO reaction	Material	biomass reaction	EPO reaction
ALA	0.403	0.045	LYS	0.304	0.019
ARG	0.247	0.029	MET	0.152	0.002
ASN	0.250	0.014	PHE	0.190	0.010
ASP	0.465	0.014	PRO	0.237	0.019
CYS	0.079	0.010	SER	0.304	0.024
GLN	0.276	0.088	THR	0.250	0.026
GLY	0.412	0.021	TRP	0.026	0.007
HIS	0.069	0.005	TYR	0.104	0.010
ILE	0.205	0.012	VAL	0.299	0.026
LEU	0.417	0.055	GLU	0.654	

Table 2. The upper bounds of constraint conditions of uptake rate corresponding to two simulations of amino acids in CHO cell biomass and EPO synthesis

Material	1th simulation	2nd simulation	Material	1th simulation	2nd simulation
ALA	−	−	LYS	0.014	0.120
ARG	0.020	0.060	MET	0.006	0.020
ASN	0.040	0.320	PHE	0.006	0.090
ASP	0.010	0.010	PRO	0.237	0.050
CYS	0.005	0.010	SER	0.050	0.050
GLN	0.067	0.800	THR	0.011	0.040
GLY	0.000	0.000	TRP	−	0.010
HIS	0.004	0.020	TYR	0.104	0.090
ILE	−	0.060	VAL	0.012	0.050
LEU	0.016	0.120	GLC[1]	0.198	8.28
O2	1.125	0.720			

[1] GLC is the abbreviation of glucose

Table 3: Number of genes, metabolites and reactions in iHepatocytes 2322 and our model

	Genes#	Metabolites#	Reactions#
iHepatocytes 2322	2322	5686	7930
Our Model	3674	3216	4284
% similarity	62	54	50

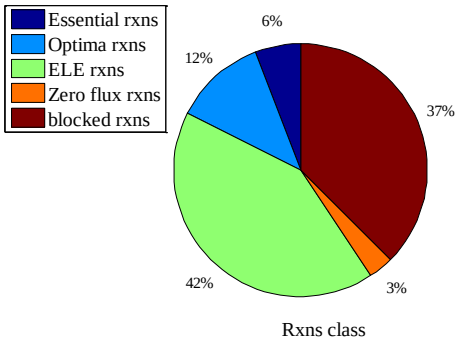


Figure 1: Graph shows classification of reactions based on essentiality, optima, Enzymatically Less Efficient (ELE), metabolically less efficient (MLE), no flux (zero flux) and blocked ones.

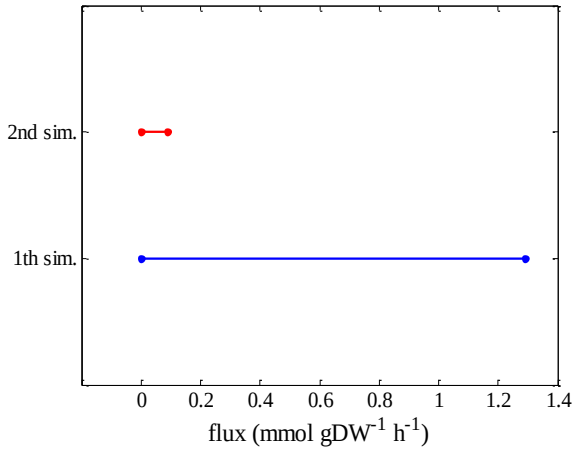


Figure 2. Flux distribution intervals for the EPO synthesis reaction. Solid blue and red lines correspond to EPO manufacture intervals on the basis of the underlying conditions of the first and second simulations, respectively, in Table 2.

Deletion of the essential genes leads to zero value of objective. When the growth is sole objective, the essential genes are related to energy sources, fatty acids, amino acids and protein productions which are required precursors for biomass production. However, when the EPO production is the sole objective, the essential genes corresponding to the pathways of essential amino acids and non-essential amino acid biosynthesis which are essential for EPO production. Finally, regarding to the tradeoff between biomass and EPO production (Maximization of "Biomass + 0.68 EPO=>"), the essential genes are the necessary genes that are vital for both objective, separately.

As the genes are responsible for expression of the enzymes related to each reaction, the similar conclusion could be drawn for essentiality of the reactions.

Investigation of essential amino acids influence

The main purpose of performing such analysis is to investigate the effects of uptake rates of essential amino acids (Arg, His, Lys, Met, Thr, Tyr, Leu, Val, and Ile) on the growth rate and EPO production. Figure 4 demonstrates the change in: a) EPO synthesis and, b) growth rate with respect to changes in uptake rates of essential amino acids. The resulting graphs for these amino acids are shown with the colored lines indicating each amino acids (Figure 4a, b).

The slope of each curve explains the sensitivity of the objective function to perturbations in the flux of amino acid uptake. In EPO production (Figure 4a), MET and LEU have fastest and slowest uptake rates, respectively. The uptake rates of VAL and THR is of interest, since the figure 4a shows that their plot are overlapped together and it means their similar effect on EPO production. Similarly in growth (Figure 4b), THR and LEU have fastest and slowest uptake rates, respectively. Although, it seems that the intervals of uptake rates of amino acids in growth case are lower than those in EPO production case which means that cell culture requires lower amounts of amino acids to achieve the optimal cell growth, whereas in optimal EPO production the cell needs much more quantities of them. Additionally, as shown in Figure 4-a, b, EPO production is more sensitive to Lys than biomass synthesis. It is hence expected that the main difference between these two objectives arises from the effect of lysine uptake.

For better comparison of the difference of amino acids uptake rates in growth and EPO production, the values objective with respect to the uptake flux values are normalized (maximum value of uptake flux become one for each amino acids) and the scaled data are depicted in figure 5. This representation shows the qualitative differences in uptake rates of each amino acid for both objectives in one plot, independent of the amount of their flux values. The solid blue line and the black dot-line are corresponding to growth and EPO production objectives. Except for MET, the uptake rates of each essential amino acids are higher for optimal growth than those for optimal EPO production. This is reasonable, since the cell should growth to produce secondary metabolite. Therefore, depletion of MET from culture media should be inhibited, because cell consume it at the same rate for growth and EPO biosynthesis.

FVA of amino acid reactions

FVA was performed by application of exchange constraints according to 1th simulation (provided in Table 2) and using optimal objectives in transition from 0% to 100 % EPO production. Figure 6 shows the plots pertaining to the optimal window of amino acid fluxes during the transition from a growth-only phenotype to EPO-only phenotype for CHO cells capable of producing EPO. The shaded areas signify the flexibility (range of minimum and maximum values) for each reaction fluxes.

The figures for non-essential amino acids display fixed flux values in transition states and it means that the biosynthesis of non-essential amino acids depend on fluxes of essential ones. Regarding the results, the conditions in first simulations provide the required amount of amino acids for this aim. Meanwhile, the essential amino acids exchange reactions have increased during the transition from 0 % to 100 % product objective. This increase in the fluxes is due to the necessity to recycle EPO to the metabolism.

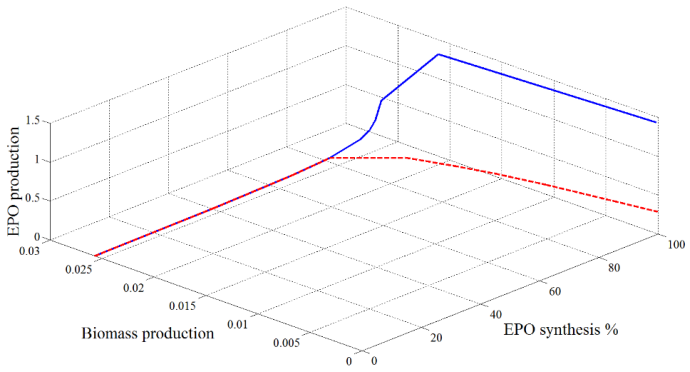


Figure 3. Trade-off between growth and production for the first and second simulations.

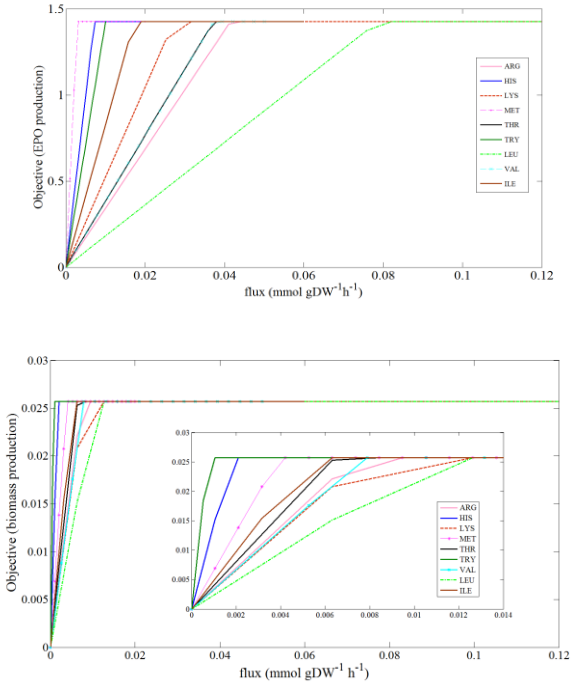


Figure 4. a. Optimal EPO production flux value as a function of the uptake rates of amino acids. **b.** Optimal growth rate as a function of the uptake rates of amino acids. The abbreviations show the supply effect of amino acids, ARG: arginine, HIS: Histidine, ILE: Isoleucine, LEU: Lucien, LYS: Lysine, MET: Methionine, PHE: Phenylalanine, THR: Threonine, TRP: Tryptophan, VAL: Valine.

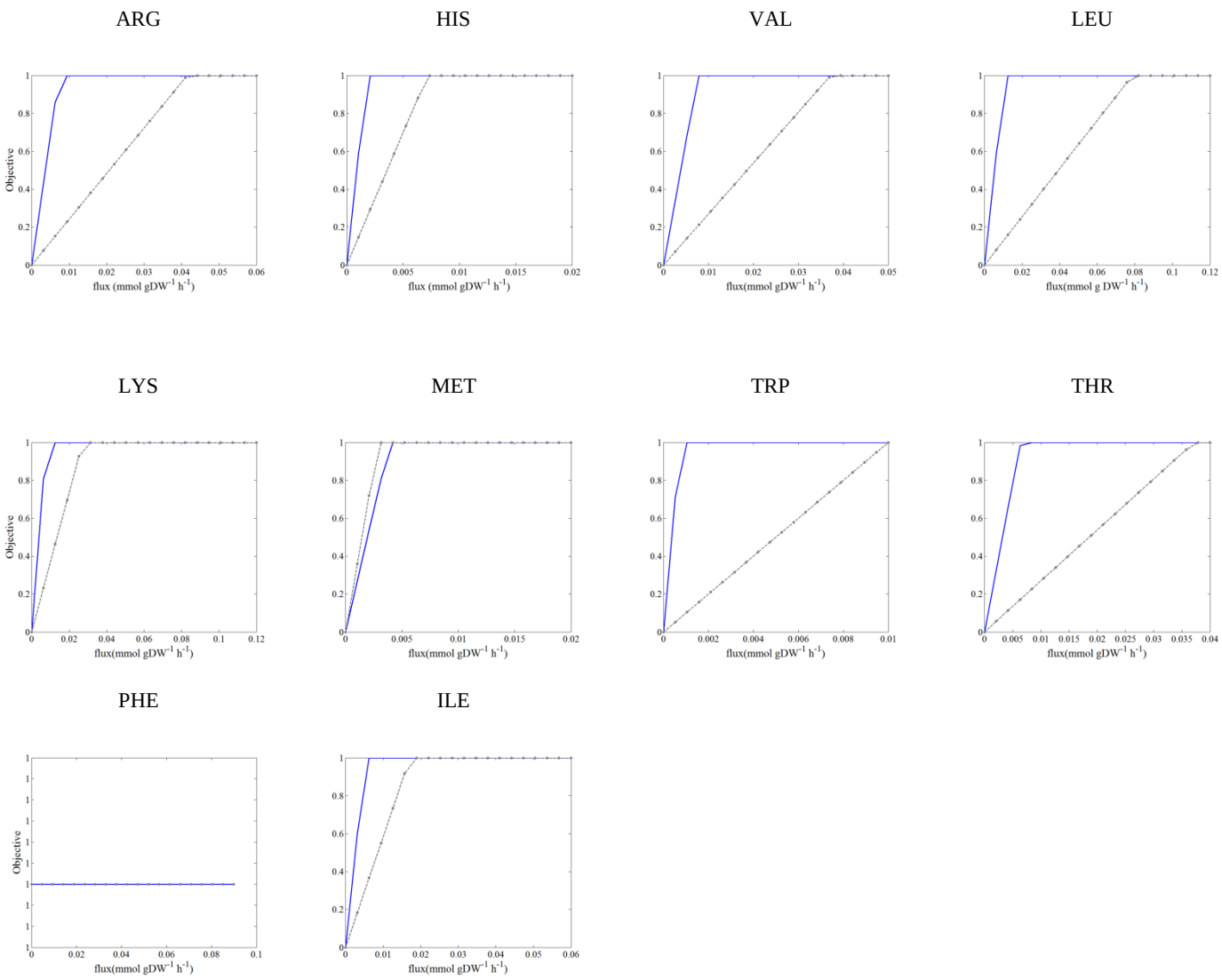
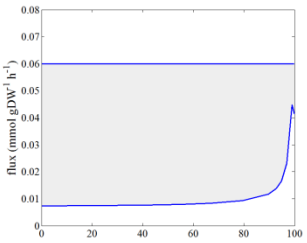


Figure 5. The normalized curves represent the computed optimal objective value with respect to the uptake rates of amino acids. The solid blue line and the gray dot line pertain to the robust curves for the growth and EPO production objectives, respectively. The abbreviations show the supply effect of amino acids, ARG: arginine, HIS: Histidine, ILE: Isoleucine, LEU: Lucien, LYS: Lysine, MET: Methionine, PHE: Phenylalanine, THR: Threonine, TRP: Tryptophan, VAL: Valine.

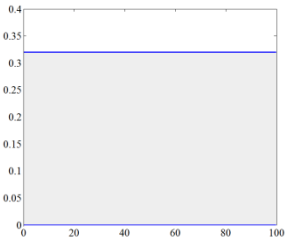
Table 4. Count of essential genes and reactions pertains to the objectives

Objective	Objective formula	Maximum objective (mmol $gDW^{-1}h^{-1}$)	Item	count
Biomass production	biomass=>	0.025	Essential genes	58
			Essential rxns	90
EPO production	EPO=>	1.429	Essential genes	12
			Essential rxns	13
Biomass + EPO production	Biomass + 0.98 EPO=>	0.020	Essential genes	60
			Essential rxns	94

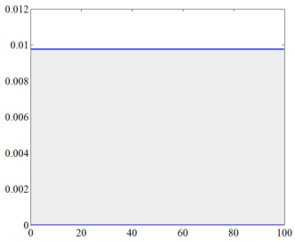
ARG



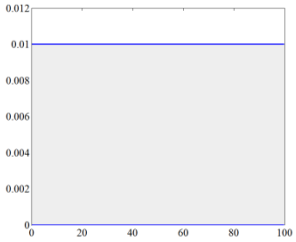
ASN



ASP



CYC



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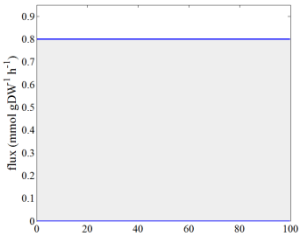

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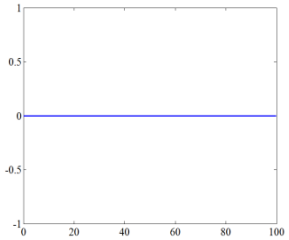

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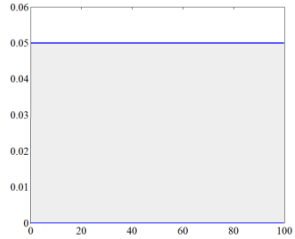

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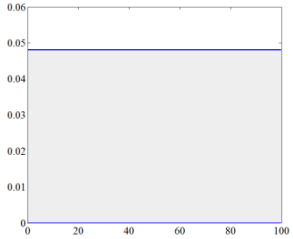

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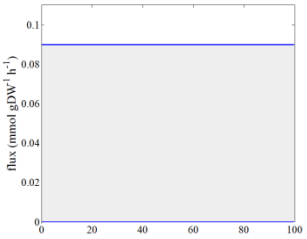

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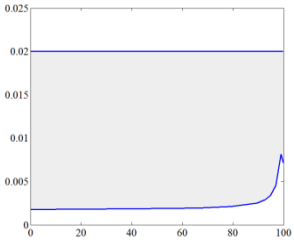

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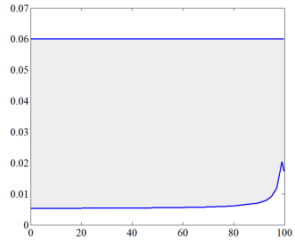

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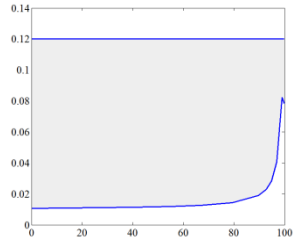

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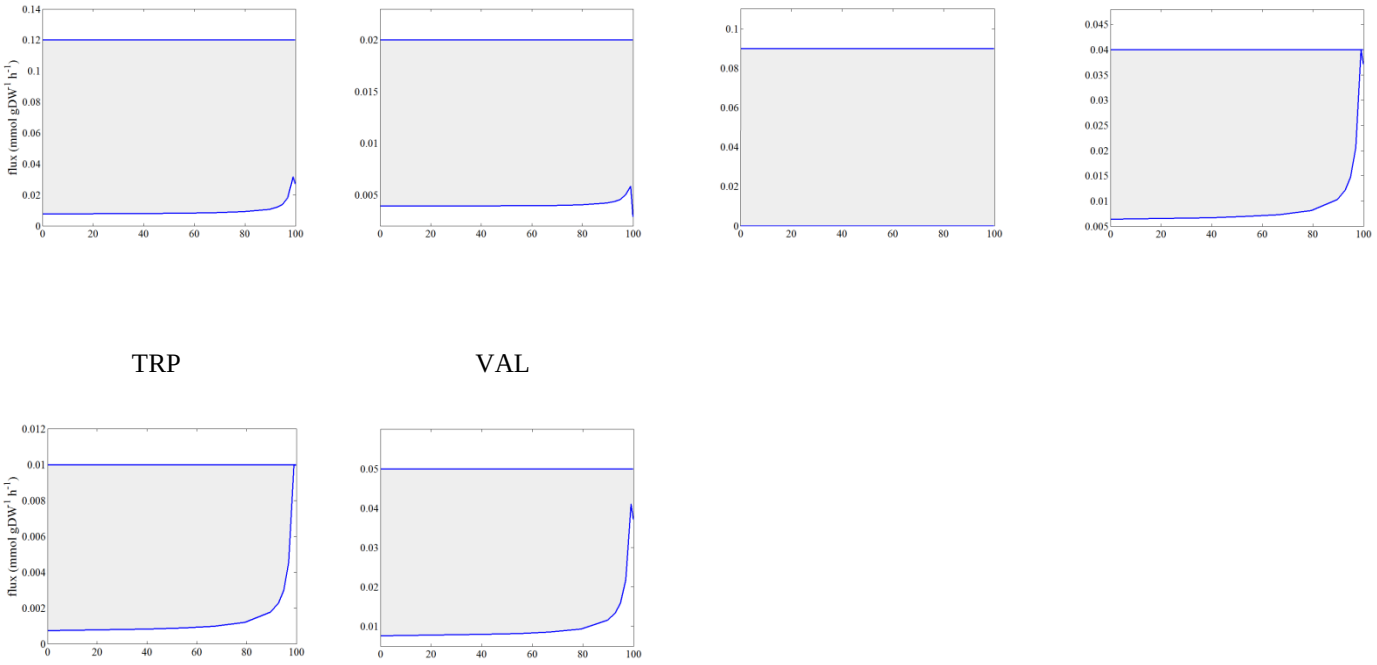

Figure 6. Plots show the required amino acid fluxes during the transition from a growth-only phenotype to a production-only phenotype for CHO cell producing EPO. The shaded areas demonstrate non-unique solutions (flux variability). The label of horizontal axis is flux (mmol gDW^{-1} h^{-1}).

DISCUSSION

Computational network reconstructions and metabolic modeling offers significant potential to identify and guide suitable culture conditions to improve the quantity and quality of recombinant proteins. In this research, variety of constraint-based analysis methods are used to investigate the behavior of Erythropoietin (EPO)-producing CHO cells and the effect of nutrients on the biosynthesis of EPO. Thus, the genome-scale metabolic network of CHO cell was reconstructed by the INIT algorithm in the RAVEN Toolbox to upgrade the metabolic network of CHO cell expressing EPO. The quality and performance of the reconstructed model was assessed to prove the applicability of this model for the subsequent analyses. To simulate the growth and EPO synthesis, the reconstructed model was subjected to FBA and Multi-objective analysis, constrained under two culture conditions extracted from the literature. Both conditions have

the same effect on growth, while the condition with higher amount of amino acids and glucose leads to more EPO production, therefore, the amount of essential amino acids and glucose have direct effect on the amount EPO synthesis. Next, the essentiality of gene and reactions of the model was assessed which demonstrates that the integration of EPO pathways requires more gene and reactions besides the growth-leading essentials.

CONCLUSION

Regarding the influence of amino acids in both growth and EPO biosynthesis, the sensitivity of essential amino acids and the flexibility of amino acid reactions was addressed which the results highlighted the more effective amino acids in growth versus EPO biosynthesis in addition to providing optimal window for supplementation of amino acids within culture media.

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