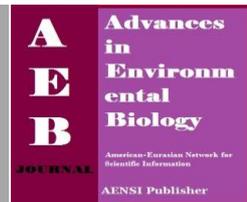




AENSI Journals

Advances in Environmental Biology

ISSN:1995-0756 EISSN: 1998-1066

Journal home page: <http://www.aensiweb.com/aeb.html>

Effects of Glutamine and Serum on IGF- Dependent Cho-K1 Cell Growth in T-Flask

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ARTICLE INFO

Article history:

Received 23 December 2013

Received in revised form 25

February 2014

Accepted 26 February 2014

Available online 25 March 2014

Key words:

CHO-K1 cells, cell growth, Insulin-like growth factor-I (IGF-I), glutamine, serum.

ABSTRACT

An effective mammalian cell culture based host system is highly desirable for production of bio-therapeutics. There are several strategies to achieve an effective system, one of which is regulation of media coupled with exploitation of inherent characteristics of cell line. In this present study, it is hypothesized that glutamine and serum may affect CHO-K1 cell growth through Insulin-like Growth Factor-I (IGF-I) pathway. The effects of glutamine and serum on IGF-dependent CHO-K1 cell growth in T-flask were studied based on three-level Full Factorial Design generated by SIMCAP+v12 (Umetrics, Sweden). The combination factor of 0.50 mM glutamine and 10 % (v/v) serum was found to be the optimal condition producing 8.87 x 10⁵ cells/ml. The CHO-K1 culture was also found to be able to grow in zero glutamine reaching 16.60 x 10⁵ cells/ml. Meanwhile, improvement of cell growth can also be achieved in reduced serum level with the presence of IGF-I protein.

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To Cite This Article: VasilaPackeer Mohamed, YumiZuhanis Has-Yun Hashim, SalfarinEzrinaMohamadSaberi, SitiNuramalis Ibrahim, Azura Amid and Maizirwan Mel., Effects of Glutamine and Serum on IGF- Dependent Cho-K1 Cell Growth in T-Flask. *Adv. Environ. Biol.*, 8(3), 831-837, 2014

INTRODUCTION

An effective host system to express therapeutic proteins is a system that is able to provide desirable outputs which may include high cell density, good growth rate, minimize consumption of media and high expression of the protein of interest. While many host systems can be used such as bacteria, plant, yeast, insect and mammalian cells, the latter is the optimal and preferred host system for the production of recombinant eukaryotic proteins for biopharmaceutical purposes. Its major benefit is the direct expression of the desired protein, including large and complex proteins like Factor VIII in the culture medium. Mammalian cell culture host also allows correct folding and posttranslational modifications for optimal biological activity of the protein produced [1,2].

In order to develop an efficient mammalian cell culture host system, there are several strategies to be focused on. This includes improved design and the ability of the host cell line to offer potential product improvements, maximization of viable cell number, improvement of medium formulation, inclusion of exogenous growth factors, implementation of high performance reactor configurations and maximization of production rate per cell [1,2]. Many earlier studies focused on a single strategy at a time. For example, reported on the development of an efficient cell line which produced the constitutive expression of Insulin-like growth factor 1 (IGF-1) leading to self promotion of the cell proliferation, (i.e. autocrine growth) [4,5]. Meanwhile, focused on media regulation where the authors compared the expression of IGF genes in serum supplemented and serum free media in relation to cell proliferation [6].

In order to develop an efficient mammalian cell culture host system, there are several strategies to be focused on. It is hypothesized that glutamine and serum may affect CHO-K1 cell growth through Insulin-like Growth Factor I-(IGF-I) pathway. We have confirmed that CHO-K1 cells expressed IGF-I gene and protein [7, 8]. In the current study, the effects of glutamine and serum on IGF-dependent CHO-K1 cell growth in T-flask were studied with the aid of Response Surface Methodology (RSM) model.

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Therefore, it is the main aim of the study to develop an efficient CHO-K1 mammalian cell based host system targeting at serum and glutamine regulated IGF-1 pathway.

MATERIALS AND METHODS

CHO-K1 Cell Line:

CHO-K1 cells (ATCC CCL-61™) were obtained from American Type Culture Collection (ATCC, USA).

Culture Media and Chemicals:

CHO-K1 cells were cultured in RPMI 1640 medium (Mediatech, USA) supplemented with glutamine and fetal bovine serum (FBS) at 5% CO₂/37°C; according to experimental design (section 2.3 and Table 2). T-75 flasks were used for cell cultivation. There were three biological replicates with eight flasks in each batch; designated for 8-hourly sampling. All cultures were initiated at a viable cell concentration of 2.00 x 10⁵ cells/ml.

Experimental Design:

The study of effects of glutamine (0.50, 3.25 and 6.00 mM) and serum (5, 10 and 15 % (v/v) on IGF-I dependent CHO-K1 cell growth were performed using a three level-full factorial model generated by SIMCAP+v12 (Umetrics, Umea, Sweden). Regression model analysis was later performed using the same software. Responses were grouped into (i) cell growth behavior comprising of Y1 (maximum viable cell number), Y2 (doubling time) and Y5 (viable cell concentration at mid-exponential phase) and (ii) IGF-I expression (gene; Y3 and protein; Y4). For responses in group (i), cells were counted using trypan blue dye exclusion method with the aid of haemocytometer. For IGF-I gene and protein expression, samples were taken at mid-exponential phase.

Quantitative PCR (qCR) on IGF-I Gene Expression:

Isolation of total RNA from cultured CHO-K1 cells (cell pellet) from sample at mid-exponential phase was carried out using Total RNA Purification kit (NORGEN, Canada), according to manufacturer's instructions. The RNA samples were stored at -80 °C until further analysis.

Prior to the quantitative PCR (qPCR), reverse transcription (RT) reaction was carried out in a final volume of 20 µl of RNA sample using SuperScript III Reverse Transcriptase Kit (Invitrogen, USA), according to manufacturer's instructions. Primer sequences for amplification of the regions of IGF-I gene (target gene) and GAPDH (reference gene) sequences were designed using Beacon designer software by Chemoscience Company, Malaysia and synthesized by 1st BASE, Malaysia (Table 1).

To analyze the expression of IGF-I gene, QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used. For this analysis, reference gene, GAPDH was used. The genes were tested with the same panel of cDNA samples. NTC (no template control) reaction serves as negative control was performed for standards and samples. Sample with the lowest IGF-I expression level was used as control for this study.

Table 1: Sequence of primers used to detect IGF-I and GAPDH gene expression in CHO-K1 cells. The accession number is given for only one representative matching sequence on the NCBI database. F=Forward and R= Reverse.

Gene	Sequence		Accession number	Size (Base pair, bp)
GAPDH	F	5'-CGACTTCAACAGCAACTC-3'	NM_008084	109
	R	5'-GTAGCCGTATTCATTGTCAT-3'		
IGF-I	F	5'-TGACATTGCTCTAACATCTC-3'	NM_010512	107
	R	5'-CCATCGCTTCTGAAGTAC-3'		

The following real time PCR protocol was used: denaturation (95.0 °C for 10 min); 3-segment amplification and quantification (denaturation at 95.0 °C for 30 s, annealing at 53.0 °C for 1 min, and extension at 72.0 °C for 30 s) which was repeated 45 times; and finally dissociation curve was developed (continuous monitoring of fluorescence at 55.0 to 95.0 °C). Baseline and threshold values were automatically determined for all plates using the MxPro3000 software (Stratagene, CA). The obtained data were analyzed and level of IGF-I expression was calculated using the comparative C_T method ($\Delta\Delta C_T$) (Applied Biosystems, 2004). For the IGF-I gene analysis, only two biological replicates were used.

Enzyme-linked Immunosorbent Assay (ELISA) for Determination of IGF-I Protein:

Conditioned media from cultured CHO-K1 cells at mid-exponential phase for all runs were collected. The analysis was carried out using Mouse/Rat IGF-I Immunoassay (R&D Systems, UK), according to manufacturer's instructions on two biological replicates.

RESULTS AND DISCUSSION

Effects of Glutamine and Serum on IGF-I Dependent CHO-K1 Cell:

Twelve experimental runs were performed according to the design (Table 2). Experiment 5, 10, 11 and 12 were the centre points. There were five responses (Y) as described in Section 2.3.

From Table 2, it can be observed that for response (Y1) maximum viable cell number, Experiment 3 (15 % (v/v) serum and 0.50 mM glutamine), reached the highest viable cell number of 1.62×10^6 cells/ml

Table 2: Design of Experiment and Response Values.

Exp No	Exp Name	Run Order	Incl/Excl	Serum	Glutamine	(Y1) Maximum Viable Cell Number (cells/ml)	(Y2) Doubling Time (h)	(Y3) IGF-I Protein Concentration at Mid-Exponential Phase (pg/ml)	(Y4) IGF-I Gene Concentration at Mid-Exponential Phase (Fold difference)	(Y5) Viable Cell Number at Mid Exp Phase (cells/ml)
1	N1	4	Incl	5	0.5	725000	23.1	17.12	38.12	725000
2	N2	5	Incl	10	0.5	887000	18	15.38	1.21	828000
3	N3	1	Incl	15	0.5	1.62×10^6	17.33	14.79	86.39	909000
4	N4	8	Incl	5	3.25	840000	28.88	16.69	242.09	760000
5	N5	9	Incl	10	3.25	513000	32.54	15.15	9.07	212000
6	N6	2	Incl	15	3.25	554000	21	15.32	467.43	471000
7	N7	7	Incl	5	6	678000	23.1	17.08	32.13	522000
8	N8	3	Incl	10	6	669000	19.25	15.59	720.86	468000
9	N9	6	Incl	15	6	853000	23.9	14.41	15.38	717000
10	N10	12	Incl	10	3.25	719000	26.66	15.48	3.63	269000
11	N11	10	Incl	10	3.25	787000	25.67	18.85	6.16	551000
12	N12	11	Excl	10	3.25	916000	18.73	16.23	42.08	396000

This is followed by Experiment 12 (10 % (v/v) serum and 3.25 mM glutamine), 9.60×10^5 cells/ml and Experiment 2 (10 % (v/v) serum and 0.50 mM glutamine), 8.87×10^5 cells/ml. Similar observation was obtained for doubling time where Experiment 3 showed the lowest doubling time, 17.33 h. However, this is followed by Experiment 2 and Experiment 12 (18.00 h and 18.73 h, respectively). To this end, Experiment 3 represented the best combination of factors to obtain the highest cell yields and lowest doubling time.

Regression Model Analysis:

Partial Least Squared (PLS) was used to fit the model; simultaneously representing the variation of all the responses to the variation of the factors. In this study, it is important to look at all responses simultaneously in order to investigate potential relationship of serum and or glutamine with IGF-I gene and protein expression leading to promotion of cell growth.

Based on the summary of fit for all responses, the R^2 ; the goodness of model fit for (Y1) maximum viable cell concentration is 0.55, (Y2) doubling time is 0.62, (Y3) IGF-I protein expression at mid-exponential phase is 0.42, (Y4) IGF-I gene expression at mid-exponential phase is 0.09 and (Y5) viable cell concentration at mid-exponential phase is 0.62.

A much better indication of the usefulness of a regression model is given by the Q^2 parameter, which is the goodness of prediction where it can estimate the predictive power of the model. For (Y2) and (Y5), it equals to 0.46 and 0.29 respectively. Meanwhile, for (Y1), (Y3) and (Y4) the values are -0.03, -0.10 and -0.04 respectively. Responses that produced Q^2 less than 0.50 indicates that model is a poor model and it cannot predict new data very well. Meanwhile, all models are statistically significant as $p < 0.05$. The models also have no lack of fit ($p > 0.05$) except for response Y4.

Regression Coefficient Plot and Variance Importance Plot (VIP):

The regression coefficients of the five models are given in Figure 1. The most important factor for (Y1), (Y2) and (Y5) is glutamine*glutamine while for (Y3) is serum. However, important factor for IGF-I gene expression (Y4) is less discernible with serum and glutamine having similar bar sizes. Thus, to improve the cell concentration, one should concentrate on increasing the twice of one-factor glutamine (glutamine*glutamine) while lowering this factor may improve the doubling time. Further, to improve IGF-I protein, one should lower the serum and to improve IGF-I gene expression, glutamine should be increased. Based on variance important plot (VIP) (Figure 2), the most important factor for overall model is glutamine*glutamine, serum and glutamine as single factor. Thus, it can be suggested that serum and glutamine may play a role in improving cell growth through IGF-I pathways albeit the non-linear correlation.

Figure 3 shows the optimal factor combinations. Three (growth behavior related) from five responses showed optimum combination of factors of very low glutamine and very high serum. When inferred to Table 2, this corresponds to 15 % (v/v) serum and 0.50 mM glutamine. However, since the high level of serum will increase the cost of media, the combination factor of 0.50 mM glutamine and 10 % (v/v) serum was chosen as the optimal factors.

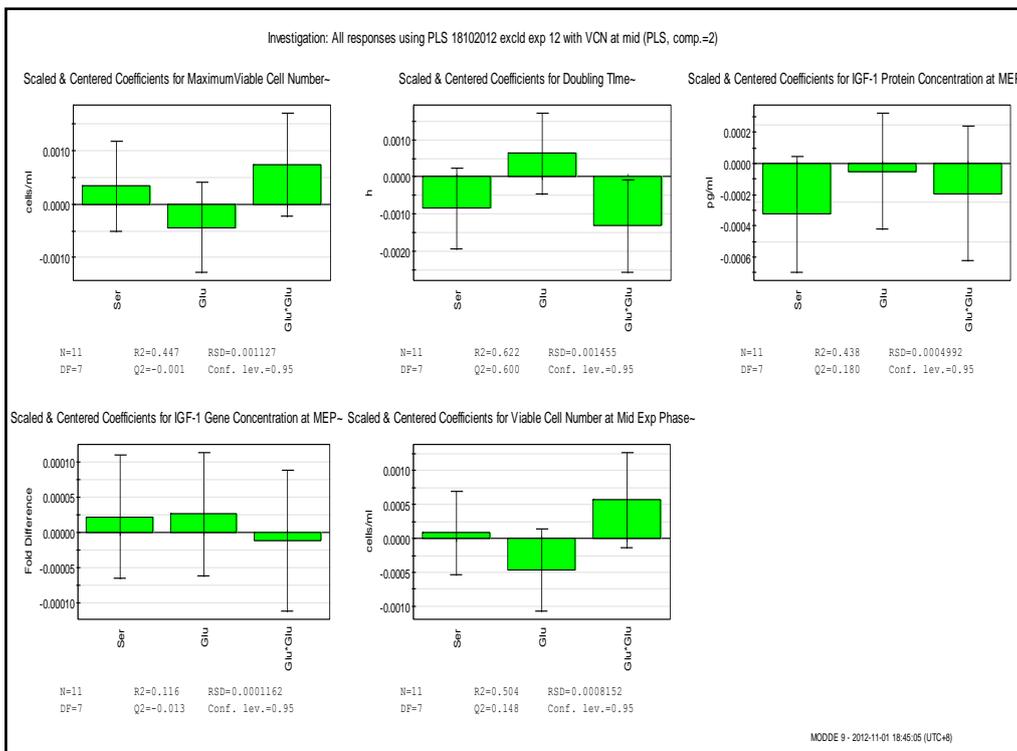


Fig.1:Regression coefficients of the interaction model.Size of bar indicates the effects and direction of bar indicates the significant results.

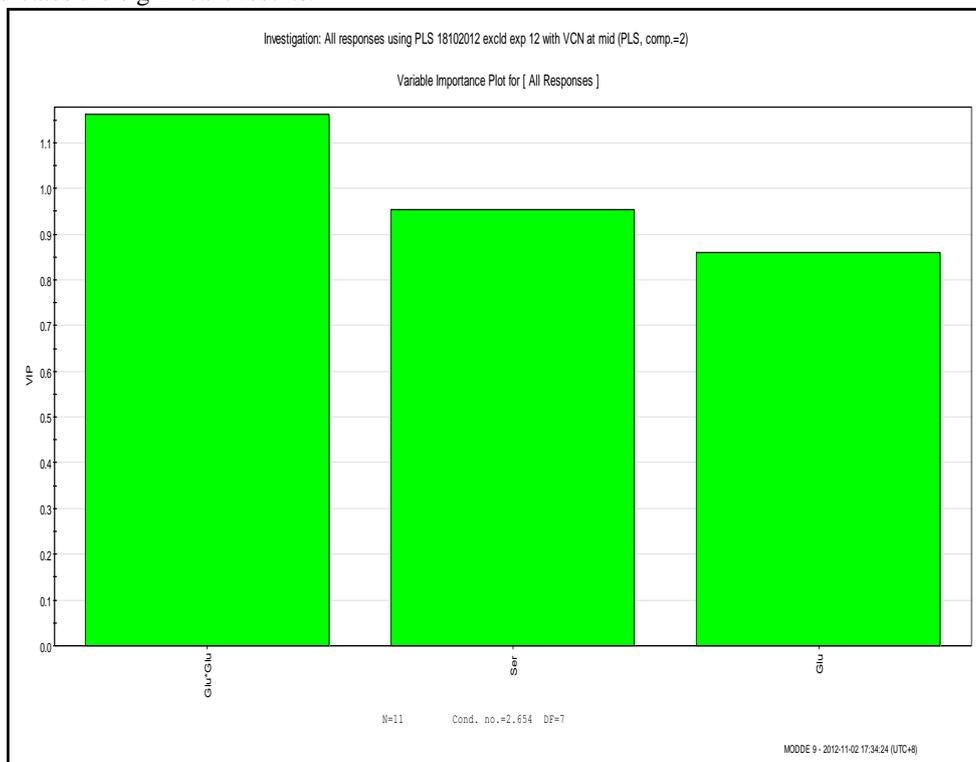


Fig. 2: Variance Important Plot (VIP).
X-axis: Factors
Y-axis: Variance Important Plot (VIP)

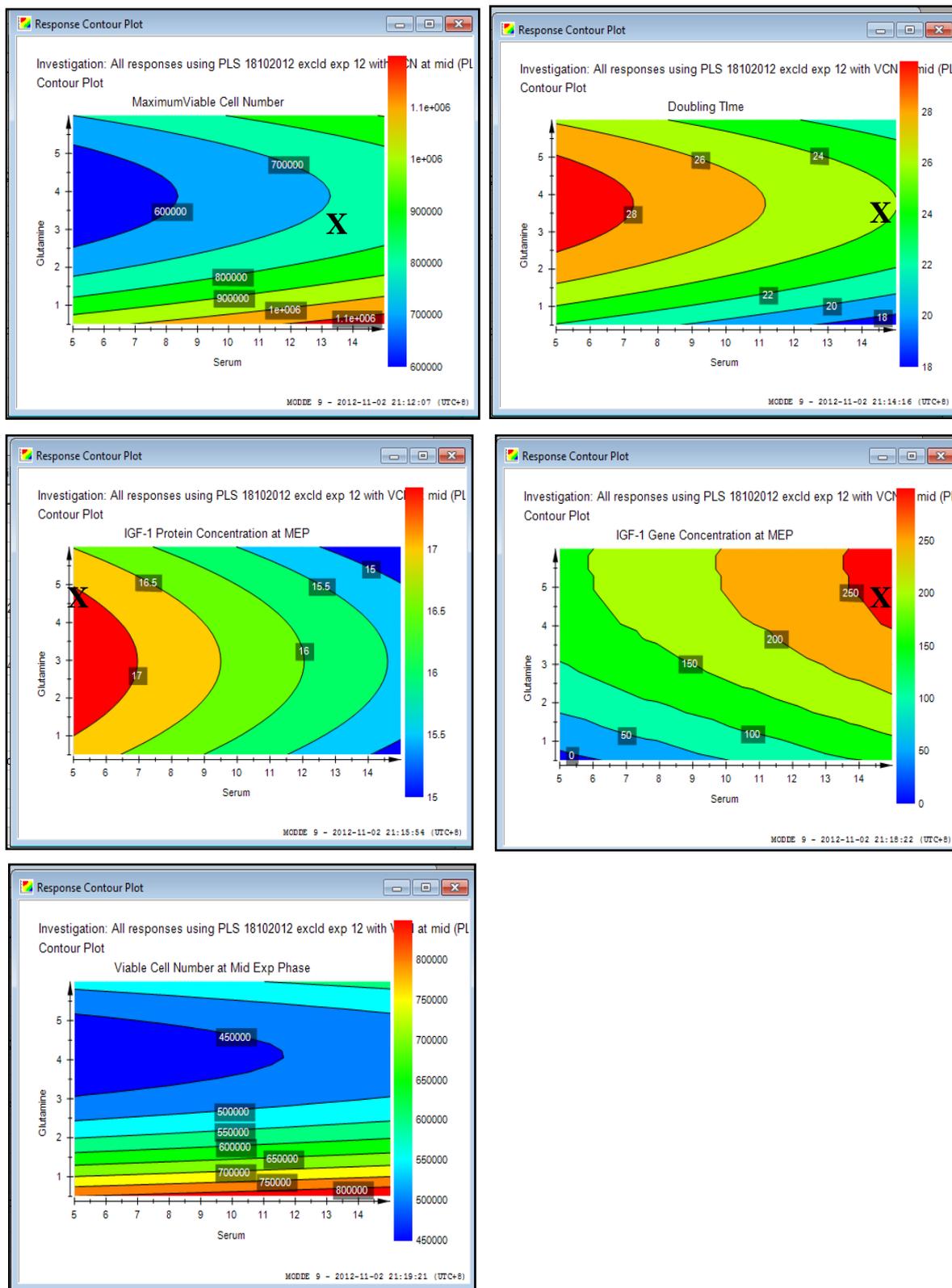


Fig. 3: Responses contour plots showing that maximum signal is obtained at low glutamine (0.50 mM) and high serum (15 % (v/v)) for Y1 (maximum viable cell number), Y2 (doubling time) and Y5 (viable cell concentration at mid-exponential phase). (X is optimal point).

Validation of Optimal Condition and Adaptation of CHO-K1 Cells in Zero Glutamine Media:

The optimal condition which is 10 % (v/v) of serum and 0.50 mM of glutamine was experimentally validated. Based on coefficient and variable importance plot (VIP) (section 3.2.1), the term glu*glu was the

most important factor in the study. Further, based on RSM (section 3.1 and 3.2), the optimal condition pointed that very low glutamine corresponds to high cell growth. Therefore, cells were then cultured in zero glutamine media to investigate the growth behavior of CHO-K1 in the absence of glutamine. Similar growth pattern for CHO-K1 cells cultured in optimal and zero glutamine conditions respectively was observed (Figure 4).

Furthermore, culture in zero glutamine gave higher maximum cell concentration by 24.70% as compared to validation of optimal condition. This suggests that the use of zero glutamine media may reduce the cost and unnecessary toxic waste [9]. On the other hand, the ability of CHO-K1 cell to synthesize glutamine [10] supports these observations.

The observations are in agreement with the regression coefficient plot (section 3.2.1, Figure 1) where quadratic glutamine acts as an important factor to enhance cell growth. Also, serum can be further reduced in order to improve cell growth (since they may have negative feedback effect where low serum may induce the expression of IGF-I protein leading to reduced doubling time, Figure 1).

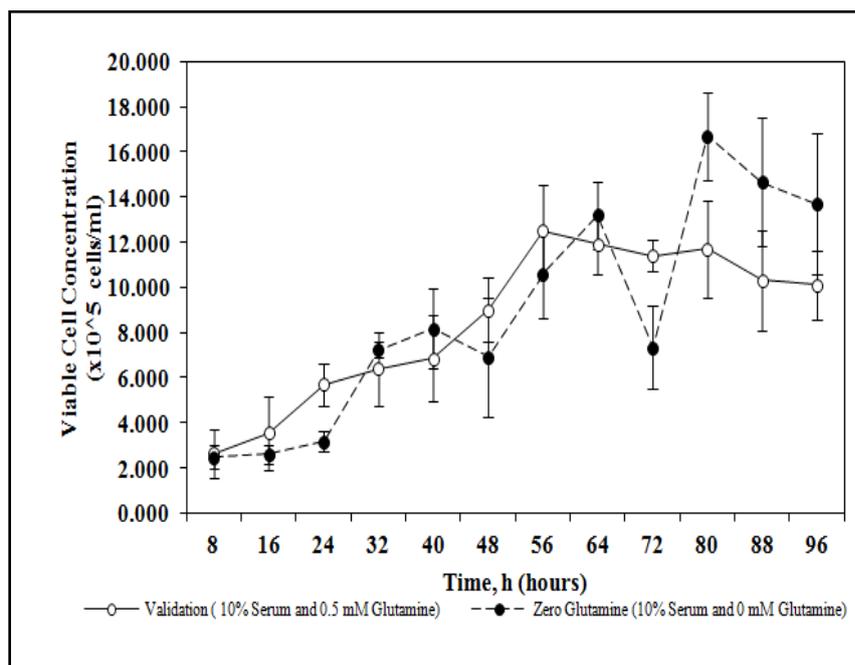


Fig. 4: CHO-K1 cell growth during batch culture in optimal condition and zero glutamine media (seeding concentration: 2.0×10^5 cells/ml; $n=3$; Mean \pm SD).

Conclusions:

The combination factor of 0.50 mM glutamine and 10 % (v/v) serum (Experiment 2) was found to be the optimal condition producing 8.87×10^5 cells/ml. The CHO-K1 culture was also found to be able to grow in zero glutamine reaching 16.60×10^5 cells/ml. Meanwhile, improvement of cell growth can also be achieved in reduced serum level with the presence of IGF-I protein.

ACKNOWLEDGEMENT

This Project is funded by the Ministry of Higher Education (MOHE), Malaysia through Fundamental Research Grant Scheme, FRGS 0409-110, which is gratefully acknowledged.

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