Suspension Culture Process of MethA Tumor Cell for the Production of Heat-Shock Protein Glycoprotein 96: Process Optimization in Spinner Flasks

Ya-Jie Tang,*†‡ Hong-Mei Li,†‡ and Jean-François P. Hamel‡

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, and Hubei Provincial Key Laboratory of Industrial Microbiology, College of Bioengineering, Hubei University of Technology, Wuhan 430068, China

Heat-shock proteins (HSPs) act like “chaperones”, making sure that the cell’s proteins are in the right shape and in the right place at the right time. Heat-shock protein glycoprotein 96 (gp96) is a member of the HSP90 protein family, which chaperones a number of molecules in protein folding and transportation. Heat-shock protein gp96 serves as a natural adjuvant for chaperoning antigenic peptides into the immune surveillance pathways. Currently, heat-shock protein gp96 was only isolated from murine and human tissues and cell lines. An animal cell suspension culture process for the production of heat-shock protein gp96 by MethA tumor cell was developed for the first time in spinner flasks. Effects of culture medium and condition were studied to enhance the MethA tumor cell density and the production and productivity of heat-shock protein gp96. Initial glucose concentration had a significant effect on the heat-shock protein gp96 accumulation, and an initial glucose level of 7.0 g/L was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity. Cultures at an initial glutamine concentration of 3 and 6 mM were nutritionally limited by glutamine. At an initial glutamine concentration of 6 mM, the maximal viable cell density of 19.90 × 10^5 cells/mL and the maximal heat-shock protein gp96 production of 4.95 mg/L was obtained. The initial concentration of RPMI 1640 and serum greatly affected the MethA tumor cell culture process. Specifically cultures with lower initial concentration of RPMI 1640 resulted in lower viable cell density and lower heat-shock protein gp96 production. At an initial serum concentration of 8%, the maximal viable cell density of 19.18 × 10^5 cells/mL and the maximal heat-shock protein gp96 production of 5.67 mg/L was obtained. The spin rate significantly affected the cell culture process in spinner flasks, and a spin rate of 150 rpm was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity. Not only the cell density but also the production and productivity of heat-shock protein gp96 attained in this work are the highest reported in the culture of MethA tumor cell. This work offers an effective approach for producing heat-shock protein glycoprotein 96 from the cell culture process. The fundamental information obtained in this study may be useful for the efficient production of heat-shock protein by animal cell suspension culture on a large scale.

Introduction

Heat-shock proteins (HSPs), which are expressed constitutively in all cells, are essential for several important cellular processes, such as protein folding, protection of proteins from denaturation or aggregations, and facilitation of protein transport through membrane channels (Hartl, 1996; Srivastava et al., 1998). A wide variety of “stressful” stimuli, such as heat-shock, ultraviolet radiation, and viral or bacterial infections, induce an increase in the intracellular synthesis of HSPs (Anderson and Srivastava, 2000; Wallin et al., 2002; Hoos and Levey, 2003). HSPs play an important role in antimicrobial, as well as autoimmune, responses and have potent effects in inducing antigen-specific immunity to bound material upon immunization (Srivastava, 1993; Srivastava and Udono, 1994; Janetzki and Srivastava, 1995; Przepiorka and Srivastava, 1998; Basu and Srivastava, 2000; Srivastava, 2002a, 2002b; Hoos and Levey, 2003).

Glycoprotein 96 (gp96) (glucose-regulated protein 94, grp94), the endoplasmic reticulum heat-shock protein 90 paralog, elicits both innate and adaptive immune responses (Srivastava, 1993, 2002a, 2002b; Nicchitta, 1998; Yang et al., 2007). Regarding the former, heat-shock protein gp96 stimulates antigen-presenting cell cytokine expression and dendritic cell maturation (Singh-Jasuja et al., 2000). The adaptive component of heat-shock protein gp96 function reflects a proposed peptide-binding activity, and consequently a role for native heat-shock protein gp96-peptide complexes in cross-presentation (Srivastava, 2002a, 2002b). It is by this mechanism that tumor-derived gp96 is thought to suppress tumor growth and metastasis. Recent data have demonstrated that gp96-elicited innate immune responses can be sufficient to suppress tumor growth and metastasis (Baker-LePain et al., 2004; Parmiani et al., 2006; Pilla et al., 2006; Oki et al., 2007).

Heat-shock protein gp96 has already been isolated and purified from a variety of tissues and cell lines (Udono and...
The preculture and culture processes except when mentioned otherwise were conducted in spinner flasks on magnetic stirrers (100 rpm) at 37 °C in a 5% CO₂ atmosphere and 95% relative humidity in CO₂ incubators.

**Initial Glucose Concentration Experiments.** For the assessment of the effect of initial glucose concentration in the culture medium on the MethA tumor cell growth, metabolism, and heat-shock protein gp96 biosynthesis, four different initial glucose concentrations were used: 4.5, 7.0, 9.5, and 12.0 g/L. The other culture medium and conditions were the same as in the above experiments.

**Initial Glutamine Concentration Experiments.** The significance of initial glutamine concentration was studied by setting the initial glutamine concentration at 3, 6, 9, and 12 mM in the culture medium. The other culture medium and conditions were the same as in the above experiments.

**Initial RPMI 1640 Concentration Experiments.** The impact of initial RPMI 1640 concentrations (e.g., 10.39 (recommended by the manufacture), 7.5, 5.0, and 2.5 g/L) were evaluated to enhance the MethA tumor cell growth and heat-shock protein gp96 accumulation during the culture process in a spinner flask. The other culture medium and conditions were the same as in the above experiments.

**Initial Serum Concentration Experiments.** The significance of initial serum concentration on the MethA tumor cell growth and heat-shock protein gp96 accumulation was studied by setting the initial serum concentration at 10%, 8%, 6%, 4%, and 0% in the culture medium. The other culture medium and conditions were the same as in the above experiments.

**Spin Rate Experiments.** To provide some insights into the potential influences of shear stress, oxygen supply, and fluid mixing on the MethA tumor cell growth and heat-shock protein gp96 production and productivity, the culture medium was optimized in spinner flasks. In addition, the significance of spin rate in a spinner flask was also investigated in detail so as to provide some insights into the potential influences of shear stress, oxygen supply, and fluid mixing on the MethA tumor cell growth and heat-shock protein gp96 biosynthesis in a bioreactor.

**Materials and Methods**

**Cell Line, Medium, and Culture Maintenance.** The MethA tumor cell line producing heat-shock protein gp96 was used in all experiments. It was maintained in the frozen state in liquid nitrogen. The frozen cells were thawed in a 37 °C water bath, then inoculated and maintained in spinner flasks with preculture medium. The preculture cells in spinner flasks were serially passaged every 48 h at the inoculum size of 2 × 10⁶ cells/mL.

The preculture MethA tumor cells in the exponential growth phase (i.e., cell viability above 95%) were centrifuged at 1000 rpm (200 g) for 10 min. The cell pellet in the precipitation was then washed with prewarmed fresh medium and distributed into the spinner flask to initiate the MethA tumor cell culture process with an initial cell concentration of 2 × 10⁶ cells/mL in order to optimize the culture medium and condition.

The preculture and culture medium except when mentioned otherwise were composed of 10% Fetal Clone I (HyClone, Logan, UT) and 10.39 g/L RPMI 1640 (Invitrogen Corporation, Carlsbad, CA) supplemented with 0.87 g/L L-glutamine (Sigma-Aldrich Corp., MO), 2.5 g/L glucose (Sigma-Aldrich Corp., MO), 1 mM sodium pyruvate (Mediatech, Inc. Herndon, VA), 2 g/L sodium bicarbonate (Sigma-Aldrich Corp., MO), and 100 IU/mL penicillin-streptomycin (Sigma-Aldrich Corp., MO).

The amount of heat-shock protein gp96 in the above supernatant was determined by the immunonodetection method. This immunonodetection method was performed according to the description in QIAexpress Detection and Handbook (Qiagen). Heat-shock protein gp96 was separated by using SDS-PAGE (7.5% Tris-HCl gel) and transferred electrophotically onto a PVDF membrane in a tank-blotting system (Bio-Rad). The primary antibody was rat monoclonal IgG anti gp94 (NeoMarkers, Carlsbad, CA) and the secondary antibody was alkaline phosphatase-conjugated goat anti-rat IgG (Sigma-Aldrich Corp., MO).
in Figure 1B, the time profiles of cell viability under different initial glucose concentrations during the MethA tumor cell culture process in a spinner flask. Symbols for initial glucose concentration (g/L): 4.5 (○), 7.0 (●), 9.5 (△), and 12.0 (▲). sharply. This suggested that, for the MethA tumor cell growth in spinner flasks, the lag phase was from the inoculation to the culture of 19 h, the exponential growth phase was from the culture of 19 h to 49 h, and the stationary phase lasted from the culture of 49 h to 73 h, after which it belonged to the cell death phase.

The kinetics of glutamine consumption under different initial glucose levels are compared in Figure 2. There was almost no significant effect of initial glucose concentration on the glutamine consumption within the range as investigated. During the lag phase, the glutamine concentration decreased fast (Figure 2A), which corresponded well to the quick increase of viable cell density (Figure 1A). During the exponential growth phase, the glutamine consumption rate was higher than that at the lag phase, which corresponded to the viable cell density increase rate at the exponential growth phase being higher than that at the lag phase. At the stationary phase, the viable cell density remained almost constant, and the glutamine was consumed slowly just for the maintenance of cell activity itself. During the cell death phase, the MethA tumor cell began to die, and stopped consuming the glutamine. So, the concentration of glutamine remained almost constant during the cell death phase. This demonstrated that the glutamine consumption corresponded well to the MethA tumor cell growth in the cell culture process. The yield of viable cell against glutamine was $1.70 \times 10^{11}$.
2.21 \times 10^{11}, 2.04 \times 10^{11}, and 1.87 \times 10^{11} viable cells/mol glutamine at an initial glucose level of 4.5, 7.0, 9.5, and 12.0 g/L, respectively. Not only the time profile of glutamate accumulation (Figure 2B), but also the kinetics of ammonia production (Figure 2C) corresponded well to the consumption of glutamine in the MethA tumor cell culture process of spinner flasks. The final concentrations of glutamate and ammonia seem to be independent of initial glucose concentration, indicating that the overall yields of glutamate and ammonia on glutamine are constant, while Hu et al. reported that reduced glucose concentration resulted in an increased glutamine consumption (Hu et al., 1987), which could increase byproduct formation from glutaminolysis, which is inconsistent with our case.

Figure 2. Kinetics of glutamine consumption (A), glutamate production (B), and ammonia accumulation (C) under various initial glucose concentrations. The symbols for initial glucose concentration are the same as those in Figure 1.

Figure 3. Time courses of glucose consumption (A), lactate accumulation (B), and pH variation (C) under various initial glucose concentrations. The symbols for initial glucose concentration are the same as those in Figure 1.

The kinetics of glucose consumption under various initial glucose concentrations is indicated in Figure 3A. The residual glucose concentration in the culture broth at higher initial glucose level was always higher than that at lower initial glucose level during the whole culture process, and the residual glucose concentration even at the lowest initial concentration (e.g., 4.5 g/L) still remained at a high level (e.g., 2.33 g/L) at the end of the culture process. The viable cell yield against glucose was 6.74 \times 10^8, 9.08 \times 10^8, 8.47 \times 10^8, and 8.58 \times 10^8 viable cells/g glucose at an initial glucose level of 4.5, 7.0, 9.5, and
12.0 g/L, and the corresponding total consumption of glucose during the whole culture process was 2.67, 2.44, 2.36, and 2.70 g/L, respectively. This indicated that the total consumption of glucose during the whole culture process remained almost constant (e.g., 2.36–2.70 g/L), despite the initial glucose concentration, and the pattern of lactate production under various initial glucose concentrations was quite similar (Figure 3B). The final lactate concentrations seem to be independent of the initial concentration of glucose, indicating that the overall lactate yield on glucose is constant, while Zielke et al. reported that increased glucose concentration led to a higher accumulation of lactate (Zielke et al., 1978), which is in contrast to our results. The time profile of pH variation under different initial glucose concentrations is depicted in Figure 3C. Before the culture of 73 h, the pH variation was similar in all four cases. From the inoculation to the culture of 19 h, pH increased from 7.10 to about 7.25, then decreased sharply until the culture of 49 h. The pH value remained almost constant (e.g., 6.93–6.98) during the culture of 49 to 73 h. After the culture of 73 h, the pH value began to decrease gradually and the pH value at lower initial glucose concentration was higher than those at higher levels.

The heat-shock protein gp96 production under various initial glucose levels is shown in Figure 4. The heat-shock protein gp96 accumulated slowly at the lag and exponential growth phase, while most of heat-shock protein gp96 began biosynthesis when the MethA tumor cell entered into the cell death phase. Compared with the kinetics of cell growth (Figure 1A), it was concluded that the majority of heat-shock protein gp96 was biosynthesized at the cell death phase. The highest heat-shock protein gp96 production titer at the initial glucose concentration of 4.5, 7.0, 9.5, and 12.0 g/L was 3.28, 4.84, 3.82, and 3.50 mg/L as obtained on the culture of 97, 115, 115, and 97 h, respectively, while the corresponding volumetric heat-shock protein gp96 productivity was 0.73, 0.94, 0.73, and 0.79 mg/L (L-day) and the corresponding specific heat-shock protein gp96 productivity was 1.54, 4.03, 2.80, and 1.90 pg/(cell-day), respectively. Not only the heat-shock protein gp96 production, but also the productivity and specific productivity of heat-shock protein gp96 obtained maximum values at the initial glucose concentration of 7.0 g/L.

The above results indicated there is no significant effect of initial glucose concentration on the MethA tumor cell growth, while initial glucose concentration affected the heat-shock protein gp96 accumulation obviously, and an initial glucose concentration of 7.0 g/L was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

**Effect of Initial Glutamine Concentration.** Animal cells are very sensitive to the toxic compounds produced by the cell itself during normal growth. Many research papers reported that lactate and ammonia are the two major waste products of cell culture, and they have inhibitory effects on cell growth and protein production rate (Adema, 1989; Chang et al., 1995; Glacken et al., 1986; Hassell et al., 1991; Omasa et al., 1992). The lactate accumulation follows the consumption of glucose, and the ammonia production follows the consumption of glutamine. As shown in Figure 1A, the MethA tumor cell stopped growing after the culture of 73 h (e.g., the stationary phase). One reason for this would be the inhibitory effect of the high concentration of toxic byproducts lactate (Figure 3B) and ammonia (Figure 2C) on the MethA tumor cell growth. Another explanation would be no more glutamine in the culture broth (Figure 2A).

On the basis of the above information as obtained, the effects of initial glutamine concentration on the kinetics of the MethA tumor cell growth and metabolites’ production were investigated in spinner flasks by setting the initial glutamine concentration at 3, 6, 9, and 12 mM in the culture medium. The kinetics of the MethA tumor cell growth under various initial glutamines is compared in Figure 5A. At the lowest initial glutamine
concentration of 3 mM, the viable cell density reached its peak of 17.53 \times 10^5 cells/mL after 56 h of cultivation and then decreased sharply after 73 h of culture, because glutamine was completely consumed at that time (Figure 6A); and the cell viability remained at a relatively higher level (e.g., above 95%) before the culture of 56 h (Figure 5B), after which it decreased sharply with the complete consumption of glutamine in the culture medium (Figure 6A). A similar experimental phenomenon was observed at an initial glutamine concentration of 6 mM. The pattern of MethA tumor cell growth was similar at relatively higher initial glutamine concentrations (e.g., 9 and 12 mM). The viable cell density and cell viability at an initial glutamine concentration of 12 mM were a little bit higher than those in the case of 9 mM after the cultivation of 68 h. The maximum viable cell density at an initial glutamine concentration of 3, 6, 9, and 12 mM was 17.53 \times 10^5, 19.90 \times 10^5, 19.13 \times 10^5, and 17.41 \times 10^5 cells/mL on the culture of 56, 56, 49, and 58 h, and the corresponding average growth rate was 0.68, 0.70, 0.79, and 0.66 d^{-1}, respectively.

The kinetics of glutamine consumption under different initial glutamine concentrations is compared in Figure 6A. The medium glutamine was exhausted on the cultivation of 50 and 95 h at an initial glutamine concentration of 3 and 6 mM, while there was 0.43 and 1.37 mM of residual glutamine at the end of culture (hour 169) under initial glutamine level of 9 and 12 mM, respectively. The yield of viable cell against glutamine was 5.18 \times 10^{11}, 4.09 \times 10^{11}, 4.31 \times 10^{11}, and 2.26 \times 10^{11} viable cells/mol glutamine at an initial glutamine level of 3, 6, 9, and 12 mM, respectively. Not only the time profile of glutamate accumulation (Figure 6B), but also the kinetics of ammonia production (Figure 6C) corresponded well to the consumption of glutamine (Figure 6A) in the MethA tumor cell culture process under various initial glutamine concentrations. Before the culture of 73 h, the concentrations of glutamate (Figure 6B) and ammonia (Figure 6C) increased quickly with the fast consumption of glutamine (Figure 6A). After that, the medium glutamine was consumed slowly, and spontaneously the glutamate and ammonia accumulated slowly. Cells at higher initial glutamine concentration experienced the higher concentrations of glutamate and ammonia during the whole culture process. Sanfeliu et al. also observed that the increased glutamine concentrations increase the production of ammonium byproducts (Sanfeliu et al., 1996). At the relatively higher initial glutamine concentrations (e.g., 9 and 12 mM), the toxic product ammonia concentration in the culture broth was higher than 5 mM (Figure 6C) after the culture of 58 h, which maybe the reason why the MethA tumor cell stopped growing.

The kinetics of glucose consumption under various initial glutamine concentrations is indicated in Figure 7A. The viable cell yield against glucose was 11.77 \times 10^8, 19.46 \times 10^8, 11.65 \times 10^8, and 9.63 \times 10^8 viable cells/g glucose at an initial glutamine level of 3, 6, 9, and 12 mM, respectively. Compared with the initial glutamine concentrations of 6 mM, more glucose was consumed in the case of 3 mM, and more lactate was accumulated (Figure 7B), and a lower pH value was observed after the cultivation of 73 h (Figure 7C). The reason for this would be that glutamine was consumed faster and was exhausted after the cultivation of 50 h in the case of 3 mM, so the cells need to consume more glucose to maintain activity. Hu et al. also reported that glutamine affects the consumption rate of glucose and higher glutamine concentrations reduce the glucose consumption rate (Hu et al., 1987). Under the initial glutamine concentrations of 9 and 12 mM, not only the pattern of glucose consumption, but also the pattern of lactate accumulation and the pH variation were similar. The pattern of glucose consumption was different from that of glutamine—only a portion of glucose was consumed and the extent of consumption was dependent on both cell growth and initial glutamine concentration.

The heat-shock protein gp96 production under various initial glutamine levels is shown in Figure 8. The highest heat-shock protein gp96 production titer at an initial glutamine concentration of 3, 6, 9, and 12 mM was 4.57, 4.45, 4.05, and 3.54 mg/L as obtained on the culture of 56, 73, 120, and 75 h, respectively, while the corresponding volumetric gp96 productivity was 1.81, 1.52, 0.74, and 1.03 mg/(L-day) and the corresponding specific
gp96 productivity was 1.86, 1.58, 2.59, and 1.29 pg/(cell·day), respectively.

At an initial glutamine concentration of 3 and 6 mM, it was concluded that the cultures were nutritionally limited by glutamine, and all the glutamine was consumed by the cultures and the cessation of growth coincided with the time of glutamine depletion. The final ammonia concentration seemed to be dependent on initial glutamine concentration, and accumulation or buildup of ammonia inhibited cell growth and heat-shock protein gp96 production and productivity. All in all, an initial glutamine concentration of 6 mM was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

Effect of Initial RPMI 1640 Concentration. Typically for a new process, using one of some complex media would require less development time for achieving adequate cell-culture performance. So, effects of initial RPMI 1640 concentration on the kinetics of the MethA tumor cell growth and metabolites’ production were studied in spinner flasks by setting the initial RPMI 1640 concentrations at 10.39 (recommended value by the manufacture), 7.5, 5.0, and 2.5 g/L in the culture medium.

Time profiles of viable cell density (A) and cell viability (B) under various initial RPMI 1640 concentrations are depicted in Figure 9, parts A and B, respectively. No net growth of MethA tumor cell occurred at the lowest initial RPMI 1640 concentration of 2.5 g/L, while the cell still maintained some viability (Figure 9B). This suggests that some concentration of certain essential component(s) from RPMI 1640 was necessary for the MethA tumor cell growth and could not be synthesized by the cultured cells of MethA tumor. From inoculation to the culture of 49 h, the increasing rate of viable cell density was enhanced with the increase of initial RPMI 1640 concentration (Figure 9A), while during the cell death stage (e.g., from hour 49 to the end of cultivation), the decreasing rate of cell viability was increased with the increase of initial RPMI 1640 concentration (Figure 9B). The reason for this was due to the corresponding higher concentration of ammonia (Figure 10C) and lactate (Figure 11B). MethA tumor cell growth at an initial RPMI concentration of 5.0 g/L appeared to be slow as the culture period continued for up to 98 h, where it was 56 or 49 h for the case of 7.5 and 10.39 g/L. Within the concentration of investigation, more biomass was obtained when higher initial RPMI 1640 concentration was used in the MethA tumor cell culture process. The maximum viable cell density at an initial RPMI 1640 concentration of 10.39, 7.5, and 5.0 g/L was 19.13 × 10^5, 12.17 × 10^5, and 8.86 × 10^5 cells/mL, and the corresponding average growth rate was 0.79, 0.62, and 0.31 d^{-1}, respectively. The results demonstrated that initial RPMI 1640 concentration had a significant effect on the cell growth in the culture process and an initial RPMI 1640 concentration of 10.39 g/L seemed to be best for MethA tumor cell growth.

The kinetics of glutamine consumption under different initial RPMI 1640 concentrations is compared in Figure 10A. At the lowest initial RPMI 1640 concentration of 2.5 g/L, the glutamine consumption was 1.86, 1.58, 2.59, and 1.29 pg/(cell·day), respectively. At an initial glutamine concentration of 3 and 6 mM, it was concluded that the cultures were nutritionally limited by glutamine, and all the glutamine was consumed by the cultures and the cessation of growth coincided with the time of glutamine depletion. The final ammonia concentration seemed to be dependent on initial glutamine concentration, and accumulation or buildup of ammonia inhibited cell growth and heat-shock protein gp96 production and productivity. All in all, an initial glutamine concentration of 6 mM was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

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was consumed slowly by MethA tumor cells because the growth of MethA tumor cells was quite poor. The medium glutamine was exhausted at the end of culture at the initial glutamine concentration of 10.39, 7.5, and 5.0 g/L, while there was 3.15 mM of residual glutamine at the end of culture under the initial RPMI 1640 level of 2.5 mM. The yield of viable cell against glutamine was $4.19 \times 10^{11}$, $2.93 \times 10^{11}$, and $1.73 \times 10^{11}$ viable cells/mol of glutamine at an initial RPMI 1640 level of 10.39, 7.5, and 5.0 g/L, respectively. This indicated that the yield of viable cell against glutamine increased with the increase of initial RPMI 1640 concentrations within the range investigated. Compared with the other three cases (e.g., 10.39, 7.5, and 5.0 g/L), the glutamate and ammonia in the culture broth were accumulated slowly in the case of 2.5 g/L (Figure 10B-C). The glutamate concentration in the culture broth was decreased with the increase of initial RPMI 1640 concentration except for the case of 2.5 g/L (Figure 10B). As shown in Figure 10C, the final ammonia concentrations seem to be dependent on both viable cell density and initial concentration of RPMI 1640. More ammonia was accumulated at higher initial RPMI 1640 concentration and more dense cultures exhibit a more rapid rise in the concentrations of waste product ammonia.

The kinetics of glucose consumption under various initial RPMI 1640 concentrations is indicated in Figure 11A. When higher initial RPMI 1640 concentration was used in the culture medium, higher initial glucose concentration was observed in the culture broth, and lower residual glucose was observed after the cultivation of 56 h. This indicated that more glucose was consumed under the higher initial RPMI 1640 concentration in the culture of MethA tumor cell. There was almost no glucose consumption at an initial RPMI 1640 concentration of 2.5 g/L, which is due to the poor growth of MethA tumor cell. The viable cell yield against glucose was $12.78 \times 10^8$, $12.25 \times 10^8$, $10.72 \times 10^8$, and $0.00 \times 10^8$ viable cells/g of glucose at an initial RPMI 1640 level of 10.39, 7.5, 5.0, and 2.5 g/L, respectively. As shown in Figure 11B, the lactate concentration under the higher initial RPMI 1640 concentration was always higher than that at the lower initial RPMI 1640 concentration. The lactate concentration in the culture broth was decreased with the increase of initial RPMI 1640 concentration except for the case of 2.5 g/L (Figure 11C). As shown in Figure 11C, the final lactate concentrations seem to be dependent on both viable cell density and initial concentration of RPMI 1640. More lactate was accumulated at higher initial RPMI 1640 concentration and more dense cultures exhibit a more rapid rise in the concentrations of waste product lactate.

**Figure 9.** Dynamic profiles of viable cell density (A) and cell viability (B) under various initial RPMI 1640 concentrations during the MethA tumor cell culture process in a spinner flask. Symbols for initial RPMI 1640 concentration (g/L): 10.39 (○), 7.5 (●), 5.0 (▲), and 2.5 (▲).

**Figure 10.** Kinetics of glutamine consumption (A), glutamate production (B), and ammonia accumulation (C) under various initial RPMI 1640 concentrations. The symbols for initial RPMI 1640 concentration are the same as those in Figure 9.
accumulation corresponded well to the glucose consumption. The final lactate concentrations seem to be dependent on both RPMI 1640 level and cell growth. The more dense cultures exhibit a much more rapid rise in the concentrations of lactate, and low lactate in the culture broth led to low pH value (Figure 11C).

The time profile of heat-shock protein gp96 production under various initial RPMI 1640 concentrations is shown in Figure 12. The production of heat-shock protein increased quickly with the increase of initial RPMI 1640 concentration from 2.5 g/L to 7.5 g/L, while there was just a small increase when the initial RPMI 1640 concentration continued to increase up to 10.39 g/L. The highest heat-shock protein gp96 production titer at initial RPMI 1640 concentrations of 10.39, 7.5, 5.0, and 2.5 g/L was 4.05, 3.93, 3.51, and 0.65 mg/L as obtained on the culture of 120, 169, 169, and 192 h, respectively, while the corresponding volumetric gp96 productivity was 0.74, 0.51, 0.45, and 0.04 mg/(L·day) and the corresponding specific gp96 productivity was 2.59, 267, 1.64, and 0.31 pg/(cell·day), respectively. This indicated that the production of heat-shock protein gp96 was significantly affected by the initial RPMI 1640 concentration. Not only the heat-shock gp96 production, but also the volumetric gp96 productivity and specific gp96 productivity increased with the increase of initial RPMI 1640 concentration within the range investigated.

From the above results, it was concluded that RPMI 1640 was essential for both MethA tumor cell growth and heat-shock protein gp96 production, and not only the MethA tumor cell growth, but also the accumulation of heat-shock protein gp96 was significantly affected by the initial RPMI 1640 concentration in the culture medium. The above data indicated that an initial RPMI 1640 concentration of 10.39 g/L in the culture medium was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

**Effect of Initial Serum Concentration.** Serum provides sterols, fatty acids, growth factors, protein stability, protein transporters, trace metals, vitamins, and shear protection to animal cell cultures (Rose et al., 2003). Removing serum without addressing these functions could result in poor cell growth and performance. There are no data on the influence of initial serum concentration on the MethA tumor cell growth and heat-shock protein gp96 biosynthesis. The effects of initial serum concentration on the kinetics of MethA tumor cell growth and metabolites’ production were investigated in a spinner flask by setting the initial serum concentrations at 10%, 8%, 6%, 4%, and 0% in the culture medium.

Time profiles of viable cell density and cell viability under various initial serum concentrations are depicted in Figure 13, parts A and B, respectively. MethA tumor cell could not grow without serum in the culture medium. Not only the viable cell density, but also the cell viability obtained in the serum-free culture medium were much lower than those obtained in the culture medium containing serum. Except for the serum-free culture medium, the growth pattern of MethA tumor cell
including the viable cell density and cell viability was quite similar despite the initial serum concentration in the culture medium. The maximum viable cell density at an initial serum concentration of 10%, 8%, 6%, 4%, and 0% was $19.13 \times 10^5$, $19.18 \times 10^5$, $17.63 \times 10^5$, $18.14 \times 10^5$, and $2.71 \times 10^5$ cells/mL, and the corresponding average growth rate was 0.79, 0.81, 0.69, 0.70, and 0.30 d$^{-1}$, respectively. The results demonstrated that serum was essential for MethA tumor cell growth, while the initial concentration of serum has no significant effect on the MethA tumor cell growth.

The kinetics of glutamine consumption under different initial serum concentrations is compared in Figure 14A. In the serum-free culture medium (e.g., the initial serum concentration of 0%), the glutamine was consumed slowly because of the poor growth of MethA tumor cell. The medium glutamine was almost exhausted at the end of culture at the initial serum concentration of 10%, 8%, 6%, and 4%, while there was 3.59 mM of residual glutamine under an initial serum level of 0%. The yield of viable cell against glutamine was $4.31 \times 10^{11}$, $3.90 \times 10^{11}$, $2.95 \times 10^{11}$, $2.78 \times 10^{11}$, and $0.24 \times 10^{11}$ viable cells/mol of glutamine at an initial serum level of 10%, 8%, 6%, 4%, and 0%, respectively. This indicated that the yield of viable cell against glutamine increased with the increase of initial serum concentrations within the range investigated. As shown in Figure 14B, the glutamate concentration in the case of 10% was much lower than that of the other four cases, and the pattern of glutamate accumulation was similar despite the initial serum concentration in the culture medium for the other four cases. As shown in Figure 14C, the ammonia in the culture broth was accumulated slowly in the serum-free culture medium, and the pattern of ammonia accumulation was quite similar for the other four cases.

The kinetics of glucose consumption under various initial serum concentrations is indicated in Figure 15A. The glucose was consumed slowly by MethA tumor cell in the serum-free medium because of the poor growth of MethA tumor cell, which was similar to the glutamine consumption. The residual glucose concentration in the serum-free culture medium was much higher than that of the other four cases. Except for the serum-free culture medium, the consumption pattern of glucose was
quite similar despite the initial serum concentration in the culture medium. This corresponded well to the MethA tumor cell growth pattern (Figure 13A). The viable cell yield against glucose was $12.78 \times 10^8$, $15.61 \times 10^8$, $14.60 \times 10^8$, $15.97 \times 10^8$, and $4.67 \times 10^8$ viable cells/g of glucose at an initial serum level of 10%, 8%, 6%, 4%, and 0%, respectively. The lactate concentration in the serum-free culture medium was always lower than that of the other four cases (Figure 13B), and the corresponding pH value was always higher than that of the other four cases (Figure 13C).

The above results indicated that the serum in the culture medium was essential for MethA tumor cell growth, and both the MethA tumor cell growth and heat-shock protein gp96 production were significantly affected by the initial serum concentration in the culture medium, and an initial serum concentration of 8% was optimal for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

**Effect of Spin Rate.** It has been noted that animal cells are sensitive to the shear force exerted on cells by fluid flow. Excessive shear force can lead to cell death. To avoid this detrimental effect of shear force, a low agitation rate is usually used in the cell culture process. The cell culture process with high cell density may lead to difficult problems, such as poor fluid mixing and oxygen transfer. By changing the spin rate during the MethA tumor cell culture process in spinner flasks, we may observe preliminary interaction between the fluid mixing, oxygen transfer, cell growth, and metabolite biosynthesis. Such experimental results may offer some useful information for further large-scale cultures.

The effect of spin rate in spinner flasks on the viable cell density is indicated in Figure 17A. The cell growth at a spin rate of 50 rpm appeared to be slow as the culture period continued for up to 86 h, where it was 56 h for the other three cultures. During the MethA tumor cell growth phase, viable cell density was increased with the increase of spin rate from 50 to 150 rpm, while there was a decrease when spin rate increased from 150 to 200 rpm. Clearly, the hydrodynamic environment corresponding to an agitation rate of 200 rpm...
caused more rapid lysis of cells than did milder agitation at 150 rpm. Similar behavior was observed by Zhang et al. in the cultivation of porcine erythrocytes (Zhang et al., 1995). The maximum viable cell density at a spin rate of 50, 100, 150, and 200 rpm was $13.85 \times 10^5$, $14.84 \times 10^5$, $19.56 \times 10^5$, and $17.27 \times 10^5$ cells/mL, and the corresponding average growth rate was 0.42, 0.65, 0.70, and 0.68 d$^{-1}$, respectively. The average growth rate remained almost constant (i.e., $0.65 - 0.70$ d$^{-1}$) when the spin rate exceeded 50 rpm. Time profiles of cell viability under different spin rates are shown in Figure 17B. From the inoculation to the end of exponential growth phase, the cell viability in the four cases was above 95%. After that, the cell viability decreased sharply. Higher spin rate led to higher shear stress in the spinner flask, which has a detrimental effect on MethA tumor cell growth, so the cell viability at higher spin rates decreased faster (Figure 17B).

The kinetics of glutamine consumption under different spin rate levels is compared in Figure 18A. There was almost no significant effect of spin rate on the glutamine consumption within the range investigated. The yield of viable cell against glutamine was $1.48 \times 10^{11}$, $2.03 \times 10^{11}$, $2.43 \times 10^{11}$, and $2.04 \times 10^{11}$ viable cells/mol of glutamine at a spin rate of 50, 100, 150, and 200 rpm, respectively. This indicated that the maximal yield of viable cell against glutamine of $2.43 \times 10^{11}$ viable cells/mol glutamine was obtained at a spin rate of 150 rpm. The accumulation of glutamate and ammonia follows the consumption of glutamine. As observed in the other experiments, not only the time profile of glutamate accumulation (Figure 18B), but also the kinetics of ammonia production (Figure 18C) corresponded well to the consumption of glutamine in the MethA tumor cell culture process of the spinner flask. The final concentrations of glutamate and ammonia seem to be independent of spin rates, indicating that the overall ammonia and glutamate yields on glutamine are constant.

Figure 17. Dynamic profiles of viable cell density (A) and cell viability (B) under various spin rates during the MethA tumor cell culture process in a spinner flask. Symbols for the spin rate (rpm): 50 (○), 100 (●), 150 (▲), and 200 (▲).

Figure 18. Kinetics of glutamine consumption (A), glutamate production (B), and ammonia accumulation (C) under various spin rates. The symbols for spin rate are the same as those in Figure 17.
The heat-shock protein gp96 production under various spin rates is shown in Figure 20. The production of heat-shock protein gp96 was increased with the increase of spin rate from 50 to 150 rpm, while there was a decrease when the spin rate increased from 150 to 200 rpm. The effect of spin rate on the heat-shock protein gp96 biosynthesis was similar with its effect on the MethA tumor cell growth. The highest heat-shock protein gp96 production titer at a spin rate of 50, 100, 150, and 200 rpm was 2.50, 3.45, 4.55, and 3.58 mg/L, as obtained on the culture of 107, 107, 107, and 86 h, respectively, while the corresponding volumetric heat-shock protein gp96 productivity was 0.48, 0.70, 0.94, and 0.90 mg/(L-day) and the corresponding specific heat-shock protein gp96 productivity was 0.63, 1.20, 1.54, and 1.14 pg/(cell-day), respectively. Not only the heat-shock protein gp96 production, but also the productivity and specific productivity of heat-shock protein gp96 obtained the maximum values at a spin rate of 150 rpm in the spinner flask.

The above results indicated that both the MethA tumor cell growth and heat-shock protein gp96 production were significantly affected by the spin rate in the cell culture process of the spinner flask, and a spin rate of 150 rpm was desirable for MethA tumor cell growth and heat-shock protein gp96 production and productivity.

**Conclusion**

A suspension cell culture process of MethA tumor cell for the production of heat-shock protein glycoprotein 96 was developed in spinner flasks for the first time. It was observed that the maximum viable cell densities as well as the amount of heat-shock protein gp96 were dependent on the medium composition and spin rate. The significance of initial glucose, glutamine, RPMI 1640, and serum concentration and spin rate in spinner flasks on the MethA tumor cell growth, metabolism, and the production of heat-shock protein glycoprotein 96 were studied in detail. As shown in Table 1, the optimal conditions for the cell growth and accumulation of heat-shock protein glycoprotein 96 were identified. In this work, not only the viable cell density but also the production and productivity of heat-shock protein glycoprotein 96 obtained in this work are the highest reported in the culture of MethA tumor cell. This offers an effective approach for producing heat-shock protein glycoprotein 96 from the cell culture process, and the scale-up study on the novel suspension culture process of MethA tumor cell is underway in the laboratory. The work is considered useful for a large-scale process for highly efficient production of glycoprotein 96 toward industrial application. The information obtained may also be beneficial to other cell culture processes, which have wide utilization and application in the health care sectors.

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Table 1. The Optimized Culture Medium and Condition for MethA Cell Growth and Heat-Shock Protein gp96 Production

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>7.0 g/L</td>
</tr>
<tr>
<td>glutamine</td>
<td>6 mM</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>10.39 g/L</td>
</tr>
<tr>
<td>serum</td>
<td>8%</td>
</tr>
<tr>
<td>spin rate</td>
<td>150 rpm</td>
</tr>
</tbody>
</table>

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References and Notes


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