Operating Conditions for Bioreactor with Animal Cells

Student ID#: 01M18106 Name: SATO, Kosuke Supervisor: KAJIUCHI, Toshio and EGASHIRA, Ryuichi

動物細胞を用いた生物化学反応器における操作条件

佐藤　孝祐

t-PA（組織プラスミノーゲン活性化因子）を生産する遺伝子が導入された Chinese Hamster Ovary（CHO）細胞および無血清培地を用いた生物化学反応器における攪拌および非イオン性高分子界面活性剤（本研究では Pluronic F68）添加の影響を調べた．緩やかな攪拌速度でも細胞の損傷は見られ，攪拌速度の増加とともに代謝活性が向上した．攪拌条件下ならびに非攪拌条件下においても Pluronic F68 を添加すると細胞増殖速度が増加する一方，代謝活性が低下した．

1, Introduction

Animal cells are extremely sensitive to fluid mechanical stresses because they lack a protective cell wall and larger than microbial cells; accordingly their shear sensitivity is widely perceived as a barrier to scale up and to carry out high oxygen supply by bubbling aeration and mechanical agitation. Many studies on animal cell damage due to mechanical agitation have shown that shear sensitivity depended on cell line and culture medium composition (Frangos *et al*., 1987, Stathopoulos *et al*., 1985, Kunas *et al*., 1990, Murhammer *et al*., 1990, Motobu *et al*., 1998). Each research used different cell line and medium composition; thus it is difficult to compare each research. For example, animal cells are possibly more fragile in serum-free medium, because addition of sera, which are added to culture medium as nutrients in many of animal cell culture, has protective effects on animal cells from shear stress (McQueen *et al*., 1989, Kunas *et al*., 1989, Handa-Corrigan *et al*., 1989, Michaels *et al*., 1991, Ramirez *et al*., 1990). Sera will be barriers to large-scale culture because of their high cost, complexity and risks of contamination. Nevertheless there are few studies about effects of agitation on cell growth, metabolism and protein productivity in serum-free medium. Therefore we studied effects of agitation on cell proliferation and metabolism in serum-free medium.

Other medium additives such as synthetic polymer surfactants (especially Pluronic F68) are used to protect cells from mechanical stresses (Kilburn *et al*., 1968, Handa-Corrigan *et al*., 1989, Goldblum *et al*. 1990, Michaels *et al*., 1991). However, few studies were done about their effects on product activity or metabolism, or their effects under static conditions. Therefore we studied effects of Pluronic F68 on cell proliferation, metabolism and t-PA (Tissue plasminogen activator) productivity on t-PA producing CHO cells under agitating and static conditions.

2, Materials and methods

The human t-PA producing cell line (CHO Tf70R) was obtained from Pharamacia Upjohn (Kind gift of T. Björing). Cells were cultured at 37 °C in a 5% CO_2 and 95% humidity atmosphere in CO_2 incubator (Heraeus, B5060 Ek-CO_2), and grown in suspension in a serum free medium (BioPro-1: BioWhittaker) supplemented with 1mM glutamine (GIBCOBRL); 20mM glucose (SIGMA); 100 U/ml penicillin-streptomycin (BioWhittaker); and 0.0-1.2 w/w% Pluronic F68 (SIGMA), either in 80ml T.C. Flasks (NUNC) or 250ml spinner flasks (Techne).

Effects of agitation and Pluronic F68 under agitating condition were studied by using spinner flask, and effects of Pluronic F68 under static condition by using T.C. flask. When spinner flasks were used, the cells grown in T.C. flask under static conditions were transferred to a spinner flask to be the cell density, 1.5×10^5 ml^{-1}. The spinner flasks were agitated by using a magnetic stirrer (TECHNE, Mcs 104s) at speed of 20-200rpm. Bubbling aeration was not carried out. All spinner flasks were sterilized by using autoclave in 45 minutes at 125℃ 2 atm. Samplings were withdrawn at 24 hours intervals for 9 days. All experiments were done more than twice with control experiments (when agitating conditions, 50 rpm without Pluronic F68; when static conditions, 0 rpm without Pluronic F68) to figure out the effects of slight differences of cell generation, initial medium compositions and so on.

The determination of cell density was carried out manually with a haemacytometer, and viability by trypan blue exclusion method. The concentration of glucose; lactate; glutamine; and total protein concentration was determined by using SIGMA Glucose Trinder Kit (SIGMA 315-100); SIGMA Kit (SIGMA 715-10); Roche Kit (Roche 0-139-092) with L-asparaginase (Roche 102-903); and Bio-Rad Protein assay (Bio-Rad 500-0001), respectively. The ammonia concentration was determined by using ammonia electrode (Mettler-Toledo). The enzymatic t-PA activities were used as an indicator of t-PA production, and measured by a colorimetric method using the synthetic peptide-p-nitroanalide (Chromozyme t-PA: Boehringer Mannheim).

3, Results and Discussion

3.1. DEFINITIONS OF EVALUATION PARAMETERS

We selected initial specific growth rate, $\mu_{r,0}$, as an indicator of cell proliferation. The $\mu_{r,0}$ [h^{-1}], was calculated from **Equation 1**, obtained by integrating equation, $dx/dt=\mu_r x$, supposing $\mu_{r,o}$ dose not change with time in the initial part of cell culture.

$$\ln \boldsymbol{x} = \boldsymbol{\mu}_{\boldsymbol{r},0} + \ln \boldsymbol{x}_0 \qquad (1)$$

However, these $\mu_{r,0}$ were possibly affected by some slight differences of initial experimental conditions; therefore specific growth rate ratio, α_μ [-], defined as **Equation 2,** was used.

$$\alpha_{\mu} = \frac{\mu_{r,0}}{\mu_{r,0,cont.}} \qquad (2)$$

The reaction rate (72 hour from beginning of cell culture) per unit cell, π_i [$\mu g \cdot h^{-1}$ (total protein), $mU \cdot h^{-1}$ (t-PA) $mmol \cdot h^{-1}$ (others)] was selected as an indicator of metabolism and t-PA production. The π_i was calculated from **Equation 3**.

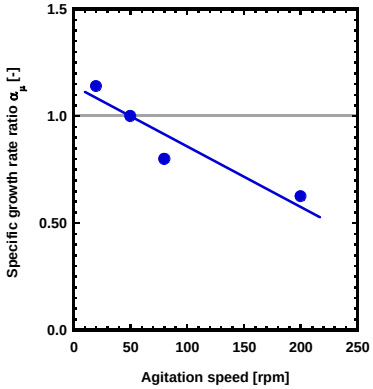
$$\pi_i = \frac{\left| \Delta C_i / \Delta t \right|}{\bar{x}_v} \qquad (3)$$

These π_i were possibly affected by some slight difference of initial experimental conditions; therefore we used reaction rate ratio, α_i [-], which is defined as **Equation 4**.

$$\alpha_i = \frac{\pi_i}{\pi_{i,cont}} \qquad (4)$$

3.2. EFFECTS OF AGITATION

Figure 3.2. shows effects of agitation on (a) cell proliferation; and (b) metabolism. The specific growth rate ratio, α_{μ}, was reduced with increment of agitation speed (Fig.3.2.(a)). This indicates that cell damage by agitation possibly occurred even relatively low agitation speed (below 200 rpm) in serum free media. The reaction rate ratio of total proteins, α_{tp} was drastically increased with agitation speed (Fig. 3.2(b)); this possibly means proteins inside cell were released by cell breakage due to mechanical agitation. The reaction rate ratios, α_{glc}, α_{lac}, α_{gln} and α_{amm}, were increased with increment of agitation speed at low agitation speed, though almost constant at higher agitation speed (Fig. 3.2.(b)); this means that the metabolic activity of cells was possibly stimulated by mechanical agitation. Frangos *et al.* (1987) investigated the relationship between shear stress and prostacycline production, and also found that increasing the flow rate stimulated prostacyclin production. Stathopoulos *et al.* (1985) also performed shear stress experiments with human embryonic kidney cells and found that shear stress caused cell morphology changes and productivity enhancement, but high shear stress reduced cell viability. We observed reduction of cell viability with agitation speed increasing (data not shown); however, we could not observed productivity enhancement: the reaction rate ratio of t-PA, $\alpha_{t\text{-}PA}$ was not changed with increment of agitation speed (Fig.3.2. (b)).



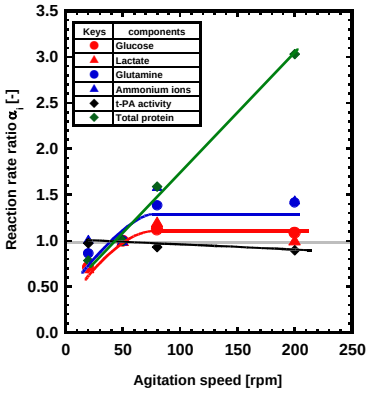


Fig. 3.2. Effects of agitation on (a) specific growth rate ratio, α_{μ} [-] (above); (b) reaction rate ratio, α_i [-] (below).

3.3. EFFECTS OF PLURONIC F68

Figure 3.3.1. shows the effects of Pluronic F68 addition under agitating conditions on (a) cell proliferation; (b) metabolism; **Figure 3.3.2.** shows the effects of Pluronic F68 addition under static conditions. The specific growth rate ratio, α_{μ}, was much promoted with adding Pluronic F68 and also reduced with increment of agitation speed (Fig.3.3.1. (a)). The reaction rate ratio of total proteins, α_{tp} was slightly increased with agitation speed but smaller than experiments without Pluronic F68 (Fig. 3.3.1. (b)); this possibly means cells were protected from cell breakage due to mechanical agitation by adding Pluronic F68, accordingly protein release from inside cell was reduced. The α_{μ} was also promoted with adding Pluronic F68 under static condition (Fig.3.3.2.). The α_{glc}; α_{lac}; α_{gln}; and $\alpha_{t\text{-}PA}$ were reduced as compared to no Pluronic F68 addition under both agitating condition (Fig.3.3.1.(b)) and static condition (Fig.3.3.2.); this was similar to experiments at low agitation speed without Pluronic F68 which were considered to be under low shear stress. Therefore, supposedly adding Pluronic F68 to cell culture medium could reduce the effects of fluid mechanical stresses not only agitating condition but also static condition.

It is generally known that cells under unfavorable culture conditions, such as low temperature (Jenkins and Hovey, 1993, Hendrick *et al*., 2001), high shear stress (Frangos *et al*. 1987, Stathopoulos *et al*. 1985), adding Na-butyrate to culture medium (Dorner *at al*., 1987, Hendrick *et al*. 2001) *etc*. have higher metabolic activity than those under favorable culture conditions. We also observed low metabolic activity and low productivity in cell culture with Pluronic F68, which was considered to be a more favorable culture condition supposing that adding Pluronic F68 to culture medium reduced the effect of fluid mechanical stresses. The reason why cells have high metabolic activity and high specific productivity under unfavorable culture conditions has not perfectly clarified yet. However we can supposedly think some kinds of cell selection occurred under unfavorable culture conditions. Lloyd *et al*. (2000) and Takagi *et al*. (2000) showed that a cell size distribution exists even in the same cell line in the same flask and specific productivity and metabolic activity clearly

increased with the size of cells. Accordingly the slight differences of sensitivity to low temperature, fluid mechanical stresses, Na-butyrate *etc.* possibly exist in each cell even in the same cell line. The relationship among sensitivity to negative influences, specific productivity and metabolic activity can probably explain the results.

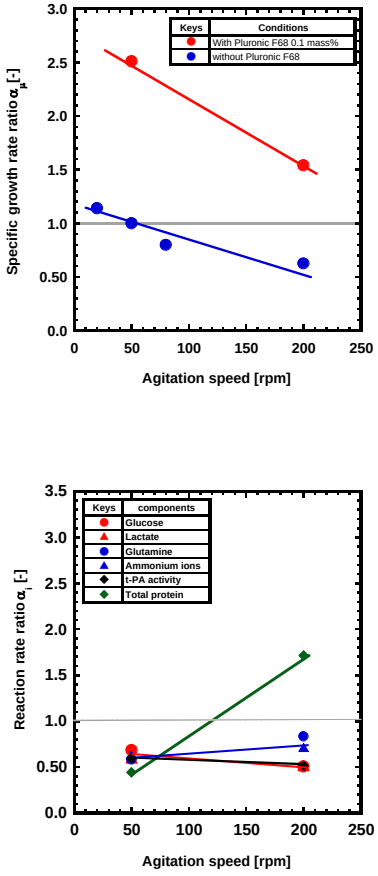


Fig.3.3.1. Effects of Pluronic F68 addition under agitating conditions on (a) specific growth rate ratio, α_μ [-] (above); (b) reaction rate ratio, α_i [-] (below).

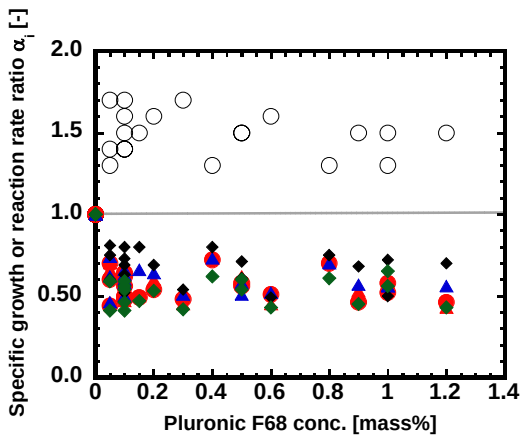


Fig.3.3.2. Effects of Pluronic F68 addition under static conditions :(○: α_μ [-]; the others are the same as Fig 3.3.1.(b).)

4, Conclusion

We showed effects of agitation and effects of adding Pluronic F68 to culture medium on CHO cells in serum free medium. Adding Pluronic F68 promoted cell proliferation under both agitating and static conditions; this implied it reduced effects of fluid mechanical stresses and fluid mechanical stresses possibly worked on cells even in static culture conditions. These phenomena were possibly related to the relationship among sensitivity to fluid mechanical stresses, specific productivity and metabolic activity. It will be an interesting topic for further research.

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Nomenclature

C	[mM or $\mu g \cdot l^{-1}$(total proteins)]:	concentration
t	[hour]:	time
x	[ml^{-1}]:	cell density
α_μ	[-]:	specific growth rate ratio
α_i	[-]:	reaction rate ratio
π_i	[$mM \cdot h^{-1}$ $mU \cdot h^{-1}$ $\mu g \cdot h^{-1}$]:	reaction rate per unit cell
μ_r	[h-1]:	specific growth rate

<Subscripts>

0	initial
amm	ammonia and ammonium ions
cont.	control experiments
glc	glcose
gln	glutamine
lac	lactate
tp	total protein
t-PA	tissue plasminogen activator (t-PA)
v	viable

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