DETERMINATION OF IGF-1-PRODUCING CHO-K1 GROWTH PHASES USING GCMS-BASED GLOBAL METABOLITE ANALYSIS

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ABSTRACT: Mammalian cell lines, in particular CHO-K1 is vital for the multibillion dollar biotechnology industry. The majority of large scale bioprocessing of commercially valuable protein biopharmaceuticals is produced using this type of cell. An ideal mammalian cell system as host for biologics production should retain efficient use of energy sources in order to boost productivity at minimum cost. Various analyses such as cell counting and monitoring of specific biochemical responses are used to provide data to enable bioprocess control in order to achieve the ideal system. Our study aimed to see whether global metabolite analysis using Gas Chromatography Mass Spectrometry (GCMS) would be a potential alternative approach in providing data for bioprocess control. In this study, we analyzed metabolites of CHO-K1 cells at different growth phases using GCMS. CHO-K1 cells producing insulin like growth factor-I (IGF1) were obtained from ATCC. Cells were grown in T-flask and incubated at 37°C/ 5% CO₂ until 70-80% confluent in RPMI 1640 media. Samples (cells and spent/conditioned media) were taken at designated intervals for routine cell counting (Trypan Blue dye exclusion method); glucose, glutamine and lactate determination (YSI 2700); IGF-1 production (ELISA kit R&D Systems, Inc); and global metabolite analysis (GCMS). Conditioned media from each time point were spun down before subjecting into GCMS. Data from GCMS was then transferred to SIMCA P+12.0 for chemometric evaluation using Principal Component Analysis (PCA). The first component, PC1 results was able to explain 36% of the variation of the data with clear separation between exponential phase and other phases (initial and death phase). This suggests that GCMS-based global metabolite analysis has the ability to capture cell growth behaviour and offered insights of factors that may influence the biological system.

ABSTRAK: Produk yang berupa sel kekal mamalia, terutamanya CHO-K1 adalah penting dan menguntungkan industri bioteknologi. Majoritinya pemprosesan protein biofarmaseutikal secara besar-besaran dihasilkan dengan menggunakan sel jenis ini. Sistem sel mamalia yang ideal sebagai hos untuk penghasilan produk ubatan harus mengekalkan penggunaan sumber tenaga secara efisien untuk meningkatkan produktiviti pada kos yang minima. Pelbagai analisa seperti perhitungan sel dan pemerhatian tindak bulas biokimia tertentu digunakan untuk memberikan data dan untuk menentukan bioproses terkawal untuk mendapat sistem yang ideal. Kajian ini dijalankan untuk mengkaji sama ada analisa metabolit global menggunakan Spektrometri Jisim Kromatografi Gas (Gas Chromatography Mass Spectrometry (GCMS)) boleh berpotensi sebagai pendekatan alternatif dalam membebalkan data untuk kawalan bioproses. Dalam kajian ini, metabolit sel CHO-K1 dikaji pada peringkat tumbesaran berbeza menggunakan GCMS. Sel CHO-K1 menghasilkan insulin seperti faktor pertumbuhan-I (IGF1) didapati daripada ATCC. Sel dibesarkan dalam kelalang-T dan dieramkan pada
37°C/5% CO₂ sehingga 70–80% konfluen dalam perantara RPMI 1640. Sampel (sel dan perantara yang digunakan/dilazimkan) diambil kiraan pada selang masa yang telah ditetapkan untuk sel rutin (kaedah eksklusi pencelu p Trypan Blue), glukosa, glutamin dan penentuan laktat (YSI 2700); penghasilan IGF -1 (kit ELISA R&D Sstems, Inc); dan analisa global metabolit (GCMS). Perantara yang dilazimkan dari setiap poin masa dikuurangkan kelajuanannya pada setiap pusingan sebelum dianalisa secara GCMS. Data daripada GCMS kemudiannya dipindahkan ke SIMCA P+12.0 untuk taksiran kemometrik menggunakan Analisis Komponen Utama (Principal Component Analysis (PCA)). Keputusan komponen pertama, PC1 berupaya menjelaskan variasi 36% data dengan pengasingan jelas antara fasa eksponen dan fasa-fasa lain (fasa permulaan dan fasa akhir). Ini menunjukkan bahawa analisa metabolit global berasaskan GCMS mampu menjelaskan tingkah laku pertumbuhan sel dan memberikan pemahaman tentang faktor-faktor yang mempengaruhi sistem biologikal.

**KEYWORDS**: CHO-K1 cells; Insulin Growth Factor-1 (IGF-1); metabolomics; metabolite profile; PCA; GCMS

1. **INTRODUCTION**

Animal cell lines have been used extensively for the production of a variety of therapeutics and prophylactic protein products including hormones, cytokines, enzymes, monoclonal antibodies, recombinant glycoproteins, viral vaccines and blood clotting factors. Mammalian cells are generally capable of secreting functionally active proteins with correct folding and post-translational modifications, unlike bacterial or yeast systems [2]. Although many simple proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) require animal cells as host system.

The natural surroundings of mammalian cells (biochemical, physiological and physiochemical) directly affect the growth of cells and their metabolism. A battery of analyses is routinely performed to provide data for bioprocess control in the desire to achieve the ideal. This includes determination of the concentration of target bioproducts and cell counting. However, these analyses require repeated sampling at multiple time points which may lead to high cost and potential contamination. Each type of analysis is very specific and has its own procedure with different consumables and apparatus which also incur cost.

Metabolomics (metabolome analysis) refers to the in-depth analysis of metabolites [3]. GCMS-based metabolomics is used to reveal important metabolic pathway by surveying large number of metabolites that may not be picked up by standard metabolic analyses and hence provide a starting point for further detailed study. GCMS also work as an excellent starting point for a non-hypothesis driven way, analyze and quantify as many metabolites as possible and generate data sets that can be used for biomarker discovery [1].

There are various analytical instrument platforms for metabolomics approach, namely nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, chromatographic and column separation, vibrational spectroscopies such as fourier-transform infrared (FTIR) and hyphenated instruments such as gas chromatography mass spectrometry (GCMS) and liquid chromatography mass spectrometry (LCMS) [4]. NMR increases the analytical reproducibility and simplicity of sample preparation but it is relatively insensitive compared to mass spectrometry technique [13]. Meanwhile, gas chromatography (GC) coupled to mass spectrometry (MS) offers high chromatic
resolution but requires chemical derivatization for many biomolecules. Only volatile chemicals can be analyzed without derivatization. MS is designed to separate gas ions according to their m/z (mass to charge ratio) value. GCMS measures mass of analytes and give information about chemical structures including metabolites. While LCMS requires no derivatization of samples and gives excellent throughput, GCMS covers a wider range of global metabolites compared to LCMS [14]. In addition, metabolite identification by GCMS is simplified when standardized retention indices and mass spectra are registered in a database.

Global metabolite analysis studies generate complex data from GCMS mass spectra, which are hard to summarize and visualize without appropriate tools. The use of chemometric tools, e.g., principal component analysis (PCA) and partial least-squares to latent structures (PLS) are therefore of great importance as these include efficient, validated, and robust methods for modelling information-rich biological data. The PCA model can indicate systematic trends within the data, i.e., clustering, time trends, etc.

Metabolomics has been used in a wide range of fields, including diagnostic marker search and etiology analysis in medical treatment, biomarker search for revealing efficacy and toxicity in drug manufacturing, and quality control in food processing [3]. Metabolomics also being used in plant science [4], yeasts-related research [5] and increasingly used in nutrition research [6]. Although metabolomics have been applied to the mammalian system in relation to disease diagnosis and therapeutic efficacy, pre-clinical drug safety assessment and pharmacology [7], it has been applied to a lesser extent in the mammalian cell culture/bioreactor area [4, 8]. As the number of therapeutic proteins produced by mammalian cell cultures in the pharmaceutical industry continues to increase, the need to improve productivity and ensure consistent product quality during process development activities becomes more significant. While the focus here is on the extracellular metabolite of CHO-K1 cell cultures, this methodology is generally applicable to quantitating intracellular concentrations and can be extended to other mammalian cell lines, as well as platforms such as yeasts, fungi, and *Escherichia coli* [16].

GCMS has proven to be an effective tool for metabolites identifications and quantifications in mammalian cell lines due to its excellent resolution, sensitivity, separation capacity, and its ability to generate a mass spectrum for each compound, allowing separation and detection of many metabolites [9-11]. In this study, we use gas chromatography coupled to mass spectrometry together with chemometric tools to investigate whether GCMS-based approach can identify the growth phases of cells in culture using spent media. This would offer alternative to bioprocessing monitoring in particular using non-invasive technique.

### 2. MATERIALS AND METHODS

#### 2.1 Media and Reagents

RPMI 1640 media were obtained from Cellgro (Herndon, VA USA); Other reagents include Fetal Bovine Serum, (GIBCO – Invitrogen, South America); Accutase in Dulbecco's Phosphate-Buffered Saline, DPBS 0.5 mM EDTA (Innovative Cell Technologies, Inc); Phosphate Buffered Saline, (Sigma-Aldrich).

#### 2.2 Cell Line
The CHO-K1 cell line, (ATCC CCL-61™) was obtained from American Type Culture Collection (ATCC).

2.3 Cell Culture and Growth Assessment

Stocks of CHO-K1 cells were revived in 25 cm² T-flask in 5 ml RPMI 1640 with 10% FBS (v/v). After several passages, the cells were then subcultured in 75 cm² T-flask (15 ml) for 3-4 days with a seeding density 2.0 x 10⁵ cells ml⁻¹, 37°C/ 5% CO₂. Cells were prepared in separate T-flask for each time point for three independent experiments. Samples were taken at 8-hourly intervals for routine cell counting (Trypan Blue dye exclusion method), biochemical responses and metabolite analysis.

2.4 Biochemical/Standard Analysis

Supernatant were collected for glucose, glutamine and lactate concentration analyses. These analyses were carried out at all designated time points (8-hourly intervals) in each condition. All experiments were carried out with 3 biological replicates for each experimental condition. Conditioned media were collected for glucose, lactate and glutamine concentrations at designated time points. Determination was carried out using biochemical analyzer YSI 2700.

2.5 IGF-1 Protein Analysis

Conditioned media were separated from cells by centrifugation at 1400 rpm in 5 minutes. The expression of IGF-1 protein was then studied using ELISA kit (R&D Systems, Inc.) based on manufacturer instructions.

2.6 GCMS Analysis

GCMS analysis was performed on a 7890A GC System (Agilent Technologies) coupled to a 5975C Inert XL MSD. Samples of supernatant (spent media) were spun down and injected (splitless mode, 10:1) onto a HP-5MS Ultra Inert (Agilent Technologies; 250 µm x 30 m x 0.25 µm film thickness) using helium (1.2 ml/min) as the carrier gas. Components were separated by isothermal chromatography for 1 min at 60°C, followed by an increase to 325°C. Mass spectra were acquired in positive ion mode using electron impact ionisation at 70 eV. The injector, MS source and MS quad temperatures were set at 250, 230 and 150°C respectively.

2.7 Multivariate Data Analysis

Multivariate data analysis was conducted in SIMCA-P+12.0. PCA scores plot of GCMS data from the spent media of CHO-K1 was generated. In this study, the principle component analysis was based on the top 50% library-matching of metabolites (qual), given by the retention time. Peak areas with low intensity were rejected.

3. RESULTS AND DISCUSSION

All experiments were carried out at 3 biological replicates for each experimental condition. Cell growth and behaviour were observed under various conditions using standard analyses and GCMS-based metabolomics approach.

3.1 CHO-K1 Cell Growth
CHO-K1 cell cultures were monitored every 8 hours for growth and viability. These data represent the average cell viability for 96 hours. Cultures were seeded at a density of $2 \times 10^5$ cells/ml in 15 ml media.

The cell growth of CHO-K1 is shown in Fig. 1. The highest saturation density of CHO-K1 is $9.70 \times 10^5$ cells/ml at 80th hour. The doubling time of CHO-K1 cells is 29 h at specific growth rate of 0.02 cells h$^{-1}$. It can be seen in the graph that there is no lag phase in cell growth of CHO-K1.

### 3.2 Biochemical/Standard Analysis

In mammalian cell culture, both glucose and glutamine can be utilized as the carbon sources to provide energy for cell growth through the catabolic pathway, and to transform to precursors or intermediates for synthesis of cellular components. While these components are vital for the mammalian cells to grow, the metabolites produced upon consumption of these components result in toxic waste products (metabolites) such as lactate [15]. Figure 2 showed the trend of glucose and glutamine consumption as well as lactate production during the CHO-K1 cell growth.

![Fig. 1: Cell Growth of CHO-K1 Cell. I=Early log phase, II=Early mid log phase, III=Mid log Phase, IV=Late log phase, V=Death phase.](image1.png)

![Fig. 2: (a) Concentration of glucose, (b) Concentration of glutamine, (c) Concentration of lactate during CHO-K1 cell growth.](image2.png)
The amount of glucose in the cell culture formulations is 2.0 g/L. In this culture, it can be seen that glucose was totally consumed after 64 hours. The high concentration of glucose is usually used because cells at high density consume a lot of glucose and the growth and maintenance of mammalian cells depend on glucose availability [19].

Meanwhile, the trend of glutamine consumption was similar to glucose. When glucose levels are low and energy demands are high, cells can metabolize amino acids for energy. Glutamine is one of the most readily available amino acids for use as energy source and it is a major source of energy for many rapidly dividing cell types in vitro. As the glucose concentration is decreased, glutamine becomes the sole energy source for cultured cells. The concentration of glutamine is regulated by the presence of glucose [17].

While glucose and glutamine are vital for the mammalian cells to grow, the metabolites produced upon consumption of these components result in toxic waste products (metabolites) such as lactate [15]. Figure 2 shows that cell growth was reduced after 72 hours with the increase in lactate during the exponential phase.

3.3 IGF-1 Protein Analysis

Figure 3 shows that the IGF-1 concentration increased during the exponential phase and decreased after the highest saturation density point. But these analyses (biochemical/standard/IGF-1 protein analyses) are difficult to promise us the concrete results in future as biomarker in culture system since the amount of consumption and accumulation of substrates in a cell growth will change associated/non-associated with the different growth culture environment setting.

![Fig. 3: IGF-1 production (pg/ml) in CHO-K1 cell growth.](image)

3.4 Multivariate Analysis

Figure 4 shows GCMS data analysed by PCA. The score plot shows that there were clear groupings of the samples in a pattern which relates to the time of sampling (growth phases). Principle component 1 (PC1) explained 36% variation of the data while PC2 only explained 15% of the variation. Based on PC1, samples were discriminated into (A) exponential phase and (B) early, late log and death phase. PC1 is most probably refers to the abundance of metabolites.
Interestingly, based on PC2, early log phase (I) and mid log phase (III) – A, are grouped together while early-mid log (II), late log (IV) and death phase (V) - B are in one group. The mass spectra of group A and B were shown in Fig. 6. We are currently analysing the phenomena to further understand the system in study.

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Figure 5 shows mass spectra at each phase of growth. There are two main dimensions of which the spectra can be analyzed. One is the complexity where the number of peak increases as the culture aged. The second dimension is related to abundance of compounds. However, in this study it is observed that each phase revealed similar metabolites but with different abundance. The abundance of compounds increased at later stages of growth. This is in contrast to a study of yeast metabolomic footprinting where yeast culture footprints were simple at initial stages and became more complex at later stages Allen et al. [18].

GCMS-based metabolomics approach using spent media as samples is a potentially valuable tool in providing biomarker for the purpose of monitoring cell culture growth and product formation. This is particularly useful for large scale production where invasive technique such as cell counting from adherent cells are not favourable. Online monitoring could be designed to complement bioreactor system where spent media is being sampled at designated time for subsequent analysis using GCMS.

In this study, it was identified that the highest abundance of compound throughout all phases is 2-cyclohexen-1-one, 2-methyl-5-(1-methylthethyl)-, (S)- which is a terpenoid compound detected at 16.20 minute (CAS Number 057397-12-3).
Fig. 5: Mass spectra of global metabolite of CHO-K1 cells at different growth phases: early-log [I], early – mid log [II], mid log [III], late log [IV] and death [V].

Fig. 6: A – early-mid log [II] and mid log [III] phase spectra, (c) B – early-log [I], late log [IV] and death [V] phase spectra.
Although common biochemical analyses such as determination of glucose, glutamine and lactate concentration are routinely performed alongside cell counting, these analyses have not been able to describe the growth phases of cells as clearly as being shown by the PCA results (Fig. 5).

Pertaining to the detection of terpenoid as the highest concentration of compound in the culture, it suggests that this compound could be used as a biomarker to predict growth phase of a similar culture system without having to conduct cell counting and other common biochemical analyses. The biomarker can also be used to predict product formation. For instance, the CHO-K1 cells in this study produced Insulin-like growth factor 1 (IGF-1) with the highest level found at 64 h (data shown in Fig. 3). Therefore the optimal level of terpenoid at 56 h would serve as a cue to the IGF production thus enable on-time downstream processing such as harvesting of IGF-1.

To this end, global metabolite analysis offers the benefits of using a single method of analysis as compared to a battery of specific analyses targeting specific compounds in the quest to maintain efficiency of cell culture system and give insights on how to improve the system when necessary.

4. CONCLUSION

The growth phases of IGF-1-producing CHO-K1 were clearly identified using gas chromatography coupled to mass spectrometry together with bioinformatics tools PCA to monitor changes in the levels of metabolites. The GCMS based global metabolite analyses may enable and provide novel insights into the biological system under study.

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REFERENCES

[1] Goodacre, R., Seetharaman, V., Dunn, W.B., Harrigan, G.G., Kell, D.B., “Metabolomics by numbers: acquiring and understanding global metabolite data”
[2] Castilho, L.R., Moraes, A.M., Augusto, E.F.P. Butler, M., “Animal Cell Technology: From biopharmaceuticals to gene therapy”. Taylor & Francis Group, New York (2008), p. 30.
[3] Yuki, S., Katsuhiro, N., Shuichi, K., Novalina, L., Hui-Loo, L.C., Haruhiko, M., “Profiling of Japanese Green Tea Metabolites by GCMS”. Shimadzu
[4] Khoo, S.H.G., Al-Rubeai, M., “Review: Metabolomics as a complementary tool in cell culture”. Biotechnology Aplication Biochemistry, Portland Press Ltd (2007), 47: p. 71-84.
[5] Pope, G.A.M., D.A., Defernez, M., Aroso, M.A.A.M., Fuller, L.J., Mellon, F.A., Dunn, W.B., Brown, M., Goodacre, R., Kell, D.B., Marvin, M.E., Louis, E.J., Roberts, I.N., “Metabolic footprinting as a tool for discriminating between brewing yeasts”. Yeast (2007), 24: p. 667-679.
[6] Gibney, M., “Metabolomics in human nutrition: opportunities and challenges”. American Journal of Clinical Nutrition (2005), 82: p. 497-503.
[7] Lindon, J.C.N., J.K., Holmes, E., “The handbook of metabolomics and metabolomics”. (2006), Amsterdam: Elsevier.
[8] Kuystermans D, K.B., Swiderek, H., Al-Rubeai, M., "Using cell engineering and omic tools for the improvement of cell culture processes”. Cytotechnology (2007), 53: p. 3-22.
[9] Fernandez, C., Fransson, U., Hallgard, E., Spegel, P., Holm, C., Krogh, M., Warell, K., James, P., Mulder, H., “Metabolomic and Proteomic Analysis of a Clonal Insulin-Producing β-Cell Line (INS-1 832/13)”. Journal of Proteome Research (2008), p. 400-411.

[10] Takeda, S., Sim, P. G., Horrobin, D. F., Sanford, T., Chisholm, K. A., Simmons, V., “Mechanism of lipid peroxidation in cancer cells in response to gamma-linolenic acid (GLA) analyzed by GC-MS(I): Conjugated dienes with peroxyl (or hydroperoxyl) groups and cell-killing effects”. Anticancer Res (1993), p. 193-199.

[11] Wojciech, F., Andreas, S., Anna, F., Clemens, A., Jochen, S., Wolfram, M., Anton, A., Jakob, T., “TD-GC-MS Analysis of Volatile Metabolites of Human Lung Cancer and Normal Cells In vitro”. Cancer Epidemiol Biomarkers Prev (2010), p. 182.

[12] Sellick, C.A., Knight, D., Croxford, A.S., Maqsood, A.R., Stephens, G.M., Goodacre, R., Dickson, A.J., “Evaluation of extraction processes for intracellular metabolite profiling of mammalian cells: matching extraction approaches to cell type and metabolite targets”. Metabolomics (2010), 6: p. 427 – 438.

[13] Griffin, J.L., “Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterisation of xenobiotic toxicity and disease diagnosis”. Curr Opin Chem Biol 7 (5): 648–54.

[14] Silas, G.V.B., Sandrine, M., Mats, A., Jorn, S., Jens, N., “MASS SPECTROMETRY IN METABOLOME ANALYSIS”. Mass Spectrometry Reviews (2005), 24, 613– 646.

[15] Provost, A.B., G., “Dynamic metabolic modeling under the balanced growth condition”. Journal of Process Control, (2004). 14: p. 717-728.

[16] Scott, A. B., Anli O., Jennifer P., Tim, A. S., Tongtong, W., A. K., Fermentanomics: Monitoring Mammalian Cell Cultures with NMR Spectroscopy. J. Am. Chem. Soc., 2010, 132 (28), pp 9531–9533.

[17] http://www.sigmaaldrich.com/lifescience/cell-culture/learning-center/media-expert/glutamine.html

[18] Allen, J., Davey, H.M., Broadhurst, D., Heald, J.K., Rowland, J.J., Oliver, S.G., Kell, D.B., “High-throughput classification of yeast mutants for functional genomics using temetabolic footprinting”. Nat. Biotech. 2003, 21(6), pp 692-696