

Full Length Research Paper

Chinese hamster ovary (CHO-K1) cells expressed native insulin-like growth factor-1 (IGF-1) gene towards efficient mammalian cell culture host system

Vasila Packeer Mohamed, Yumi Zuhanis Has-Yun Hashim*, Azura Amid, Maizirwan Mel, Abdul Razak Kamarulzaman, Muhammad Adil Ab Wahab and Salfarina Ezrina Mohmad Saberi

Bioprocess and Molecular Engineering Research Group (BPMERU), Department of Biotechnology Engineering, Kulliyah of Engineering, International Islamic University Malaysia (IIUM), P.O. Box 10, 50728, Kuala Lumpur, Malaysia.

Accepted 7 November, 2011

Insulin-like growth factor-1 (IGF-1) has been shown to promote cell proliferation and inhibit apoptosis of cells. These are two characteristics of mammalian cell culture which may lead to high density cell culture producing optimal desired yield of bioproducts. An inherent secretion of IGF-1 protein from host cells into the culture media is hypothesized to enable reduction or removable of serum from culture media, thus reducing cost. This study was set to investigate the IGF-1 gene expression in Chinese hamster ovary (CHO-K1) cells. The cells were first cultured in T-flask with three independent experiments. An 8-hourly sampling for responses (glucose, lactate, total protein and biomass) was done. PCR-based method was performed to study the expression of IGF-1 gene. To this end, it was confirmed that CHO-K1 cells used in this study expressed IGF-1 gene. The study also provides the baseline data on kinetics and biochemical responses of CHO-K1 cell growth. Together, the data would be particularly useful for further studies using CHO-K1 cells as efficient mammalian cell culture host system to produce biologics.

Key words: Chinese hamster ovary (CHO-K1) cells, insulin-like growth factor-1 (IGF-1) gene, polymerase chain reaction (PCR)-based method, gene expression.

INTRODUCTION

Animal cell lines have been used extensively for the production of a variety of therapeutic and prophylactic protein products including hormones, cytokines, enzymes, antibodies and vaccines. They offer the advantage of reproducibility and ability to perform complex post-translational modification, leading to biological active form of therapeutics having the potential for large scale production. In particular, Chinese hamster ovary (CHO) cells have been known as the industry's premier host cell for the production of bioproducts due to its adaptive ability

to the system in question and its ease of maintenance (Jayapal et al., 2007). Apart from cell line used, other factors such as media and process conditions also affect the mammalian cell culture system and its production of desired biologics. Media composition may be regulated to achieve optimal performance at the minimum cost. Serum, which supplies growth promoting compounds is the most expensive ingredient in a media composition. While the current trend in large scale production is to use serum-free media, this type of media is very specific to the host system, thus could not be used as 'platform' media. There are several approaches in the serum free media development, one of which is to supply identified ingredients commonly found in serum into the culture media. Another approach is to use cell line that produces such compounds that promote growth and proliferation of cells, thus reducing or removing the need to supply serum.

Insulin-like growth factors (IGFs) are compounds com-

*Corresponding author. E-mail: yumi@iium.edu.my. Tel: 603 61965767. Fax: 603 61964442.

Abbreviations: IGF-1, Insulin-like growth factor-1, CHO-K1, Chinese hamster ovary.

monly found in serum. The IGF signaling has been recognized to play an important role in the development and growth of many tissues as well as having mitogenic and anti-apoptotic effects on normal and transformed cells (Yakar et al., 2002).

The IGF system comprises a complex network of ligands (IGF-1 and IGF-2), receptors (IGF-1R, IGF-2R and insulin receptor) and six IGF binding proteins (IGFBP1–6). IGF-1 and IGF-2 are highly homologous to each other (62% amino acid identity) and to insulin (INS) (Pacher et al., 2007).

Baserga (1993) reported that recombinant CHO cells constitutively expressing IGF-1 (and IGF-1R) were able to grow in serum free media without the addition of exogenous growth factor which would in part reduce cost. Meanwhile, Sunstrom et al. (1998) described the ability of Super-CHO cells to constitutively express IGF-1 and transferrin in sufficient amounts to support long-term, stable growth without the addition of exogenous growth factors, thus making it an ideal host for the production of recombinant biopharmaceutical. Although, previous *in vitro* investigations have demonstrated expression of IGF-1 in cell lines derived from mesenchymal and epithelial tissues as well as cancers (Sekyi-Otu et al., 1995), IGF-1 gene has been reported not to be normally expressed in CHO cells while IGF-1R has been reported to be below the detection limit (Sunstrom et al., 1998). There are many strains of CHO cells with different expression system such as glutamine synthetase (GS), episomal and others. Specifically, we hypothesized that if CHO-K1 cells (with GS expression system) expressed native IGF-1 gene, the cell line is potential to be used as host system which reduces or removes the need to supply serum, thus reducing cost.

Therefore, the objective of this study was to confirm the expression of IGF-1 by PCR-based method in CHO-K1 cells.

MATERIALS AND METHODS

Cells and cell culture

CHO-K1 cells (ATCC CCL – 61TM) were obtained from American Type Culture Collection (ATCC). The cell line possesses glutamine synthetase (GS) expression system which is very useful for transfection of recombinant genes (ATCC). The cell line was maintained in Ham's F-12 medium (Mediatech, USA) supplemented with 10% (v/v) fetal bovine serum (FBS (Invitrogen, USA) at 5% CO₂/37°C. T-75 flasks (Orange Scientific, Belgium) were used for cell cultivation. All cultures were initiated at seeding concentration of 20 x 10⁴ cells/ml and experiments were carried out in three independent experiments.

Biochemical responses/metabolic analysis

Analytical methods (cell number)

Cells were counted using trypan blue dye exclusion method with the aid of haemocytometer. Biomass was determined using cell dry weight method while total protein content was analyzed using

Protein Assay kit (Bio-Rad, USA).

Metabolite determinations

Glucose and lactate concentration were determined by employing the YSI 2700 analyzer (Yellow Spring Instrument, USA).

Specific rate calculations

Specific growth rate (μ) and doubling time (t_b) were calculated using the formula described by Butler (1996). Meanwhile, the specific nutrient consumption rate, q_s and specific product formation rate, q_p were calculated using the following equations (from Hu, 2003):

Specific nutrient consumption Rate, q_s :

$$q_s = - \left(\frac{1}{x} \right) \left(\frac{ds}{dt} \right) \quad (1)$$

Where, x is the viable cell concentration and s is the substrate concentration.

Specific product formation Rate, q_p :

$$q_p = \left(\frac{1}{x} \right) \left(\frac{dp}{dt} \right) \quad (2)$$

Where, x is the viable cell concentration and p is the product concentration.

Total RNA extraction

Isolation of total RNA from cultured CHO-K1 cells at six different time points (representing the early and late lag phase; early and middle exponential phase; stationary phase and death phase) were 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.

RNA quantification

Total RNA concentration was quantified using NanoPhotometer (IMPLEN, Canada) by measuring the extinction of RNA at 260 nm. Additionally, the OD_{260/230} and the OD_{260/280} ratio representing RNA purity were examined. RNA quality was assessed by calculating the A260/A280 ratio to determine possible DNA and protein contamination.

Reverse transcription and first strand cDNA synthesis

The reverse transcription (RT) reaction was carried out in a final volume of 20 μ l using SuperScript III Reverse Transcriptase Kit (Invitrogen, USA), according to the manufacturer's instructions. A master mix (MM) was used for reverse transcription to minimize pipetting errors.

PCR primers

Primers for amplification of regions of the IGF-1 gene as target gene and GAPDH as reference gene sequences were designed and chosen using NCBI, Primer Bank (Wang and Seed, 2003) and

Table 1. The sequence of primers used for PCR assays of CHO-K1.

Gene		Sequence	Accession number
IGF-1	F	5'-CTGGACCAGAGACCCCTTTGC-3'	NM_010512
	R	5'-GGACGGGGACTTCTGAGTCTT-3'	
GAPDH	F	5'-AGGTCGGTGTGAACGGATTTG-3'	NM_008084
	R	5'-TGTAGACCATGTAGTGGTCA-3'	

F = Forward and R = reverse; *The accession number is given for only one representative matching sequence on the NCBI database (<http://www.ncbi.nlm.nih.gov/>). IGF-1, Insulin like growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Primer 3 (Rozen and Skaletsky, 2000) databases. Sequences of primers used in this study are depicted in Table 1. Primers were synthesized by First Base (1st BASE, Malaysia).

Standard PCR assays

Each amplification reaction contained 1x reaction buffer; 0.2 mM dNTPs mix; 2.5 U *Taq*DNA polymerase; 2.0 mM MgCl₂ (Invitrogen, USA), 0.3 Mm of each primer (1st BASE, Malaysia), cDNA template of samples and RNase free water were used, respectively, as negative control for the amplifications. Amplification profiles were as follows: pre-denaturation for 2 min at 94.0°C; 1 cycle, denaturation for 30 s at 94°C, annealing for 30 s at 66.0°C (IGF-1) and 60.0°C (GAPDH), extension for 1 min at 72.0°C; 35 cycles, and final extension for 10 min at 72.0°C; 1 cycle.

PCR fragment analysis

Analysis of amplified cDNA fragments were electrophoresed on 2% agarose gels in 1X TAE buffer, and bands were visualized by ethidium bromide staining and UV transillumination.

RESULTS AND DISCUSSION

CHO-K1 growth profile

The CHO-K1 cells culture exhibit a typical exponential growth pattern (Figure 1). In the four-day period of study, CHO-K1 cells increased from 20 x 10⁴ cells/ml to reach maximum of 87 x 10⁴ cells/ml at 48 h. The specific growth rate (μ) and doubling time (t_D) are shown in Table 2. The results are in agreement with a non-recombinant CHO cells used by Percell Biolytica (retrieved from www.percell.se, 2010), where the μ was 0.058 h⁻¹ and t_D was 12 h. However, the maximum cell density obtained in Percell Biolytica was 60 x 10⁵ cells/ml.

This is about seven times higher than the maximum cell density obtained in this study. The higher cell number is probably due to the volume of culture media used (500 ml spinner vessel in Percell Biolytica as compared to 75 cm² T-flask in this study).

Biochemical responses

The analyses on biochemical responses were determined

for selected responses (biomass, total protein content, lactate and glucose concentration) as shown in Table 3. Biomass and total protein content give information on the ability of the cells to maintain their growth (Figure 2). In addition, biochemical analyses such as glucose and lactate demonstrate the metabolic changes that occur within the cells.

Glucose is consumed by the CHO cells in culture as the main energy sources. Glucose is principally metabolized by glycolysis to form pyruvate, which may be converted to lactate, then may enter the citric acid cycle, before it is oxidized into carbon dioxide (CO₂) and water (Freshney, 1994).

However, the rapid consumption of glucose in culture far beyond its requirement for supporting the cells metabolism results in faster depletion of carbon and energy source in the media, and accumulation of toxic wastes product such as lactate and ammonium. Consequently, this will lead to cell death (Altamirano et al., 2000).

As such, in this study, specific rate of glucose consumption and its corresponding rate of lactate production are of considerable value (Table 4). However, since there is still residual value of glucose after cells reach maximum cell density (Figure 3), the glucose concentration supplied to the system may be reduced to achieve minimum wastage but maintaining optimal cell growth.

RNA extraction and quantification

Ratios at OD_{260/280} for all six samples examined using NanoPhotometer (IMPLEN, Canada) ranged from 2.18 to 2.35 (data not shown). All samples were tested in triplicates. The closest ratio to 2.0 was the indicative of pure RNA.

Detection of IGF-1 and GAPDH gene in CHO-K1

PCR-based method was used to obtain optimum annealing temperature for the gene of interest (IGF-1) and reference gene (GAPDH). The presences of IGF-1 gene (269 base pairs) at all time points studied were evident in Figure 4 although at different thickness of bands as compared to the control gene (GAPDH, 123 base pairs).

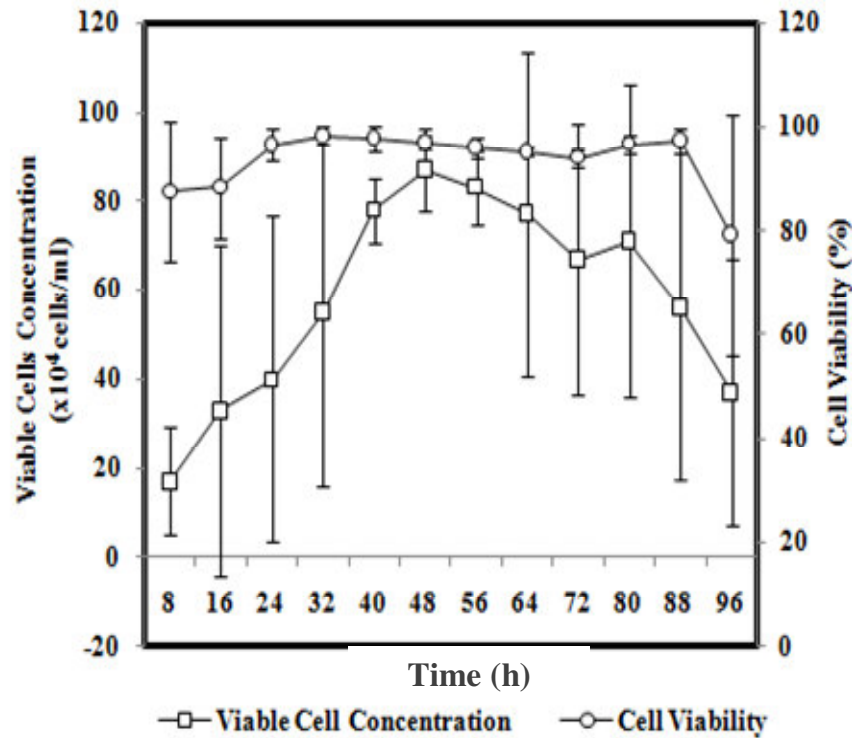


Figure 1. CHO-K1 cell growth during batch culture and percentage of viable cell (seeding concentration: 20×10^4 cells/ml; $n = 3 \pm SD$).

Table 2. CHO-K1 cells specific growth rate, μ and doubling time during batch culture (seeding concentration: 20×10^4 ; $n = 3 \pm SD$).

Maximum viable cell concentration ($\times 10^4$ cells/ml) mean \pm SD	Specific growth rate, μ (h^{-1}) mean \pm SD	Doubling time, t_d (h) mean \pm SD
$87 \times 10^4 \pm 9.098$	0.058 ± 0.020	11.000 ± 3.416

Table 3. CHO-K1 cells, maximum cell and related concentration during batch culture.

Concentration	Maximum concentration	Time (h) (where maximum concentration is reached)	Range
Viable cell concentration (cells ml^{-1})	87×10^4	48	17 to 87×10^4
Biomass (g/L)	1.000	72	0.27 to 1.00
Total protein content ($\mu g/ml$)	128.986	96	(-39.556) to 128.986
Glucose (g/L)	0.055	56	0.055 to 0.151
Lactate (g/L)	0.222	88	0.043 to 0.222

The different thickness of the bands may be related to the growth stage where the samples were taken and this warrants further investigation.

In contrast to a study by Sunstrom (1998), we success-

fully showed that CHO-K1 natively expressed the IGF-1 gene. This information is vital for future recombinant strategies to engineer efficient mammalian cell culture system, in particular targeting at IGF-1 axis. The study

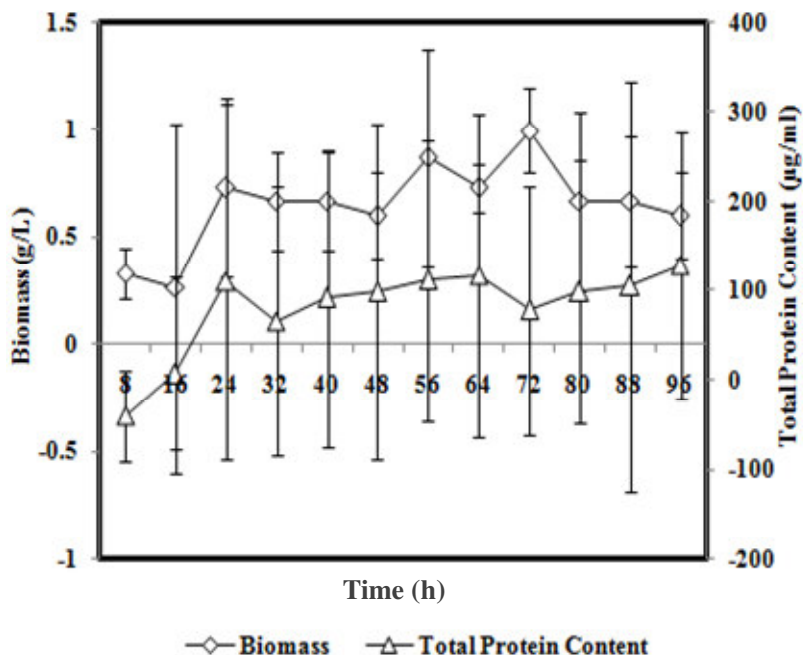


Figure 2. CHO-K1 cell biomass and total protein content concentration during batch culture (seeding concentration: 20×10^4 cells/ml; $n = 3 \pm SD$).

Table 4. Specific rates of nutrient consumption and product formation.

Response	Specific rate	Mean \pm SD
Glucose	Specific nutrient consumption rate, q_s (h^{-1})	-0.002 ± 0.001
Lactate	Specific product formation rate, q_p (h^{-1})	0.003 ± 0.001

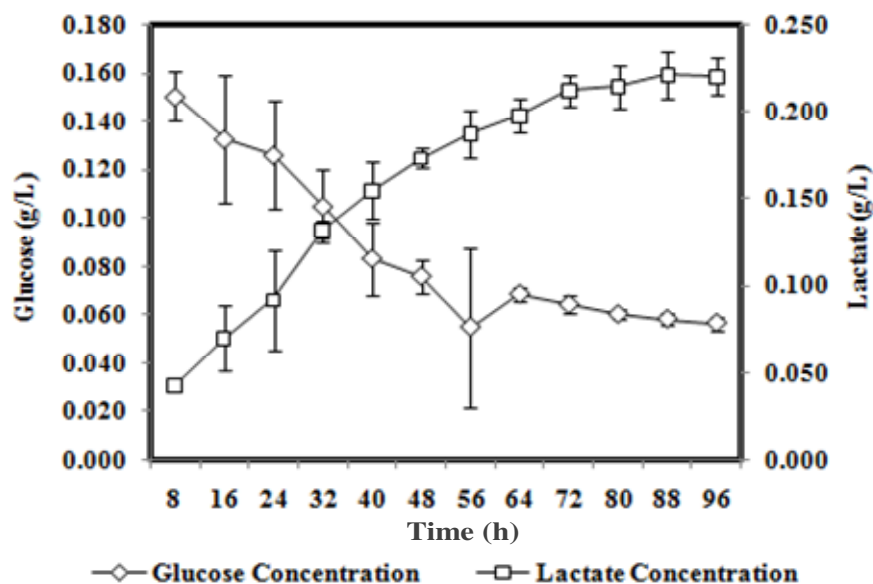


Figure 3. CHO-K1 cell glucose and lactate concentration during batch culture (seeding concentration: 20×10^4 cells/ml; $n = 3 \pm SD$).

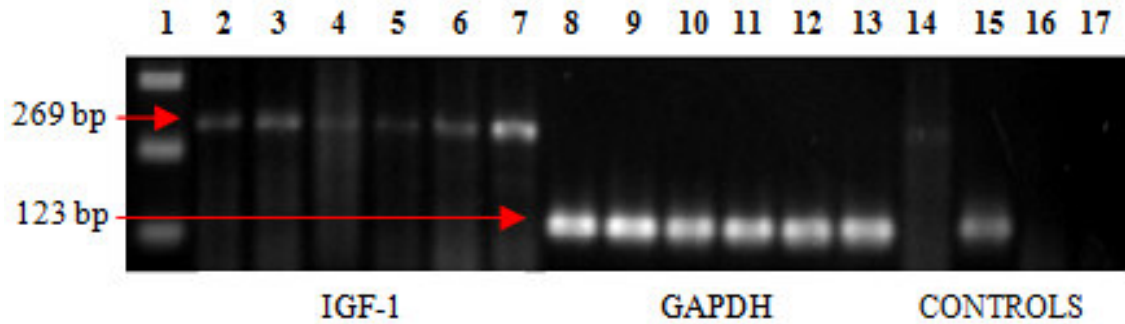


Figure 4. IGF-1 and GAPDH gene amplification using PCR (an exemplary image of one biological replicate). Lane 1: 100 bp DNA Ladder; (IGF-1) lane 2: sample at 16 h; lane 3: 32 h; lane 4: 40 h; lane 5: 48 h; lane 6: 64 h; lane 7: 88 h; (GAPDH) lane 8: 16 h; lane 9: 32 h; lane 10: 40 h; lane 11: 48 h; lane 12: 64 h; lane 13: 88 h; lane 14: IGF-1 positive control; lane 15: GAPDH positive control; lane 16: IGF-1 negative control and lane 17: GAPDH negative control.

also provides the baseline data on kinetics and biochemical responses of CHO-K1 cell growth. Together, the data would be particularly useful for further studies using CHO-K1 cells as efficient mammalian cell culture host system that is able to produce optimal yield of biologics with minimum or zero dependence on serum.

ACKNOWLEDGEMENT

This Project was funded by the Ministry of Higher Education (MOHE), Malaysia (Fundamental Research Grant Scheme, FRGS 0409-110).

REFERENCES

- Altamirano C, Paredes C, Cairo JJ, Godia F (2000). Improvement of CHO cells medium formulation: simultaneous substitution of glucose and glutamine. *Biotechnol. Progr*, 16: 69-75.
- Baserga R (1993). Cell lines which constitutively express IGF-I and IGF-I R. US Patent #5262308.
- Butler M (1996). *Animal cell culture and technology: the basics*. IRL Press: New York.
- Freshney RI (1994). *Culture of animal cells: a manual of basic technique*, 3rd edn. Wiley J. Liss, New York, pp 85-98.
- Hu WS (2003). *Stoichiometry and kinetics of cell growth and product formation*. Minnesota.
- Jayapal KP, Wlaschin KF, Hu WS, Yap MGS (2007). Recombinant protein therapeutics from CHO cells – 20 years and counting. CHO consortium. SBE Special Edition (October 1, 2007). www.aiche.org/uploadedFiles/SBE/Publications/CHO.pdf
- Pacher M, Seewald JM, Mikula M, Oehler S, Mogg M, Vinatzer U, Eger A, Schweifer N, Varecka R, Sommergruber W, Mikulits W, Schreiber M (2007). Impact of constitutive IGF1/IGF2 stimulation on the transcriptional program of human breast cancer cells. *Carcinogenesis*, 28(1): 49-59.

Percell Biolytica: Growth of Recombinant and Non-recombinant CHO cells. Application note 113. Retrieved 6th March 2010, from www.percell.se

Rozen S, Skaletsky (2000). Primer3 on the WWW for the General users and for Biologist Programmers. In: Krawets S, Misener S (eds), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Humana Press, Totowa, NJ, 365-386.

Sekyi-Otu A, Bell RS, Ohashi C, Pollak M, Andrulis IL (1995). Insulin-like Growth Factor 1 (IGF-1) Receptors, IGF-1, and IGF-2 Are Expressed in Primary Human Sarcomas. *Cancer Res*, 55: 129-134.

Sunstrom NA, Baig M, Sugyiono D, Gray P (1998). Recombinant insulin-like growth factor-I (IGF-I) production in *Super-CHO* results in expression of IGF-I receptor and IGF binding protein 3. *Cytotechnol*. 28: 91-99.

Wang X, Seed B (2003). A PCR Primer Bank for Quantitative gene Expression Analysis. *Nucleic Acids Res*. 31(24): 154, 1-8.

Yakar S, Wu Y, Setser J, Rosen JC (2002). The Role of Circulating IGF-1. *J. Endocrine*, 19(3): 239-248.