Kasetsart J. (Nat. Sci.) 34 : 79 - 84 (2000)

The Influence of Serum Concentration on tPA Production of CHO Cell

Teerapatr Srinorakutara[1] , Jin-Ho Jang[2] , Mutsumi Takagi[2] and Toshiomi Yoshida[2]

ABSTRACT

The effect of initial serum concentration on cell growth, and the production of tissue plasminogen activator (tPA) is investigated across batch fermentation of a Chinese hamster ovary (CHO) growing in newborn calf serum (NCS) medium. It was found that initial serum concentration influenced on CHO cell growth rate and cell yield. At high-serum content, the uptake rate of glucose decreased although the specific growth rate was higher, indicating a serum component becoming growth limiting. An average glucose uptake rate in 10% (v/v) NCS medium was 13.84 mg/10^6 cells/h as in 1% (v/v) NCS medium was 18.17 mg/10^6 cells/h. It was also found that the tPA or the maximum tPA productions increased with initial serum concentration. At 7.12% NCS, the maximum tPA production gave the highest value (5.55 mg/l). The effect of initial serum concentration on the main product was similar to that of major by-product (e.g. lactate and ammonia).

Key words: serum medium, tPA, CHO cell, animal fermentation

INTRODUCTION

Tissue culture medium is always supplemented with serum to provide the cell with necessary growth factors and trace mineral. The composition of serum is complex and ill-defined. More importantly, serum is the most expensive raw material in cell culture medium. In addition, serum proteins contaminate the product and subsequently increase purification costs. Therefore, it is desirable to reduce the amount of serum or other growth factors used in the production of the desired product while maximizing volumetric productivity.

Reduction of the percentage of NCS in the culture medium is an important aim in animal cell culture, because of improved economy, easier purification of products (e.g. tPA) and a better defined production process (van der Pol *et al.*, 1990).

While these media are highly optimized, a lower cost alternative is desirable for large-scale protein production, which is also serum-free and supports reasonably good cell growth and recombinant protein production. Also, when specific formulas are desired, it is useful to know what the necessary components are for designing a simple yet effective medium without a painstaking optimization process. Other investigators have sought to replace the serum to reduce cost, reduce lot-to-lot variability in serum, reduce potential risk

[1] Thailand Institute of Scientific and Technological Research, 196 Phahonyothin Road, Chatuchak, Bangkok 10900, Thailand.
[2] ICBiotech, Osaka University, 2-1, Yamada-oka, Suita, Osaka 565, Japan.

of contamination by mycoplasma, and reduce the protein load in the supernatant to facilitate downstream processing of recombinant proteins (Donaldson and Shuler, 1998).

In this study, the effect of initial serum concentration on growth rate, maximum cell yield and tPA production rate on the production of tPA was studied on CHO cultured in spinner bottles.

MATERIALS AND METHODS

Cell Line, medium and cultivation methods

CHO ATCC CRL-9606, a Chinese hamster ovary (CHO) cells producing tPA used in this study was kindly provided by International Center of Biotechnology (ICBiotech), Osaka University, Japan. The original culture medium consisted of 10.64 g/l Ham's F-12 (ICN Biomedicals Inc., Ohio, USA) supplement with 10% (v/v) NCS (GIBGO Laboratories Life Technologies Inc., USA), 500 nM methotrexate (MTX, Sigma), 1.18 g/l $NaHCO_3$ (Wako Pure Chemical Industries Ltd., Japan), and 200 µl of 1,000 units penicillin G or 1 mg streptomycin (Sigma). The inoculumn was prepared from stock culture of cells scaled to 10 ml of 10% (v/v) NCS medium in 55 cm^2 petridishes. The petridishes were then incubated in CO_2 SANYO MCO-345 incubator (SANYO, Japan) at 37°C, 5% CO_2 concentration for 3 days. Cells in petridishes used as seed were fully-grown to 8.8×10^5 viable cells/ml before introduction to the four 100-ml spinner bottles (HARIO Co., Ltd., Tokyo, Japan). Four 100-ml spinner bottles with working volume of 30 ml of serum free medium contained the NCS at concentration of 1, 2, 5, and 10%(v/v) respectively and were then put into a $CuSO_4$ solution bath with magnetic stirrer SW-600S (NISSIN Scientific Corp., Japan). The $CuSO_4$ solution bath was controlled at 37°C and 70 rpm. In order to control pH, the $CuSO_4$ solution bath was surface-aerated with humidified air containing 5% CO_2 through a 0.2 mm membrane airfilter (Millipore). CO_2 concentration was daily checked using "FYRITE" Bacharach (Bacharach Inc., Pittsburgh, USA). The initial cell concentration in spinner bottles was approximately 2×10^5 cells/ml.

Analytical Methods

Sample consisting of 750 µl suspended cells was taken daily, and was divided into 2 parts. The 1st part, 250 µl suspended cells, was used for determination of cell concentration and viability by tryphan blue exclusion on a hemacytometer. The 2nd part, 500 µl suspended cells, was centrifuged using TOMY high-speed refrigerated microcentrifuge MX-150 (TOMY TECH USA Inc.) at 4°C, 1000 rpm for 4 min. The supernatant was then used for determination of glucose and lactate concentrations using biochemistry analyzer YSI 2700-D SELECT (YSI Incorporated, Yellow Springs Instrument Co., Ltd., Yellow Springs, OHIO 45384-0279, USA). The remaining supernatant was aliquoted and stored at –20°C for later analysis of ammonia, glutamine, and tPA contents.

An enzymatic method was used to determine ammonium concentration using a NH_3 kit (Wako Pure Chemical Industries Ltd., Japan).

Glutamine and glutamate concentrations were determined using biosensor BF-4 (Oji Scientific Instruments, Hyogo, Japan).

The Biopool Imulyse tPA (Biopool international, CA, USA), an immunoassay (ELISA) for the quantitative determination of single-chain and two-chain tPA antigen in human plasma or in other biological fluids, such as cell culture supernatants, was used to determine tPA concentration.

Determination of specific growth rate and metabolic quotients

The specific growth rate, μ, assuming

negligible cell lysis, was calculated from data collected during the exponential growth phase and is defined as follows:

$$\mu = \frac{1}{x_v}\frac{dx_t}{dt} \quad \text{..... (1)}$$

Where x_v denotes the concentration of viable cells and *t* denotes the cultivation time. The specific metabolic quotient calculations for glucose consumption and lactate formation (q_{Glu} and q_{Lac}, respectively) were also based on data collected during the exponential phase of growth. They are defined as follows:

$$-q_{Glu} = \frac{1}{x_v}\frac{d[Glu]}{dt} \quad \text{..... (2)}$$

$$q_{Lac} = \frac{1}{x_v}\frac{d[Lac]}{dt} \quad \text{..... (3)}$$

where [Glu] and [Lac] are glucose and lactate concentrations respectively.

Glutamine spontaneously decomposes following first-order kinetics to pyrroilidone carboxylate and ammonia (Tritsch and Moore, 1962). The specific glutamine composition and ammonia production rates (q_{Glu} and q_{NH4+}, respectively) were determined by accounting for the degradation of glutamine at 37°C (Glacken *et al*., 1988; Ozturk and Palsson, 1990).

$$-\frac{d[G\ In]}{dt} = k[G\ In] + q_{G\ In}X_v \quad \text{..... (4)}$$

$$\frac{d\left[NH_4^+\right]}{dt} = k[G\ In] + q_{NH_4^+}X_v \quad \text{..... (5)}$$

where [Gln] is the glutamine concentration (mM or mg); $[NH_4^+]$ is the ammonia ion concentration (mM or mg); *k* is the first-order rate constant for glutamine decomposition (h^{-1}). Since the first-order decomposition rate varied with serum and medium components (Miller *et al*., 1988; Ozturk and Palsson, 1990) the values of *k* were measured experimentally.

The yield coefficients of glucose consumed to lactate produced and of glutamine consumed to ammonia produced ($Y_{Lac/Glu}$ and $Y_{NH4^+/Gln}$, respectively) are defined as follows:

$$Y_{Lac\ /\ Glu} = \frac{q_{Lac}}{q_{Glu}} \quad \text{..... (6)}$$

$$Y_{NH_4^+} = \frac{q_{NH_4^+}}{q_{G\ In}} \quad \text{..... (7)}$$

The specific tPA productivity, q_{tPA} was based on the data obtained from each batch cycle:

$$q_{tPA} = \frac{[tPA]-[tPA]_0}{\int_0^{tc} x_v dt} \quad \text{..... (8)}$$

where [tPA] and $[tPA]_0$ denote the concentration of tPA and the initial concentration of tPA for each batch cycle, respectively.

RESULTS AND DISCUSSION

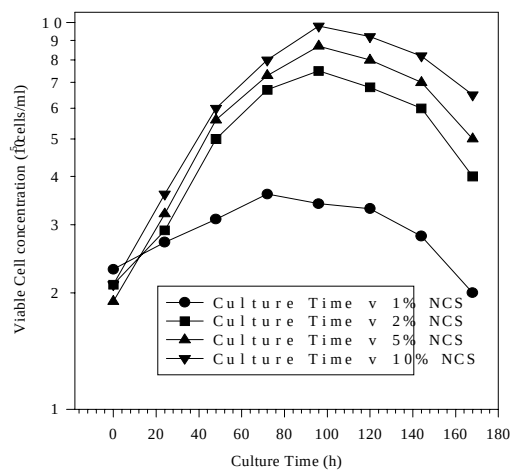

Figure 1 The growth curves for CHO ATCC CRL-9606 cultivated in four spinner bottles containing 1, 2, 5, and 10% (v/v) initial NCS concentrations.

The growth curve for each spinner bottle is presented in Figure 1. Clearly, the concentration of serum component(s) plays an important role in both CHO cell growth rate and cell yield. Only a slight reduction in viable cell number occurred when the serum content was reduced from 10 to 2%. The cell concentration rapidly decreased, however, from 2% to 1% FCS, indicating a serum component becoming growth limiting. The maximum cell yields were due to initial serum level, indicating stoichiometric as well as kinetic limitation by serum component (s) (Dalili and Ollis, 1989).

Glucose and glutamine, major carbon and energy sources in most cell culture media, required for cell growth, were measured during the cultivation. The major byproducts, lactate and ammonia, were also measured. The glucose and lactate concentrations during the cultivation are shown in Figure 2. The glucose levels decreased markedly during the exponential growth, and glucose utilization was accompanied by a

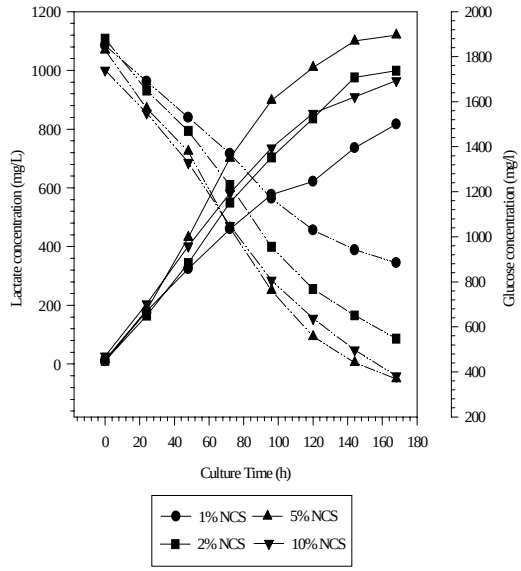

Figure 2 Glucose consumption (dotted lines) and lactate production (solid lines) of CHO ATCC CRL-9606 cultivated in four spinners bottles containing 1, 2, 5, and 10% (v/v) initial NCS concentrations.

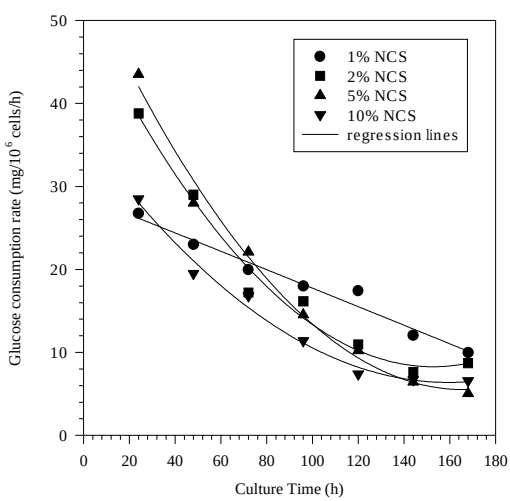

Figure 3 Glucose uptake rate of CHO ATCC CRL-9606 cultivated in four spinner bottles containing 1, 2, 5, and 10% (v/v) initial NCS concentrations.

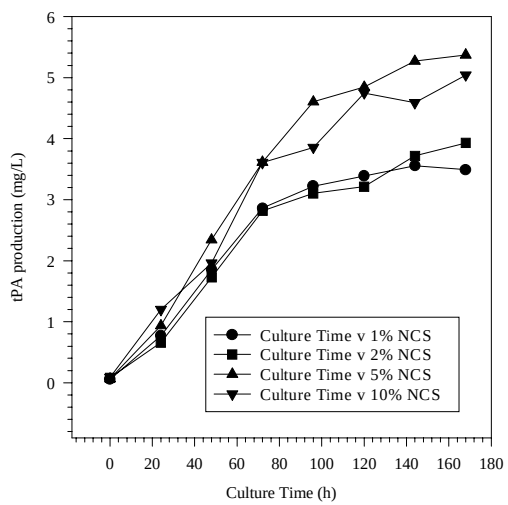

Figure 4 tPA production of CHO ATCC CRL-9606 cultivated in four spinner bottles containing 1, 2, 5, and 10% (v/v) initial NCS concentrations.

corresponding accumulation of lactate. Glucose was not a limiting nutrient for cell growth in all culture conditions tested.

With high-serum medium, the uptake rate of glucose decreased (Figure 3), although the specific growth rate was higher (Fig. 1). This result indicated that the cells utilize glucose more efficiently in high-serum medium. An average glucose uptake rate of the cells in 10% (v/v) NCS medium was 13.84 mg/10^6 cells/h, while the average glucose uptake rate increased to 18.17 mg/10^6 cells/h in 1% (v/v) NCS medium.

Although tPA production (Figure 4) or maximum tPA production (Figure 5) increased with initial serum concentration and reached the highest maximum tPA production (5.5498 mg/l) at 7.12 % (v/v) NCS, at higher serum content some factor(s) might become inhibitory for tPA production. The effect of initial serum concentration on the main product (tPA) was similar to that of the major by-products (e.g. lactate and ammonia). Concentration of tPA and lactate gave the highest values at 5% (v/v) NCS (Figure 4) as the highest ammonia production was at 10% (v/v) NCS (Figure 6). Concentration of serum at 5% (v/v) NCS may be a more suitable value for producing the tPA and lactate.

Next to sugar, glutamine is the most abundant constituent of tissue culture media (Eagle, 1955). It has been long established that the metabolism of glutamine can provide significant quantities of energy in mammalian cells (Reitzer *et al*., 1979). This cellular energy is produced via total or partial oxidation of glutamine. The complete oxidation of glutamine to CO_2 occurs via the TCA cycle, whereas its partial oxidation is accomplished by a linear pathway, which involves several intermediates of the TCA cycle. The end product of

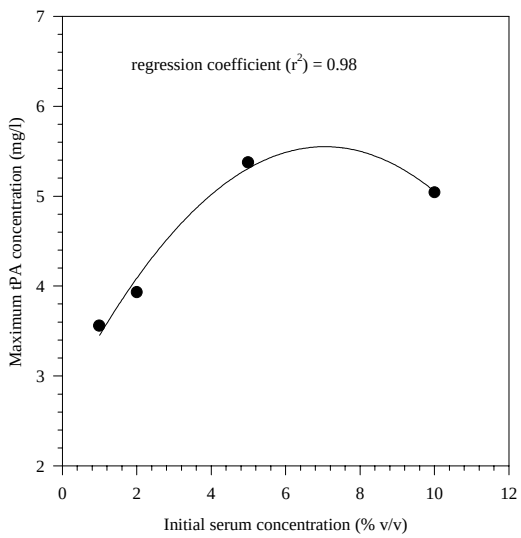

Figure 5 Relationship between initial serum concentrations and maximum tPA concentrations for cultivation of CHO ATCC CRL-9606 grown in spinner bottles. The line shown is regression of initial serum concentration against maximum tPA concentration, with a regression coefficient of $r^2 = 0.98$.

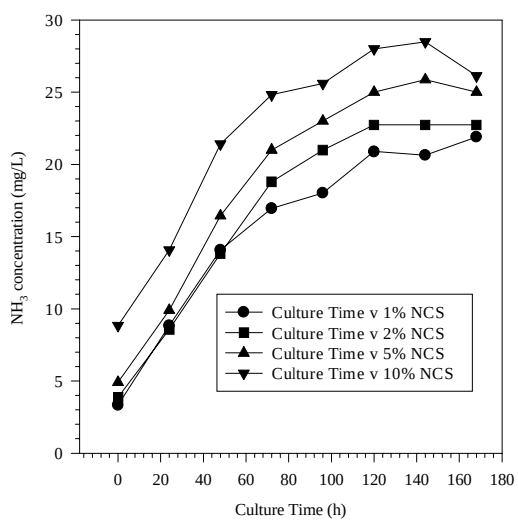

Figure 6 Ammonia (NH_3) production of CHO ATCC CRL-9606 cultivated in four spinner bottles containing 1, 2, 5, and 10% (v/v) initial NCS concentrations.

this pathway is pyruvate and /or lactate. By analogy to naming the oxidation of glucose to lactate as glycolysis, McKeehan (1982) has coined the term glutaminolysis for the partial oxidation of glutamine. Data for concentration of glutamine and glutamate in spinner bottles containing 1, 2, 5, and 10% (v/v) NCS determined as a function of time were not shown in this report because some data was lost. However, from the remaining data it showed that the concentration of glutamine decreased with time.

Unfortunately, amino acid was not analyzed in this study because of limitation of research time. Therefore, no amino acids data lead to lacking of information concerning available effect of amino acid components on such as tPA production and so on.

ACKNOWLEDGEMENT

The authors wish to thank the UNESCO, for financial support throughout this work. The authors express their sincere thanks to the technical staff of the international center of biotechnology (ICBiotech) for providing the facilities and technical assistance throughout this work. Finally, the authors would like to thank Ms Toshiko Nagai and Ms Kumiko Gojo for financial arrangement.

LITERATURE CITED

Dalili, M. and D.F. Ollis. 1989. Transient kinetic of hybridoma growth and monoclonal antibody production in serum-limited cultures. Biotechnology and Bioengineering 33 : 984-990.

Donaldson, M.S. and M. L. Shuler. 1998. Low-cost serum medium for the BTI-Tn5B1-4 insect cell line. Biotechnology Progress 14 : 573-579.

Eagle, H. 1955. Nutritional needs of mammalian cells in tissue culture. J. Biol. Chem. 214 : 839.

Glacken, M.W., R.J. Fleischaker, and A.J. Sinskey. 1988. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures for mammalian cells. Biotechnology and Bioengineering 28 : 1376-1389.

Lee, G.M., A. Varma, and B.O. Palsson. 1991. Production of monoclonal antibody using free-suspended and immobilized hybridoma cells: effect of serum. Biotechnology and Bioengineering 38 : 821-830.

McKeehan, W.L. 1982. Glutaminolysis in animal cells. Cell Biol. Int. Rep. 6 : 635.

Miller, W.M., C.R. Wilke, and H.W. Blanch. 1988. Transient responses of hybridoma metabolism to changes in oxygen supply rate in continuous culture. Bioprocess Eng. 3 : 103-111.

Ozturk, S.S. and B.O. Palsson. 1990. Chemical decomposition of glutamine in cell culture media type, pH, and serum concentration. Biotechnol. Prog. 6 : 121-128.

Reitzer, L.J., B.M. Wice, and D. Kennel. 1979. Evidence that glutamine, not sugar, is the major energy source for culture HeLa cells. J. Biol. Chem. 254 : 2669-2674.

Tritsch, G.L. and G.E. Moore. 1962. Spontaneous decomposition of glutamine in cell culture media. Exp. Cell Res. 28 : 360-364.

van der Pol, L., G. Zijlstra, M. Thalen, and J. Tramper. 1990. Effect of serum concentration on production of monoclonal antibodies and shear sensitivity of a hybridoma. Bioprocess Engineering 5 : 241-245.

Received date : 12/05/99
Accepted date : 8/11/99